Springer

Handbook of Odor

Buettner

Editor

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Springer Handbook of Odor

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With 458 Figures and 122 Tables

Springer

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Foreword

More knowledge is needed!
(*Wir können wissen, wir müssen wissen*)

Around odors there is a wealth of very different jobs, but all are wonderful when they are done with passion. Passion is the common denominator for all three of us, even if we have different activities. What activities, by the way? Some analysis is needed first in order to see more clearly, so that we can identify more precisely what has to be done for the *odor realm*.

The three of us agree: Rembrandt was an artist and he was not a house painter; on the other hand, when doing his job, a house painter does not look for emotion, and he or she has to be a technician, even if there is some artistic component involved. The most ineffable Rembrandt painting is appealing.

We don't say that technique is better than art, or that art is better than technique, because the evaluation criteria are not the same in the two fields. Should we personally prefer art to technique, as is frequently proposed? Probably not, because sometimes we need to paint walls, and Rembrandt is useless in this regard, and sometimes we need something different, and Rembrandt becomes interesting.

All this shows that our choices have to be carefully analyzed, and this discussion is particularly important in the realm of odors, because it is a place where preferences are ubiquitous. Of course, it is easy to understand that *It smells good* indeed means *Personally, I like this odor*, so that we can ask the question of the legitimacy of a personal preference, even if this comes from a master perfumer. How is his preference more important than that of a non-specialist? This is a first question ..., which we propose not to answer. Our goal here will be this one: ask questions, because we would love you to think about them and propose your own answers.

Coming back to jobs, the vision proposed here consists in considering that there are different jobs, in the areas of art, technique, technology, science. In order to avoid hierarchy, let's consider them in alphabetical order: art, science, technique, and technology.

Some people, such as Maurice Roucel, with odors, or the French chef Pierre Gagnaire, with cooking, are reluctant to call themselves artists, because they fear pretentiousness, but facts are always the most important. As we said before, Rembrandt was not a house painter, and his project was emotion, even if it included, of course, mastering the technique, and a commercial component.

In art, there is probably a passion for *beauty*, as Pierre Kurzenne says, and much apprenticeship work is needed, because work never ends in the quest for perfection in art. We slog and we work. Passion frequently means being able to see the intimate interest of the work. But with respect to beauty, the issue is huge.

In the kitchen, the issue is not to look at dishes, but to eat them. Here *good* means *beautiful to eat*; with music, it means beautiful to hear (we don't care if the pianist is well dressed or not). And one can easily understand that with odors, the issue is to make *beautiful odors* as well, which means odors that we admire.

Beauty is, then, the main question in art, and it is interesting to observe that it has had thousands of definitions in the past centuries, but it always had something do to with emotion and culture. This holds for odors; the project of the *odor artist* can be to please, to make happy, to anger, to seduce ..., but never to leave you indifferent.

For Plato, art was bad, because representing (reproducing the odor of mango, of olive oil, ..., etc.) meant a double detachment from the truth, but at the same time, the philosopher could not escape discussing art and he had to tackle difficult questions, such as the fascination for perfect representation, like the painting of grapes that appeared so *true* that birds were confused!

Anyway, Plato was refuted by Aristotle! *The* odor of *the* raspberry does not exist: any particular raspberry has a particular odor, and this means that the production of *the* raspberry odor is a lost battle. We can only produce one chosen odor among an infinity thereof. This is the old debate of realism against idealism.

Which particular odor of raspberry does the perfumer or the fla-

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vorist want to interpret and why? This is a question of individuals, but also of space, time, and … Moreover, concerning reproduction, we have to admit that the copy is never the original, which is why one of us (Hervé This) proposes that the new *note by note* way of cooking (see below) should not reproduce, but instead invent. It is the same with synthesizers in music: it is sure that one can reproduce the sound of a violin, but why? If one loves music, why not discover unknown sounds, timbers, and music?

Let's move to technique now. Here, the project is very different, because the goal is to make, to build. *Techne*, in Greek, means *to do*. For this kind of activity, the criteria are precise reproduction, regularity, and care. After decades of studying culinary productions, it appeared to one of us that care is the most important parameter of technicians, because it means giving pleasure, creating a kind of social osmosis through the product.

Here, two possibilities exist. One is to reproduce what was done in the past, by ancestors who lacked our modern information on chemistry, physics, and biology; the other is to use all modern information in order to get new skills and, often, to reach new products.

With odors, these skills can be used in various cases, such as perfumes, flavors, consumer care products, technical preparations, etc. But in all cases, the issue is to deal with mixtures of odorant compounds (i. e., compounds which can link – in one way or another – to olfactory receptors) interacting with a *matrix*, from which components of the mixture are released, at different velocities. For perfumes, this definition is clear, but for flavors? Indeed, if we eat a raspberry, we perceive the flavor of this raspberry. By definition, flavor is the synthetic sensation based on many different perceptions such as consistency, color, taste, odor, trigeminal perception, and others, because we discover more and more every year, such as the specific perception of long unsaturated fatty acids on taste buds, or of calcium ions, …; and we're probably not finished.

Odor is one component only, but the status of odorant compounds in food is more than odor, because many foods have an odor as well as a taste and a trigeminal effect! It was frequently published in the past that odor was the most important component of flavor. This is not true, as is easily demonstrated by eating after burning one's mouth with a hot potato, for example: all the flavor is lost, even if the odor remains. Let's keep in mind that it is not necessary to diminish some (other perceptions) to make others (odors) appear more important. If we recognize this, we will be able to better include odor in flavor!

Here, let's conclude by coming back to the idea that new information makes new techniques. It is the goal of this *Handbook of Odors* to give as fresh information as possible, and this is why this book is important: new information equals modernized techniques.

However, the issue of improving technique is, indeed, the goal of *technology*, from *techne* and *logos*. One of us published a whole book in order to propose that there are two kinds of technology, one called *local* (the technician, or someone near him, proposes new, more rational ways of doing) and one *global*, for which the engineer looks for results of science, selecting the useful ones and transferring them to the technical field. Here again, this *Handbook* is important because by including chapters from specialists among the best, it gives the basis for innovation. Innovation, the keyword for the industry!

And finally we look at science. Here we need to give an explanation, because there is much confusion about it. Indeed, the word *science* means knowledge, and this is why it is legitimate to speak of the *science of the shoemaker*, or the *science of the cook*, or the *science of the perfumer* … However, the science that we need to discuss here is very different: now, we restrict the general meaning to the *sciences of nature* or *natural philosophy*.

For sciences of nature, the goal is to discover the mechanisms of phenomena, using the scientific method based on:

1. Observing a phenomenon
2. Measuring it, which produces a lot of numerical data
3. Grouping these data into *laws* (i. e., equations)
4. Producing *theories*, i. e., explanations quantitatively compatible with the data
5. Making theoretical predictions which are experimentally tested, and so on, forever.

One could ask if there is a link between science and art, and one of us (Hervé This, certainly a scientist), after decades of close friendship and work with Pierre Gagnaire (certainly an artist), answers with a very strong *no*, even if there has been an intense relationship between artists and scientists, and common characteristics such as intuition, liberty, curiosity, enthusiasm … Indeed, the goals of science and art are different (mechanisms on the one hand, emotion on the other), as well as the *ways* (*methodon*, in Greek, method) in which they are achieved.

The method of science was described above; with art it is based on intuition, experience, personal emotion, the desire to communicate, … If a scientist wants to move toward art, he or she has to get away from science to technology, whereas the artist who would like to move toward science has to go through technique. No relationship between art and science, but rather re-

lationships between the applications of sciences (very different from the sciences) and the technical component of art (very different from art, even if it is needed).

All this being said, all of us three agree that one very important component of our activities is to think correctly and use good vocabulary, taking the utmost care with semantics. Very frequently, in building odors, young artists don't find the precise *words*, and this has technical consequences because they cannot select the right raw materials. For sciences of nature, also, words are very important, and the great Antoine Laurent de Lavoisier, the creator of modern chemistry, quoted Condillac in order to show why nomenclature was the basis for advances: sciences explore phenomena, but we have to think of the phenomena in order to study them; in order to think we need words, and this is why, Lavoisier said, you cannot improve science without improving words and vice versa.

By the way, all of us three also agree that with respect to odors or flavors, we would love to have more scientific knowledge.

Imagine that you make an odor without taking into account some trigeminal effect. Imagine that you make food without pepper: the dish would not be what it could be. Imagine that you don't know about some particular interaction effects of odorants on olfactory receptors: again, the result would not be what it should be. In music, it is as if the piano had only a limited number of notes. We need more information on the relationship between the molecular structure of odorant compounds and the odors of such compounds. We need more information about *odor release*, which means the physical and chemical description of the diffusion of odorants inside and outside matrices, including supramolecular associations. We need more information on the perception of odorants and the various interactions, before smelling them, and on the hedonistic interpretation of the olfactory perceptions. Of course, all this holds for food!

Perfumers succeed empirically with their current methods, for example making more vanilla note with bergamot, instead of increasing the quantity of vanillin! This is what two of us (Pierre Kurzenne and Maurice Roucel) name *contrast*. Perfumers also use metaphoric words such as *hot* or *sparkling*, and in the future it could be interesting to better understand what this is.

Concerning all this research, the past makes promises for the future. For odors, Nobel prizes have already been awarded to advances in the field (terpenes, olfactory receptors), but so much remains to be done! At this point, in particular, the prediction of the odor of a particular chemical structure is out of reach, and the effect of mixtures of odorants remains elusive. Of course, the exploration of God's shop is important, but we have to recognize that *nature*, in spite of our ideas, is far from perfect! Isn't plague natural, as are as volcanoes, tsunamis, and so on? If we use clothes, buildings, and perfumes, it is because – in a way – we are fighting against nature … because human beings belong to culture.

It is strange, in this regard, to classify odors into poisonous and non-poisonous ones. It is also strange that some odorant compounds are not as *they should be*. Why are lead salts sweet, yet toxic? Why are some bitternesses accepted (think of beer), yet often associated with toxic alkaloids? The same holds for some odorants!

Let's finish with the issue of regulation. It is clear that the public should be protected –but from what? Let's first recognize that there is a big difference between danger (a knife is dangerous) and risk (the knife can be used to kill, but if it is in a closed drawer, there is no risk). Danger is everywhere, but we have to reduce risks. In this regard, it is certainly good to know the dangers and the exposures in order to make regulations – but such rules should focus only on risk, not on hazard.

Now, coming back to the issue of nature/culture, we should say that no product of the perfume or flavor industries is natural, because it was produced! Let's remember that something is natural when it was not transformed by human beings. If you extract an essence from a flower, for example, the flower had to be cultivated, and the essence had to be extracted. It's no longer natural. But who cares: some products of culture are much better than natural products. Remember Rembrandt, Matthias Grünewald, Zao Wo-Ki, Bach, Mozart, Debussy, etc.

Moreover, in this regard, the issue of *note by note cooking* is probably important for the development of the odor industry. This new way of cooking was proposed for the first time in 1994 by one of us (Hervé This) and it is the culinary equivalent of synthetic music. Instead of using flutes and violins, one uses pure waves, in order to make sounds, after which music is composed. For cooking, the elementary units are not sound waves, but rather compounds, and note by note cooking does not use traditional ingredients (animal and plant tissues), but rather pure compounds, from which dishes are made.

This proposal is important for regulation because it kills the need to make particular categories for additives and flavorings: compounds used for making food would simply be *food ingredients*. Where will we find the needed compounds? Of course they can be extracted or synthesized, and we have to tell this to the public! Extracted or synthesized, vanillin is always vanillin, and water is always water. Of course, today a part of

the public *fears* chemistry, but, indeed, it is because it does not understand it. One way to circumvent fear is to make it desirable, trendy, fashionable, even forbidden. This is how Augustin Parmentier succeeded in making the French public eat potatoes, before the French Revolution: he invited the king to eat them! This strategy has been used to implement note by note cooking.

Let's imagine that we succeed in developing this new culinary trend. How shall we cook? A good way being explored today is to design the shapes and consistencies, then to design color, taste, odor and trigeminal perception, as we add spices to traditional dishes. However flavorists know too well that knowledge is needed in order to get the desired sensation, because of the chemical and physical interactions with the various compartments of the matrix. Chefs will have to learn, which means that flavorists' collaboration will be needed. Of course, one could imagine using flavorings as we use aromatic herbs and spices, but wouldn't it be much more interesting to use pure solutions of particular odorants (for example, a very small concentration of 1-*cis*-hexen-3-ol in ordinary oil), or *kits* of odors, in order to make entirely new flavors (*edible perfumes*)? By experience, chefs are not ready to have flavorists take the lead on their productions, which means that the first, traditional solution of using flavorings is probably not the future. If kits or pure solutions are the future, the odor industry has to be ready to make entirely new products.

Finally, after this long tour, we have to recognize that life is wonderful, in particular because the world of odors is fascinating. About art, for technique, for technology, for science, there are so many open questions! Questions are promises of answers – if we work with passion! Passion for odors, passion for imagination, passion for emotions, passion for knowledge ...

In mathematics, the great David Hilbert said *Wir müssen wissen, wir werden wissen* (*we must know, we will know*)!

Pierre Kurzenne
Maurice Roucel
Hervé This

Preface

Olfaction in humans is a sensory dimension that is often underestimated or overlooked entirely. The sense of smell is commonly believed to play only a minor role in human perceptual experiences, which is an understandable misconception when considering the everyday dominance of vision and hearing for communication, augmented by touch for interaction with the immediate surroundings. Moreover, these more prominent sensory modalities are known to exert both physiological and psychological influences on humans (e.g., sound/noise, light/vision, and temperature/climate) that can have an impact on our general wellbeing. In addition, our sense of smell is typically considered to be inferior to olfaction found elsewhere in the animal kingdom; for example, numerous species are able to trace the path of an odor source over great distances, or habitually undergo behavioral responses that are strongly modulated by volatile chemicals, e.g., pheromone-type compounds, that may play a role in aggression, mating, or rearing offspring. An absence of scientific proof does not preclude such remarkable capabilities in humans, yet there is widespread disregard for the importance and impact of the human sense of smell. Seemingly, this most likely relates to its association with *primitive*, animal-like behavior and potentially uncontrollable effects and responses that are elicited by smells, which are considered to be less rational and, therefore, more appropriate for the animal kingdom; surely the higher intellect of homo sapiens is not prone to behavioral responses from such primitive influences!

Yet, there is more to the human sense of smell than meets the nose, so to speak. There is an ever increasing body of evidence from ground-breaking research discoveries that demonstrates the crucial role that smells play in shaping our lives. From birth onwards we learn to interact with our environment using our sense of smell. Evolutionary processes have engendered a multifaceted communication that is supported – even dominated – by olfaction. This might be in the form of the smells that nurture the relationship between mother and child or influence partner selection, aromas that form our food preferences, or odors that warn us of dangers.

In the modern world we are increasingly exposed to smells that were not encountered by our ancestors. These are ubiquitous in our present-day environments and are met in all aspects of daily life, with sources ranging from manmade materials, industry, transport, household products, etc.; the list is practically endless. The outcome of this constant evolutionary process in material, product and application development is that we have generated a tolerance or even unawareness to numerous *modern* smells despite their often pervasive and abundant nature. By contrast, smells that relate to our appreciation of products such as foods have attracted intense scientific interest spanning decades, with the earliest discoveries dating back to when chemistry was still in its infancy. Specifically, pleasantly-smelling raw materials and compounds considered to be attractive to humans (e.g., as body scents or room fragrances) were at the focus of early research, sometimes with enormous efforts made to recover and enrich substances to a sufficient extent to enable a chemo-analytical elucidation of the underlying odorous molecules; such efforts were especially laborious and time-consuming at a time when the analytics and respective instrumentation were still rudimentary. Nowadays, an array of methods are at our disposal to resolve even the most complex of odor mixtures and decode the structures of individual molecules at extremely low concentrations; thus, rather than the insufficient sensitivities and resolutions limiting early research in this field, present odorant analytics might be considered as searching for a needle in a haystack, with individual odorants present amongst a forest of competing signals.

With emerging progress in the biochemical, biomedical, and neurosciences, research in olfaction has subsequently expanded to include a strong focus on the impact of smells on humans. This new direction has revealed important insights into how smells are perceived, processed and memorized, and how odor impressions influence our everyday lives. Nevertheless, the nature of numerous odorants remains unknown, as does their influence on perception, physiology and wellbeing. This is especially true for the modern smells that are encountered on a daily basis at home, at work, or when out and about.

A comprehensive treatment of smells – from ancient to modern and rare to common – in relation to the impact they have on our lives currently does not exist. This handbook aims to bridge this gap by aligning the senso-chemo-analytical characterization of smells encountered by mankind, tracing the diverse routes of po-

tential formation and release pathways, and elucidating the perceptual hedonic, behavioral, and physiological responses of humans to such odors at different stages of life. This book is intended to build a foundation for a hitherto widely overlooked area of research that has wider ramifications for human life, and to instigate intensified interdisciplinary discussions as a catalyst for gaining further insights and discoveries.

Andrea Buettner
Munich, Germany
October 2016

About the Editor

Andrea Buettner read for an undergraduate degree in food chemistry at Ludwig Maximilian University of Munich, Germany, and then completed her postgraduate and postdoctoral research at both the German Research Center for Food Chemistry (DFA) and the Technical University of Munich from 1995 to 2002. Following her habilitation to qualify for full professorship in 2007 she was appointed and still holds two concurrent positions, both as Founder and Head of the Department of Sensory Analytics at the Fraunhofer Institute for Process Engineering and Packaging IVV in Freising, Germany, and of the Odor and Aroma Research Group at Friedrich-Alexander-Universität (FAU) Erlangen-Nürnberg, Germany. Andrea has received several accolades for her research, including the Kurt-Täufel Prize for young scientists from the Food Chemistry Division of the German Chemistry Society (2010), the Young Investigator Award from the Food and Agricultural Division of the American Chemical Society (2011), the Danone Innovation Prize (together with Caroline Siefarth, 2012), and the Nutricia Science Award (2013). Since 2012, Andrea has held a position as Full Professor of Aroma Research at FAU in Erlangen.

Andrea's expertise encompasses the characterization of the main odor triggers in aromas, flavors, and common odors. Specifically, Andrea is renowned for identifying and characterizing the primary odor triggers in typical aromas, flavors, fragrances, and smells that are associated with everyday life. Amongst other things, her work involves disentangling and subsequently reconstructing characteristic smells, from food aromas to modern man-made materials, based on combined chemo-analytical and human-sensory characterization of the molecular odor constituents. Her specialized field extends to characterizing odorant release via on-line monitoring of distribution and delivery processes. This particular aspect of her work, for example, has provided insights into the importance of the combined effects of the food matrix, saliva, mucosa, mastication, and swallowing on flavor release and perception. In view of physiological processes related to odorant exposure and uptake, recent investigations have targeted pharmacokinetic aspects of odorant inhalation, absorption, biotransformation, and elimination via breath, urine, sweat, and human milk. Accordingly, Andrea's research aims to raise awareness of smell and odor dimensions in human life, especially in common, everyday situations. Her goal is to cross-link technological, chemical, or physiological aspects and to provide novel solutions in odor research. Novel or modified techniques, methodologies, processing technologies, analytical tools, and approaches related to smell are further outcomes of her research strategy, with the ultimate aim to nourish interdisciplinary cross-talk on a topic that links chemical, analytical, material, and process engineering sciences with the fields of sociology and socio-ecology, psychology, and physiology.

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List of Abbreviations

1-D	one-dimensional
2-D	two-dimensional
2-DG	2-deoxy-D-glucose
3-D	three-dimensional
3M2H	3-methyl-2-hexenoic acid
4-D	four-dimensional

A

AAT	alcohol acyl transferase
ABC	ATP-binding cassette
ACC	anterior cingulate cortex
ACD	allergic contact dermatitis
ACP	acyl carrier protein
ADH	alcohol dehydrogenase
ADI	acceptable daily intake
AEDA	aroma extract dilution analysis
AHI	apnea-hypopnea index
ALA	alpha-lipoic acid
ALDH	aldehyde dehydrogenase
AM	autobiographical memory
AMP	adenosine monophosphate
AMP	adenosine 5′-monophosphate
AMRE	axillary malodor releasing enzyme
ANOVA	analysis of variance
AO	anterior olfactory nucleus
AOB	accessory olfactory bulb
AOP	adverse outcome pathway
APC	anterior piriform cortex
APCI	atmospheric pressure chemical ionization
APET	added portions exposure technique
API-MS	atmospheric pressure ionization mass spectrometry
APLI	atmospheric pressure laser ionization
ARP	Amadori Rearrangement Product
ARS	acetone reference standard
ATP	adenosine triphosphate

B

BDI	Beck depression inventory
BDNF	brain-derived neurotrophic factor
BLA	basolateral amygdala
BMI	body mass index
BOLD	blood-oxygen-level-dependent
BOTT	basic odor term test
BSA	bovine serum albumin
BTTT	basic taste term test

C

CA	Cornu Ammonis
cAMP	cyclic adenosine monophosphate
CANN	cortical-based artificial neural network
CCCRC	Connecticut Chemosensory Clinical Research Center test
CD	cyclodextrin
CE	carboxyl esterase
CI	chemical ionization
CNG	cyclic nucleotide gated
CNS	central nervous system
COA	conditioned odor aversion
CREB	cAMP responsive element binding
CSERP	chemosensory event-related potential
CSIA	compound-specific isotope analysis
CWA	chemical warfare agent

D

DAG	diacylglycerol
DES	diethyl sulfide
DESI	desorption electrospray ionization
DG	dentate gyrus
DHE	dynamic headspace extraction
DMAPP	dimethylallyl diphosphate
DMDS	dimethyl disulfide
DMS	dimethyl sulfide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNPH	dinitrophenylhydrazine

E

e-nose	electronic nose
EBC	exhaled breath condensate
EC	entorhinal cortex
ee	enantiomeric excess
EEG	electroencephalography
EESI	extractive electrospray ionization
egr-1	early growth response protein 1
EH	epoxide hydrolase
EI	electron ionization
ELDI	electrospray-assisted laser desorption
EMEM	Eagle's modified essential medium
EMG	electromyography
EOG	electroolfactogram
EOS	emotion and odor scales
er	enantiomeric ratio
EROM	European reference mass odor
ERP	event-related potential
Es-GC	enantioselective gas chromatography
ESP	epithio-specifier proteins
EST	expressed sequence tag
EtSH	ethanethiol

F

FAD	flavin adenine dinucleotide
FD	flavor dilution

FDSS	functional drug screening system
FDT	flow drift tube
FFI	food freshness indicator
FGE	flavoring group evaluation
FID	flame ionization detection
FLEC	field and laboratory emission cell
fMRI	functional magnetic resonance imaging
FPD	flame photometric detection
FT	Fourier transform
FTIR	Fourier transform infrared
FWHM	full-width at half maximum

G

GABA	γ-amino butyric acid
GC	gas chromatography
GC-FID	gas chromatography with flame-ionization detection
GC-IRMS	gas chromatography isotope ratio mass spectrometry
GC-O	gas chromatography-olfactometry
GC-PO	GC-pedestal olfactometer
GC-RO	GC-recomposition-olfactometry
GC-SNIF	GC-surface of nasal impact frequency
GEOS	Geneva emotion and odor scale
GFP	green fluorescent protein
GG	Grüneberg ganglion
GPCR	G protein coupled receptor
GPMT	guinea pig maximization test

H

HCC-HS	high concentration capacity headspace
HD	homeodomain
HEK	human embryonic kidney cell
HLA	human leukocyte antigen
HLB	hydrophilic-lipophilic balance
HPLC	high performance liquid chromatography
HRIPT	human repeat insult patch test
HS	headspace
HS-LPME	headspace liquid-phase microextraction
HS-SPME	headspace solid-phase microextraction
HS-STE	headspace sorptive tape extraction
HSSE	headspace sorptive extraction
HTS	high-throughput screening
HVP	hydolysed vegetable protein

I

IAMS	ion attachment mass spectrometry
IAT	implicit association test
IBMX	isobutyl methyl xanthine
IC50	half-minimal inhibitory concentration
IGF	insulin-like growth factor
IL	interleukin
IMR	ion-molecule reaction
IMS	ion mobility spectrometry
IPP	isopentenyl diphosphate
IR	infrared
IRMS	isotope ratio mass spectrometry
IT	ion trap
IT-MS	ion trap mass spectrometry
ITE	individual threshold estimate

J

JND	just noticeable difference

K

KC	K-complex
Kegg	Kyoto Encyclopedia of Genes and Genomes
KSOM	Kohonen self-organizing map

L

LAB	lactic acid bacteria
LAESI	laser-assisted electrospray ionization
LBP	LPS-binding protein
LC	liquid chromatography
LDL	low density lipoprotein
LLE	liquid-liquid extraction
LLNA	local lymph node assay
LOD	limit of detection
LOQ	limit of quantitation
LOT	lateral olfactory tract
LOX	lipoxygenase
LPP	late positive event-related potential
LPS	lipopolysaccharide

M

MALDI-ESI	matrix-assisted laser desorption electrospray ionization
MAP	modified atmosphere packaging
MCC	multicapillary column
MCS	multiple chemical sensitivity
MDGC	multidimensional GC
MDR	multidrug resistence
MDT	mediodorsal thalamic nucleus
MEG	magneto-encephalogram
MeSH	methanethiol
MEST	mouse ear swelling test
MHC	major histocompatibility complex
MICA	metabolism of ingestion correlated amount
MLF	malolactic fermentation
MOB	main olfactory bulb
MoE	margin of exposure
MOE	main olfactory epithelium
mOR-EG	mouse olfactory receptor protein
MOSFET	metal oxide semiconductor field effect transistors
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSDI	maximized survey-derived daily intake

MSG	monosodium glutamate
mTAMDI	modified theoretical added maximum daily intake
MUFA	monounsaturated fatty acid
MUP	major urinary protein

N

NIF	nasal impact factor
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
NOEL	no observed effect level

O

O	olfactometry
OAV	odor activity value
OB	olfactory bulb
OBP	odor binding protein
OCAM	olfactory cell adhesion molecule
ODR	odorant response
ODT	odor detection threshold
OE	olfactory epithelium
OERP	olfactory event-related potential
OET	open epicutaneous test
OFC	orbitofrontal cortex
OIRS	odor intensity referencing scale
OMP	olfactory marker protein
OR	olfactory receptor
ORN	olfactory receptor neuron
ORP	olfactory receptor protein
ORT	odor recognition threshold
OSN	olfactory sensory neuron
OTH	odor detection threshold
OU	odor unit

P

PA	proton affinity
PAD	pleasure, arousal, and dominance
PAH	polycyclic aromatic hydrocarbons
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PC	piriform cortex
PCA	principal components analysis
PCC	posterior cingulate cortex
PCR	polymerase chain reaction
PDMS	polydimethylsiloxane
PDO	protected designation of origin
PE	polyethylene
PEA	phenylethylalcohol
PEEK	polyether ether ketone
PET	positron emission tomography
PFC	prefrontal cortex
PFPD	pulsed flame photometric detector
PFS	passive flux sampler
PI	perceived intensity
PIB	polyisobutylene
PID	photoionization detector
PLC	phospholipase C
PMMA	polymethylmethacrylate
PP	polypropylene
PPC	posterior piriform cortex
premox	prematurely aged
PS	polystyrene
PTFE	polytetrafluoroethylene
PTR	proton transfer reaction
PU	polyurethane
PUFA	polyunsaturated fatty acid
PUT	provocative use test

Q

QC	quality control
QOD	questionnaire olfactory disorder
QoL	quality of life
QRA	quantitative risk assessment

R

RAS	retronasal aroma simulator
REM	rapid eye movement
RIA	retention index allowance
RNA	ribonucleic acid
ROAT	repeated open application test
ROS	reactive oxygen species
RTL	retention time locking

S

S-HS	static headspace
SAFE	solvent assisted flavor evaporation
SAR	structure–activity relationship
SCFA	short-chain fatty acid
SDE	simultaneous distillation extraction
SEC	size exclusion chromatography
SESI	secondary electrospray ionization
SID	selected ion detection
SIDA	stable isotope dilution analysis
SIFDT	selected ion flow drift tube
SIFT	selected ion flow tube
SIM	selected ion monitoring
SIRA	stable isotope ratio analysis
SND	sinunasal disease
SNP	single nucleotide polymorphism
SOA	secondary organic aerosol
SPE	solid phase extraction
SPME	solid phase micro extraction
Src	sarcoma
SVHC	substance of very high concern
SVOC	semivolatile organic compound

T

TA	titratable acidity
TAAR	trace amine associated receptor
TAGA	trace atmospheric gas analyzer
TD	thermal desorption

TDS	temporal dominance of sensation
tERP	trigeminal event-related potential
TIC	toxic industrial compound
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TM	transmembrane
TMR	targeted memory activation
TNF	tumor necrosis factor
TOF	time-of-flight
TPOA	taste potentiated odor aversion
TPS	terpene synthase
TRP	transient receptor potential
TRPC	transient receptor potential channel
TSS	total soluble solids
Tu	olfactory tubercle
TVOC	total amount of volatile organic compounds

U

UPSIT	University of Pennsylvania smell identification test
URTI	upper respiratory tract infection
UTA	untypical aging off-flavor
UV	ultraviolet
UV–Vis	ultraviolet-visible

V

V-SMOW	Vienna Standard Mean Ocean Water
VMR	volume mixing ratio
VNO	vomeronasal organ
VOC	volatile organic compound
vPvB	very persistent and very bioaccumulative
VR	vomeronasal receptor
VSC	volatile sulfur compound
VSN	vomeronasal sensory neuron
VUV-SPI	vacuum ultraviolet single-photon ionization

W

WOF	warmed-over-flavor

1. History of Odor and Odorants

Wilhelm Pickenhagen

Smell is the oldest sense of living species on our planet and has allowed communication between species from the beginning of life on earth. Odor has always fascinated mankind even in prehistorical times and the use of scented materials is documented since early history. Materials used were extracts from odorous natural products until the advent of organic chemistry. Progress in analytical methods allowed the isolation and structure identification of odor impact compounds from natural extracts and subsequent syntheses made it possible to produce these compounds on an industrial scale for use in many applications of modern perfumery.

1.1 Defining Odor and Odorants

Chemical communication, namely a signal initiated by a chemical compound emitted by a donor, received by a receptor and recognized as such, is one of the foundations of the development of life on earth.

While the two chemical senses, namely the senses of taste and smell, are directly elicited by a chemical entity perceived by a receptor, all other senses (sight, hearing, and touch) recognize the very first signal by physical forces such as light, sound, temperature, or pressure, respectively. A cascade of biochemical reactions then amplify and transmit these signals to the brain where they can be analyzed as such.

In this context, it might be useful to define what an odor is, namely the impression in the brain elicited by the recognition of a (mostly) volatile chemical by an odorant receptor. It follows that a chemical (or molecule) can be considered as an odorant if it fulfills the following conditions:

1. It has to bind to an odorant receptor.
2. It has to result in the odorant receptor transmitting the recognition to the brain.
3. The brain has to recognize it as a signal that can be interpreted.

1.2 The Chemical Senses

While the origin of biological life on Earth is not exactly known, some hypotheses do exist [1.1]. What is certain however is that primitive forms of life began in an aqueous environment where sound and optical impressions were very limited. The so-called chemical senses were of essential importance for survival long before species with some form of brain and capabilities of reflection developed. The chemical senses are thus the oldest senses in the development of life on earth, and allowed primitive species to find food, sexual partners, and warned them for potential predators by recognizing certain low molecular chemicals in their surroundings [1.2].

As life on earth became more complex, when single cells developed into multicell organisms, a primitive brain form developed capable of recognizing and an-

alyzing different odor impressions. During evolution, these increasingly complex species eventually left the aqueous environment, and in order to survive, developed new senses of hearing and vision. Because treatment of such new stimuli could no longer be handled by the primitive brain, the neocortex developed which allowed for the compilation of these new sensory signals as well as endowed the more complex species with other capabilities still found today in the *modern brain*. Nevertheless, the perception of odors is still, even in humans, relayed directly in the limbic lobe, which has also be shown to be the neural substrate of emotional behavior [1.3]. Because of this, olfaction is the only sense capable of suddenly retrieving long forgotten memories by a single sniff of a specific scent. This phenomenon led the famous French mathematician Blaise Pascal to the desperate, but true statement *The nose knows things that the mind does not understand* [1.4].

1.3 History of Use of Odorants

1.3.1 Prehistory

If the use of fire to transform food material is considered the first direct production of odorous material, then the use of odorants by hominids by far precedes the period which we call *human history*, and in fact the existence of *Homo sapiens sapiens* themselves.

The oldest described traces of fire not ignited by lightning have been found at a site called Gesher *Benot Ya'aqov* in Israel and are dated back to about 790 000 years. The authors of this discovery discuss the probability that these fires were created by hominids belonging either to *Homo erectus*, *Homo ergaster, or even to an archaic Homo sapiens* [1.5].

During the evolution to *Homo sapiens* some of the pure instincts for survival changed slowly into reflective habits and needs, that is, into cultural rites of different kinds. The ability of recognition and interpretation of olfactory stimulation is one of the most important differences between humans and other living creatures. Use of odors as cultural acts is much older than written history. Indeed, odorous signals initiated by sacrificial burning of different objects were ways to address Gods and ancestors to ask for clemency or to thank for their kindness. *Pro fumum*, odors that were created by smoke is often considered the oldest form of perfumery.

One of the oldest known prehistorical rites is dated back to about 60 000 years. During the excavation of the *Shanidar* cave in northern Iraq, the remains of nine *Homo sapiens neanderthalensis* were discovered in a depth of about 7 m. The surrounding soil contained seeds of a total of eight different kinds of flowers. These flowers of local origin are quite odorous. It was shown that these flowers had neither grown in the cave nor had they been transported there by animals; it has thus been concluded that they were added at the burial of these bodies, indication that already at that time odorous plants were used to honor the dead [1.6].

1.3.2 Early History

The oldest sources of history stem from Mesopotamia, the land between Euphrates and Tigris, the area considered by many as the cradle of humanity. It is believed that it is the place where earthly paradise was located; Genesis 2.7 describing that creation of human life occurred through a sniff [1.7]:

> *the Lord God formed the man from the dust of the ground and breathed into his nostril the breath of life and man became a living being.*

The oldest known epic in human history, the *Gilgamesh Epic*, was found in this region of Mesopotamia. This is written on 12 clay tables and is dated somewhere between 2750 and 2500 BC Indeed in the 11th table, the first father Utnapishtim thanks for being rescued from the deluge [1.8, 9]:

> *I offered incense in front of the mountain-ziggurat,*
> *Seven and seven cult vessels I put in place*
> *And into the bowls I poured reeds, cedar, and myrtle. The gods smelled the sweet savor,*
> *And collected like flies over a sacrifice.*

Mesopotamian cuneiform tablets contain some of the oldest written documents describing the use of odorants. For example, in order to honor the sun God Baal in the Assyrian capital Nineveh, frankincense was burned at about 3000 BC. During the time of Hammurabi around 1700 BC about 2000 talents of frankincense were burned every year in the temple of Bel in Babylon [1.10]. Moreover, the use of odorants was known to exist in the Persian Empire. For example, virgins that were selected to be presented to king Xerxes had to complete twelve month of beauty treatment, six months with oil of myrrh, and six months with perfumes and cosmetics [1.11]. Even Esther had to undergo the same treatment before she was presented to the king and became his preferred wife and queen [1.12].

Odorants were not only used for religious rites but also for private purposes. Priests, royalties and other dignitaries generously used perfumes. The king honored special subjects by putting an odorous wreath on his head, the same way people of today are honored with a medal. The Babylonian king Nebuchadnezzar II built the Hanging Gardens of Semiramis, considered as one of the seven wonders of the antic world, for his wife Amyris. Mainly cypress, mimosas, roses, and lilies were planted there because their perfumes were preferred by the queen [1.13].

At around the same time as in *Mesopotamia*, that is, about 5000 years ago, odors and odorants played an important part in the cultural life of historical Egypt. The Gods by definition emitted an attractive odor whereas common people needed odorants to smell nicely. It is said that in the legend of God Amun, who appears as pharaoh in order to marry his queen, the latter used his odor as the most important attribute to conquer her. It is documented in a text on papyrus that [1.13]:

> *the queen is awakened by the fragrant breath of the God and she smiled in his presence. He came directly to her and fell in love. He gave her his heart and stepped in front of her to show her his divine nature. When she realized his beauty she rejoiced and feelings of love went through her body. The whole palace was filled with the odor of the God; all his scents were those of Punt.*

Punt was considered as the place of origin of the scent of Gods.

Incense burning of different materials was practiced three times a day in Heliopolis, residence of the sun God Ra: frankincense at sunrise, myrrh at noon and kyphi at sunset. Kyphi, a mixture of 16 ingredients was considered as the most agreeable scent belonging to the Gods [1.14].

Gods played an important part of daily life in ancient Greece. The sensory impression of odors was as inexplicable as the nature of the Gods... and there were many. At one time there was an estimate of about 30 000 of them. It followed that the origin of odors was considered as divine. Many myths connect the odor of different plants and flowers with different Gods. For example, Aphrodite kept vigil over Hector's corps night and day and anointed him with ambrosial oil of roses [1.14], and Circe traps Odysseus with the aid of seductive perfume [1.8, p. 107]. Despite the belief in the divine origin of odors the Greeks already knew about the attraction of human odor to the opposite sex. The sweat of wrestlers after the fight was collected with creams and oils, and these mixtures were commercialized [1.13, p. 205] as were other fragrances. Perfumery shops were popular meeting points for all kinds of gossip, and even the cynic Diogenes consented from time to time to place his home, the famous barrel, close to these shops. He used fragrant oils only for his feet, arguing that if only the head is anointed with perfume it would escape into the air and only the birds would benefit from it. However, the use of perfumes was not without harsh critics. The famous philosopher Socrates said that [1.8, p. 112]:

> *if a slave and a free man are oiled with scent they both smell the same but the mark of a free man should be his smell after work and exercise.*

After the Romans had conquered the territories of the Near East, they began to learn about and to adopt the luxuries of Greek lifestyles, including use of odorants, and in some cases excessively. Roman women who belonged to a class that would today be considered as socialites had their special slaves who were instructed to carry out cosmetic and hair care, as well as perfumery tasks. Hygienic care was indeed well developed: the citizens of Rome had public baths at their disposal where they would, after their bath, be anointed with scented oils. Emperor Nero and his wife Poppea Sabina used excessive amounts of scented materials, especially at their receptions and banquets. Nevertheless, there were also critics of the extensive overuse of odorous products including the consuls Licinius Crassus and Julius Caesar who eventually forbade the sales of these products [1.8, p. 116].

The Bible is a rich source of information about historical trade and use of odorous materials. Genesis 37, 25 describes the sale of Joseph by his brothers to midianite merchants who transported spices, balsam, and myrrh from Gilead on their way down to Egypt, where the use of scents for sacrifices and personal care was highly developed. After the exodus of the Jewish population from Egypt, *Moses* received many commandments, one of them ordering the construction of an altar as described in detail in Exodus 30, 1–6. The use of this altar is regulated in Exodus 30, 7–10, where Aaron is ordered to:

> *burn fragrant incense on the altar every morning when he tends the lamps at twilight so incense will burn regularly before the Lord for the generations to come.*

Exodus 30, 23–25 is a recipe for making *the sacred anointing oil* consisting of:

> *500 shekels of liquid myrrh, half as much of fragrant cinnamon, 250 shekels of fragrant cane, 500 shekels of cassia and a hint of olive oil.*

This formula could be considered as one of the oldest *captive* products, that is, products protected by law and

which can only be used by unique people for special purposes because in Exodus 30, 33 it is stated:

> *whoever makes perfume like it and whoever puts it on anyone other than a priest must be cut off from his people.*

Exodus 30, 34 describes another recipe consisting of gum resin, onycha, galbanum, and pure frankincense, also for one special purpose: *consider it holy to the Lord*. Use of myrrh and frankincense are mentioned 22 times in bible texts [1.15] and *Wolfgang Pilz* lists in his collection *Frühe Weltmacht: Parfumerie und Kosmetik* [1.16] about 50 citations from the bible that describe botanical origins of odorous plants and use of them as perfumes.

With the slow fall of the Western Roman Empire around 480 A.C. the cultural development of the Occident came to a sort of a stillstand which lasted until the middle Ages. At the same time the Byzantine Empire grew out of the East Roman Empire till 1453, the fall of Constantinople. During these about 10 centuries, the influences of the surrounding Islamic world became an important factor for the cultural development of this area. Already about 800 BC the inhabitants of the Arabian Peninsula countries cultivated myrrh, frankincense, and other aromatic materials which they also exported to the developed countries around the Mediterranean. The culture of making and using sophisticated odorous materials was highly developed and described in many tales. Based on the knowledge of the discoveries and philosophical interpretation of these by the Greeks and the Egyptians, the Arabs began to discover and develop new techniques through experimentation, just to mention distillation, sublimation, filtration, and – very important – the production and concentration of ethanol (*Arab. al koh'l*) from wine. The application of these tools and techniques gave rise to the isolation of a multitude of odorous materials from different scented raw materials. The technique of steam distillation, an Arab invention, is still a very important method to isolate volatile materials from their matrix.

1.3.3 The Middle Ages till Beginning of Industrialization

It took a while until the culture of using scented materials reached Western Europe. It is dated to the Merovingian period. The first products, mainly spices, were shipped up the Rhone river from the Mediterranean before a more active trade, especially with Venice, developed. At the end of the eighth century, the Benedictine monk *Ansegis* compiled methods for the cultivation of aromatic plants for medicinal and flavoring uses which were published as instructions in Charlemagne's *Kapitular* [1.17]. The well-equipped monasteries of that time used these instructions to cultivate mainly dill, marjoram, mint, parsley, rosemary, and sage. The Abbes *Hildegard von Bingen* (1098−1179) is one of the first scientists to describe the use of these products and their extracts for therapeutic use.

The European participants of different Crusades starting in 1095 discovered the use of the highly developed scented materials of the Near East of which they brought back samples as gifts for their ladies. One of the first ones is probably *Eau de Chypre*, a composition of extracts of two plants indigenous to Cyprus, rose and styrax. This Mediterranean island had been conquered by *Richard Lionhart* during a crusade.

Cultivation and extraction of plants for use as therapeutics that had started at the end of the 8th century [1.17] in the developed gardens of different European monasteries became more and more sophisticated and at around the beginning of the 14th century, these mixtures, the recipes of which were kept highly secretive, became items of trade. *Eau d' Ange*, a composition of aqueous extracts of rose, orange, styrax, and rue was sold by the mobile traders of that time. Another product, *Aqua Regina Hungarica*, is probably the first bestseller in the history of perfumery. It is said that this product, an aqueous extract of mainly rosemary, was given by a monk or even an angel!! [1.18] to the than 70-year-old highly arthritic Queen Elizabeth of Hungary, who, thanks to the regular use of this product recovered health and youth to the extent that the Polish king asked her for marriage.

Probably the most famous and still existing of all waters is *Eau de Cologne*. Its origin most probably dates back to an Italian immigrant to the German city of Cologne. *Giovanni Paolo de Feminis*, originally from the province of Novara brought with him a recipe based on a mixture created by the Abbes of the Monastery of Santa Maria Novella of Florence which he called *Eau admirabilis de Cologne* [1.18]. To underline the uniqueness of this product, he searched and obtained a document from the medicinal faculty of the University of Cologne, dated 13th of January 1727, certifying that it had the property of renewing, refreshing, and reviving the human life spirit [1.18]. *Eau admirabilis de Cologne* became a commercial success and *Feminis* engaged his nephews *Johann- Maria* and *Johann-Antonius Farina* as his partners who developed the commerce under their name. During the 7-year war, French officers that got acquainted with this product brought it to Paris where it became popular around 1760. The commercial success encouraged different manufacturers to engage in its production and sale, one of which was *Ferdinand Mülhens* who manufactured *Eau de Cologne* in his house in the Glockengasse in Cologne. Following

a decree of 1794 by the French occupants of Cologne all of the 7440 houses of the city were numbered and the house of *Mülhens* was given number 4711, which is the origin of the still used commercial name *Eau de Cologne 4711* [1.19]. It is said that *Napoleon* was very lavish with *Eau de Cologne*, using about two bottles a day, so he took large quantities with him in his campaigns.

The preparation of extracts of natural products, especially strong smelling flowers, expanded from the monasteries to regions of abundant starting materials, especially in the town of Grasse in Southern France where it developed from laboratory quantities to industrial dimensions. In 1768, *Antoine Chiris* founded the first of these factories in Grasse making extracts from natural products that were used for the composition of perfumes. Paris became the center of creation and a number of specialized boutiques opened their commerce. One of the first were *Jean Francois Houbigant* in 1775 as well as *Michael Adams* whose establishment *La Reine de Fleur* later became the famous *House of Piver*. *Pierre-Francois Lubin* created the famous *Eau de Lubin* around 1790. *Napoleon*, in addition to his campaigns to conquer Europe also changed the judicial system in his native France. The Code Napoleon, introduced in 1804, is still the base of law in France. One of his decrees of 1810 defines the term *Perfume* in which a perfume had to be distinguished from products for pharmaceutical use and no longer be used for internal applications. Composition of products for medicinal applications had to be declared whereas the formula for perfumes remained a company secret, encouraging the foundation of several perfumery companies, one of which was created by *Pierre-Francois Guerlain*, a Frenchman who had studied chemistry and pharmacy in England. He opened his store as *parfumeur-vinaigreur* in 1828 in the Rue Rivoli in Paris [1.20], creating products that were much in fashion at the Parisian societies. Trends of the time were mainly reconstitutions of flower scents and compositions thereof [1.21] *Guerlains* first successes such as *Senteur de Champs* and *Esprit de Fleurs* were compositions of light floral themes, heavy flower, and animalic notes were not in fashion [1.21]. The so-called soliflores [1.22], reconstitutions of single flower scents appeared and dominated the market for a while, just to mention *Rose* and *Jasmin* by *Molinard* (1860), *Rose Jaqueminot* and *Jasmin de Corse* by *Coty* (1906), *Violette Pourpre* by *Houbigant* (1907), *Narcisse Noir* by *Caron* (1912), *Gardenia* by *Chanel* (1925), and *Le Muguet de Bois* by *Coty* (1942) [1.23]. All these creations were compositions of different extracts of natural products already made by industrial procedures.

1.3.4 The Advent of Organic Chemistry and Its Contribution to Perfumery

The beginning of organic chemistry is generally considered *Friedrich Wöhler's* publication of the conversion of ammonium cyanate into urea, a synthesis of an organic molecule found in living organisms from an inorganic salt [1.24], even if he had, already 4 years earlier, synthesized oxalic acid by hydrolysis of dicyane [1.25]. These discoveries initiated the abandon of the *vitalism*, a philosophy believing that organic molecules could only be synthesized – or better created – by living organisms, even if it still took a while to be recognized as not valid.

What are the connections between these discoveries and modern perfumery? As described earlier, creation of perfumes consisted of the skillful composition of different extracts of odorous natural extracts. The nascent organic chemistry enabled scientists to analyze the composition of natural products, identify the molecules of odor impact, and subsequently synthesize those for the use in perfume compositions. This statement is of course an extreme abbreviation of these developments which took a long time and is still going on today as one of the main branches of research in perfumery science.

Even before *Wöhler's* discoveries systematic analyses were executed on natural products. *Jaques-Julien Houton de Labadière* showed that turpentile oil consisted only of carbon and hydrogen and that the atomic proportions of these were 5 to 8 [1.26]. Later, compounds of natural origin having these elementary proportions were called *terpenes*. The French scientist and later agricultural minister *Dumas* developed methods that helped to separate natural extracts into simple hydrocarbons and into their derivatives containing sulfur, nitrogen, and/or oxygen. However, analytical methods only allowed isolating and identifying compounds that occurred in larger quantities in the natural extracts. As an example, *Buignet* described in an extensive publication a series of analyses to distinguish between different species of strawberries [1.27]; the volatile part that imparts the odor of these fruits is mentioned in only some sentences concluding that the odor is due to volatile materials that occur in very small quantities and that it would need a *very large quantity of starting material to determine the exact chemical nature of these extracts*. This statement is to be considered as very optimistic, even with today's sophisticated methods for the analysis of volatile materials not all trace odorous materials have been identified.

One of the first impact odorants isolated and identified was cinnamaldehyde **1** out of cinnamon oil by *Dumas* and *Peligot* in 1833 [1.28]. Benzaldehyde **2**, the

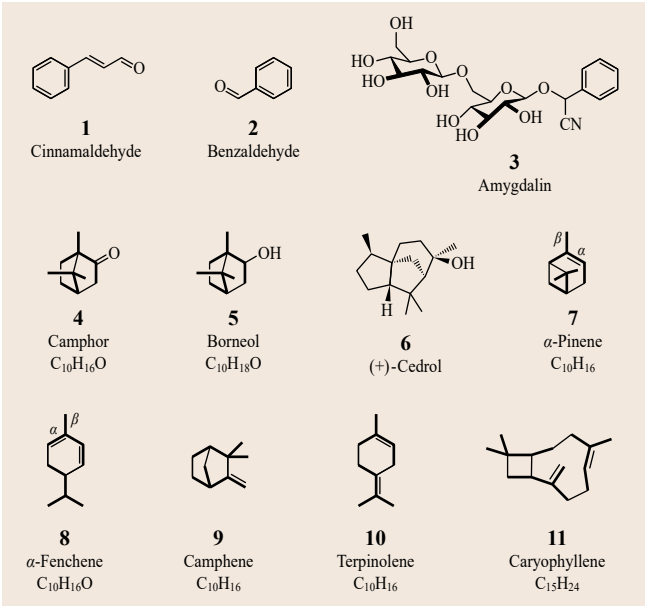

Fig. 1.1 Odorants isolated from natural sources and identified in the early times of organic chemistry

odorous principle of bitter almond oil and cherry flavor is to be considered the first synthetic odorous material after *Wöhler* and *Liebig* could show that it results from the enzymatic cleavage of amygdaline **3** that occurs in the stones of fruits like plums, cherries, peaches, and almonds. Analytical techniques improved over time so that odor impact compounds could be isolated from odorous natural products and their chemical structures could be elucidated. The first compounds thus isolated were those that are solid at room temperature and could be separated from their matrix by crystallization such as camphor **4**, borneol **5**, and cedrol **6**. Further improvement of distillation techniques like vacuum and column distillation as well as specific derivatization allowed the isolation and structure elucidation of liquid compounds. Pioneer in these techniques was Otto Wallach who, in the years 1884–1920 published from his laboratory at the University of Göttingen more than 100 papers describing the isolation and identification of many important monoterpene compounds, inter alia, α-pinene **7**, α-fenchene **8**, camphene **9**, terpinolene **10**, as well as some sesquiterpenes like caryophyllene **11** (Fig. 1.1).

1.3.5 Beginning of Modern Perfumery

Modern perfumery however began when these natural products to be used in compositions could be made by other methods than by extracting them from their natural sources. This area began with the synthesis of coumarine **12** by *Perkin* [1.29] in 1868. This product, smelling of freshly cut grass had been isolated earlier from tonka beans by *Wöhler* [1.30]. Improvement of the procedure by *Tiemann* and *Herzfeld* [1.31] made it possible to produce this product on an industrial scale to be used in perfumery. One of the first successful uses of coumarine resulted in a perfume called *Fougère Royal* by *Houbigant*, marking a family of scents called fougère which are still alive in modern perfumery; well-known examples are *Drakkar Noir* in 1982 and *Cool Water* in 1988. Coumarine is still used in about two thirds of all new perfumes. Vanillin **13** is the aromatic principle of vanilla pods in which it occurs at about 2%. Because of the costs of isolation from an already expensive starting material it could not be used in perfumery. The structure of **13** had been elucidated by *Tiemann* and *Haarmann* after they had obtained a crystalline alcohol by treating coniferine with emulsine, a hydrolytic enzyme, followed by oxidation with potassium bicromate in sulfuric acid [1.32]. This method not only allowed to obtain the pure product to determine its chemical structure but could be also considered

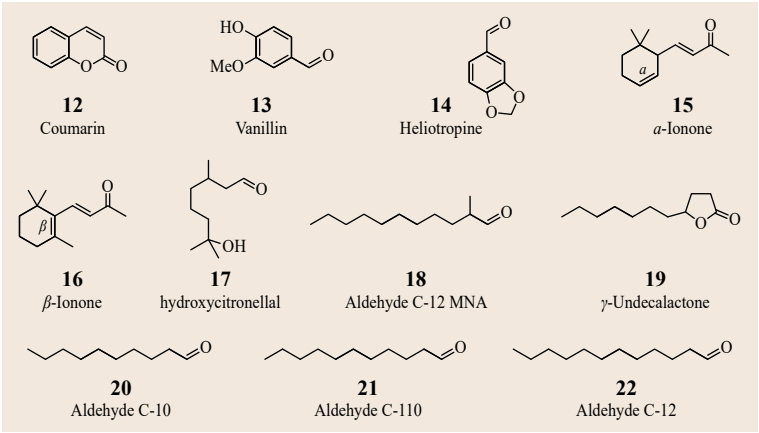

Fig. 1.2 Naturally occuring odorants available through chemical syntheses

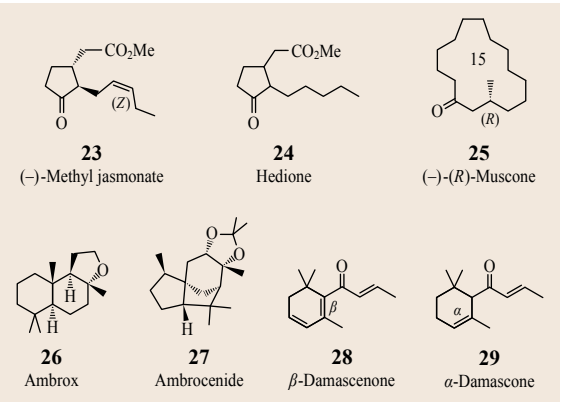

Fig. 1.3 Important natural and non-natural odorants obtained by chemical syntheses

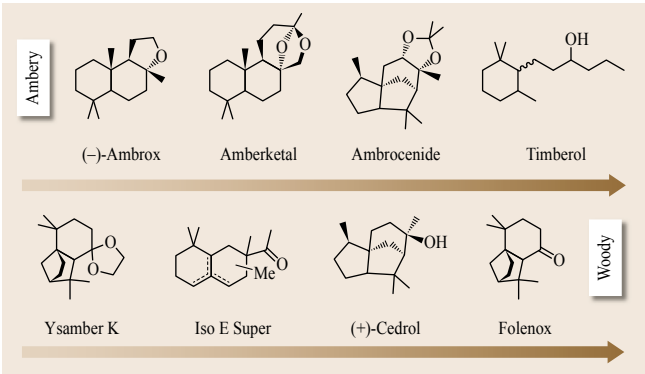

Fig. 1.4 Conversion from ambery to woody notes on the example of eight commercial odorants

as an industrial method. *Haarmann*, who originally came from Holzminden close to the Solling mountain in Germany where pine wood, the starting material for coniferine, is in great abundance, took on this task and founded *Haarmann's Vanillinfabrik* to produce **13**. A completely new synthesis of this product was developed again by *Tiemann* using a reaction discoverd by *Reimer*; this reaction known as the Reimer–Tiemann *Reaction*. Later on *Reimer* joined *Haarmann* to found *the Haarmann & Reimer Corporation* in Holzminden, one of the oldest industrial Flavor & Fragrance Companies. It acts now under the name of *Symrise* after the merger with *Dragoco* also from Holzminden in 2002.

In 1889, the French perfumer *Aimé Guerlin* created the perfume *Jicky* in which he used synthetically produced vanillin **13**, heliotropine **14**, and coumarine **12**. This creation is to be considered as a turning point in perfumery because it was the first time a perfume was not the recreation of a natural product, the so-called soliflores, but it allowed new interpretations of harmonized odor accords not necessarily occurring in nature [1.33]. Extracts of the violet flower were very much appreciated for perfume creation but because of its limited availability too expensive for general use. To identify the smelling principle of this flower, *Tiemann* and *Krüger* extracted the similar smelling but much cheaper orris root oil to discover the ionones **15** and **16** as the odorous principle [1.34]. Their synthesis was straightforward so the material became generally available and is still a basic material for the creation of flowery perfumes.

The scent of the popular lily-of-the-valley flower was very desirable to be obtained but its extraction from the natural source was not possible. In the beginning of the 20th century, three different groups nearly simultaneously succeeded in the synthesis of hydroxycitronellal **17**. *Knoll* in Germany in 1905, *Givaudan* and *Firmenich*, both in Switzerland in 1906 and 1908 respectively marketed this product under different names and qualities because of different byproducts due to their different synthetic procedures that alter the smell. The first significant use was by Houbigant in the famous perfume *Quelques Fleurs* in 1912. *Cylosia*, the product from *Firmenich* is still in popular use for flowery perfumes. Another flowery product not found in nature, aldehyde MNA, methyl nonyl acetaldehyde **18**, was discovered around the same time and is still very much in use. Around 1920, fruity notes became popular after the success of the perfume *Mitsouko* by *Guerlain* who used the so-called aldehyde C-14 which actually is γ-undecalactone **19** and has a very pronounced odor of ripe peach.

A historial trend setting event in modern perfumery was the creation of *Chanel No 5* by *Beaux*. He added to a mixture of flowery notes like ylang ylang, vanilline and musks high amounts of the straight chain aldehydes decanal **20**, undecanal **21**, and dodecanal **22**, which by themselves have a pronounced unpleasant fatty odor of candlewax. This combination became a great success and *Chanel No 5* is still today one of the most appreciated and sold perfumes.

Jasmine flower notes are very important in modern perfumery. Until around 1960, extracts from jasmine flowers were used which were very expensive and could thus be used only in very small quantities in limited applications. The turnaround came after the isolation and identification of methyljasmonate **23** by *Demole* et al. [1.35]. Its synthesis proved to be quite expensive but *Demole* could show that the dihydro derivative **24** had very close odor properties to the natural product, that is its boosting effect to floral notes. The product, called *Hedione* was first introduced by *Roudnitska* in *Eau Sauvage* in 1962. Due to its success and the steady improvement of its synthesis *Hedione* is now available

at a price that allows its use even in applied perfumery for soaps and detergents. It is not exaggerated to say that today *Hedione* is used in every perfume.

Musk odorants are in wide use in perfumery (Fig. 1.2). Analysis of the extracts from the musk deer glands secretion, that was initially used in compositions, allowed *Walbaum* in 1906 to identify a ketone $C_{16}H_{30}O$ which he called muscone as the smelling principle [1.36]. It was *Ruzicka* who 20 years later identified this compound as (−) 3-methylcyclopentanone **25** [1.37]. With this discovery, he overturned *Bayer's* theory of too much strain in carbocyclic rings greater than 12 carbon atoms [1.38] and for which he was awarded the Nobel Price in Chemistry in 1939. A carbocyclic ring ketone smelling of musk was isolated by *Sack* from the glands of the civet cat and identified by *Ruzicka* as cycloheptadecanone [1.39]. Another musk-smelling compound was isolated by *Kerschbaum* from ambrette seed oil and identified as 7-*cis*-hexadecen-16-olide [1.40]. Numerous macrocyclic and noncyclic compounds exhibiting a musk odor have since been discovered and are in use in modern perfumery [1.41].

Ambra notes are appreciated in perfumery. The first product to be used was ambergris, a mixture extracted from a pathological metabolite from the sperm whale which floats on the surface of the oceans after excretion. This extract contains a number of terpenoic structures that are degradation products from the triterpene ambreine, discovered by *Lederer* [1.42]. The high interest in amber notes initiated research into the discovery of amber-smelling compounds from other sources. Among the several synthetic compounds available today for perfume creation are Ambrox **26**, Cetalox, which is racemic **26** and the extremely powerful Ambrocenide **27** [1.43].

Woody notes are much used in perfumery. A stepwise conversion from strong amber, slightly woody to slightly amber-strong woody has been established by *Pickenhagen* using a series of different chemicals used in perfumery [1.44] (Fig. 1.4).

The invention of gas chromatography by *James* and *Martin* in 1952 [1.45] and its systematic application allowed the isolation and structure elucidation of strong smelling compounds that occur only in traces in nature. An example with wide ramifications for perfumery is the discovery of an important odorant in Bulgarian rose oil (*rosa damascena* Mill) by *Demole* et al. when they isolated a compound by preparative gas chromatography and determined its structure as 1-(2,6,6-trimethylcyclohexa-1,3-dienyl) but-2-en-1one **28**, which was named β-damascenone [1.46]. Synthetic work around this structure showed that the isomer α-damascone **29** is from a perfumistic standpoint the most interesting of different isomers tested. It is interesting to note that this product had been synthesized in another context before by *Ohloff* and *Uhde* [1.47] but its perfumistic value had not been recognized. The use of **29** led to a series of new types of perfumes of which *Poison* by *Dior* with an extremely high amount of 0.04% was the most successful trendsetter (Fig. 1.3).

Further improvement of gas chromatography and its hyphenation with analytical methods like mass-spectrometry and nuclear magnetic resonance spectroscopy allowed the isolation and identification of a plethora of new natural substances, many of them are now used as synthetic materials in perfumery. Today, the perfumer has for the creation of new and original perfumes an arsenal of about 3000 commercially available compounds at his disposal, most of them of synthetic origin. In the future, many more will be discovered and used, confirming the prediction of *Ernest Beaux*, the creator of the famous Channel No. 5 who stated [1.48]:

> *the future of perfumery is in the hands of chemists. We will rely on them to find new odorants to make original new accords.*

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Part A

Molecular

Part A Molecular Aspects and Formation Pathways

Odorous compounds are typically volatile or semi-volatile in nature and have low molecular weight, the majority being below 300 u. Despite this apparently limited range, odorous stimuli belong to a broad variety of substance classes that comprise diverse structural moieties such as ester, carbonyl, alcohol functions, bearing thio and other heteroatomic groups, having aromatic or aliphatic forms, with the potential of incorporating heteroatomic constituents in the aromatic structures. Odorants may be even-chained or branched, may be saturated or exhibit different degrees of unsaturation, and can contain double bonds or steric centers that can dictate the odor character and potency according to their steric properties. Even minimal differences can induce altered smell properties, as is encountered between certain enantiomers of the same molecule, thereby requiring that the exact structure and its formation pathways must be thoroughly resolved in order to be fully understood. Moreover, the complexity of possible combinations may be increased further by the combination and formation reactions of different structural moieties within one molecule.

A range of pathways have evolved in nature for communication purposes. The transmission of potent smells can elicit specific responses in recipients, and conversely, the latter have developed the means to detect these as signaling molecules. Odors generated and released in the plant kingdom, for instance, can serve as cues to insects and animals; fruity odors indicate a source of carbohydrate energy, and floral scents act as pollinator attractants. Animal-like smells can also induce specific responses in certain species, e.g., in conspecifics or in predator–prey relationships, and cell damage in plants, e.g., by herbivore feeding, may generate volatile compounds as a by-product of the plant's defense mechanism, which might further serve as a distress signal and attract predators of the insects damaging the plant. Apart from these biochemically driven routes, odorants may be also generated by predominantly chemical pathways. Thermal and pyrolytic processes or oxidation are amongst such reaction cascades that are often highly complex. We savor such aromas in baked and roasted foods, but specific burnt impressions tell us that heat treatment was too intense. Often, there is a complex interplay between several biochemical and chemical pathways, further increasing the number and combinatory complexity of odorous molecules being formed. This book section comprises contributions that compile numerous substance classes and their structural specifics, primarily in view of those that are of high importance to humans. Moreover, current knowledge of the most prominent pathways involved in their formation is provided, linking different mechanistic routes to present a fundamental understanding of how odorants come to exist in our world.

2. Biosynthesis of Plant-Derived Odorants

Matthias Wüst

Plants produce thousands of structurally diverse volatile signal compounds to attract pollinating insects and seed dispersing animals. These compounds are often perceived by humans as a specific fruit or vegetable aroma. Many of these volatiles serve also as defense substances against fungi, bacteria, viruses, and herbivores. The knowledge of precursors and pathways leading to the formation of volatiles in fruits and vegetables has considerably progressed during the last years because of the use of molecular and biochemical techniques. In vitro characterization of the heterologously expressed enzymes has helped clarify the pathways of volatile formation. This chapter will, therefore, provide an overview of biosynthetic sequences and construction mechanisms that are illustrated in most cases using detailed reaction schemes. The various compounds are predominantly ordered according to the biosynthetic pathway that is used in plants to synthesize them and are grouped into carbohydrate-, lipid-, and amino-acid-derived odorants, terpenoids, and glycosidically bound odorants.

2.1 Biosynthesis of Plant-Derived Odorants

The following chapter gives an overview on the biosynthesis of odorants, the metabolic sequences leading to various selected classes of plant natural products that can be perceived by humans due to their volatility and odor activity. Various odorants are predominantly ordered according to the biosynthetic pathway that is used in planta to synthesize them – a concept that has been adopted from several successful textbooks on natural product chemistry and biochemistry [2.1, 2]. Special emphasis was put on biosynthetic sequences and construction mechanisms that are illustrated in most cases using detailed reaction schemes. The molecular biology of the enzymes that catalyze these reactions, plant physiological aspects, and genetic engineering of biosynthetic pathways are clearly not within the scope of this chapter. Nevertheless, where appropriate, some remarks and references on these topics are given in the text, which allow the interested reader to gain more insight into these quickly developing fields of research.

2.1.1 Biological Functions of Plant-Derived Odorants

Plants produce thousands of structurally diverse volatile signal compounds to attract pollinating insects and seed dispersing animals and mediate interactions with other plants. Many of these volatiles serve also as defense substances against fungi, bacteria, viruses, and herbivores and are stored in specialized tissues like glandular trichomes or oil ducts [2.6]. Indeed, from a plant's point of view, human beings are nothing else than seed dispersers or herbivores that are able to perceive these compounds as a specific fruit or vegetable aroma. More recent studies have shown that some of these volatiles

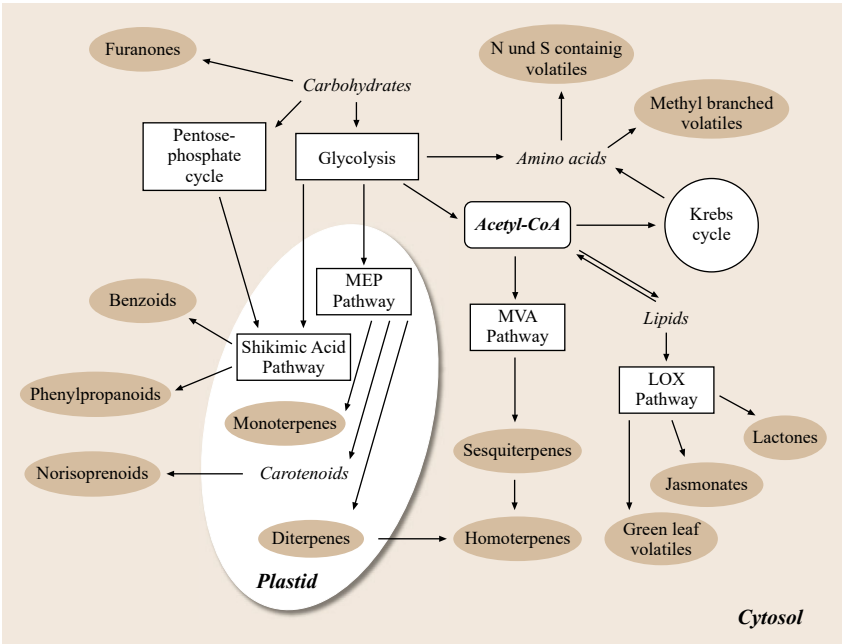

Fig. 2.1 Metabolic pathways leading to the biosynthesis of plant-derived odorants (after [2.3]). MEP: methylerythritol phosphate; LOX: lipoxygenase; MVA: mevalonic acid; CoA: coenzyme A

Table 2.1 Groups of plant secondary metabolites (after [2.5])

Type of secondary metabolite[a]	Approximate number of known structures[b]
Alkaloids	21 000
Terpenoids, all classes	22 000
Monoterpenes (C_{10})	2500
Sesquiterpenes (C_{15})	5000
Diterpenes (C20)	2500
Triterpenes, steroids, saponins (C_{30}, C_{27})	5000
Tetraterpenes (C40)	500
Non-protein amino acids	700
Amines	100
Cyanogenic glycosides	60
Glucosinolates	100
Alkamides	150
Lectins, peptides, polypeptides	2000
Flavonoids, tannins	5000
Phenylpropanoids, lignin, coumarins, lignans	2000
Polyacetylenes, *fatty acids*, waxes	1500
Polyketides	750
Carbohydrates, organic acids	200

[a]Types in **bold** contain a large number of volatiles; types in *italic* serve as precursors for volatiles
[b]The Dictionary of Natural Products on DVD [2.4] contains more than 230 000 compounds contained in over 68 000 entries. An entry may contain variants and derivatives. Variants may include stereoisomers, for example (*R*)-form, *endo*-form; members of a series of natural products with closely related structures such as antibiotic complexes. Derivatives may include hydrates, complexes, salts, classical organic derivatives, substitution products and oxidation products etc.

also function as signal molecules in tritrophic plant-herbivore–carnivore interactions. The emitted volatiles may attract the herbivore's enemies, such as parasitoids or predators, which actively reduce the number of feeding herbivores [2.7]. This has often been termed as *plant's cry for help*.

Table 2.2 Common abbreviations for cofactors and substrates

ACP	Acyl carrier protein
ADP	Adenosine diphosphat
AMP	Adenosine monophosphat
Ara	Arabinose
ATP	Adenosine triphosphate
B	General base
CDP	Cytidine diphosphate
CMP	Cytidine monophosphate
CoA	Coenzyme A in structures
CoQ	Coenzyme Q (ubiquinone)
CTP	Cytidine triphosphat
Cys	Cysteine
Cyt	Cytochrome
DMAPP	Dimethylallyl diphosphate (pyrophosphate)
Enz	Enzyme
FAD	Flavin adenine dinucleotide (oxidized form)
FADH2	Flavin adenine dinucleotide (reduced form)
FMN	Flavin mononucleotide (oxidized form)
FMNH2	Flavin mononucleotide (reduced form)
FPP	Farnesyl diphosphate (pyrophosphate)
Fru	Fructose
Gal	Galactose
GF	Green fluorescent proteine
GFPP	Geranylfarnesyl diphosphate (pyrophosphate)
GGP	Geranylgeranyl diphosphate (pyrophosphate)
Glc	Glucose
Gln	Glutamine
Glu	Glutamate
GPP	Geranyl diphosphate (pyrophosphate)
GSH	Reduced glutathion
GSSG	Oxidized glutathione
HA	General acid
HMG-CoA	Beta-hydroxy-beta-methylglutaryl coenzyme A
HSCoA	Coenzyme A
IPP	Isopentenyl diphosphate (pyrophosphate)
Met	Methionine
NAD	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NPP	Neryl diphosphate (pyrophosphate)
P_i	Inorganic orthophosphate
P	Phosphate in structure
PEP	Phosphoenolpyruvate
PLP	Pyridoxal phosphate
PP_i	Inorganic pyrophosphate
PP	Pyrophosphate in structure
Rha	Rhamnose
SAM	*S*-adenosyl methionine
TPP	Thiamine pyrophosphate
UDP	Uridine diphosphate
UDP-gal	Uridine diphosphate galactose
UDP-gluc	Uridine diphosphate glucose
UMP	Uridine monophosphate
UTP	Uridine triphosphate
Xyl	Xylose

2.1.2 Primary and Secondary Metabolism

Most of the odorants described in this chapter are so-called secondary metabolites derived from building blocks that are generated by the fundamental processes in primary metabolism like glycolysis, Krebs cycle, pentose phosphate cycle, and others. These fundamental reaction sequences are not shown in detail in this chapter with the exception of the methylerythritol phosphate (MEP) pathway, which is surprisingly often not yet discussed in general biochemistry textbooks. As opposed to plant primary metabolites, plant secondary metabolites occur in a very high structural diversity as illustrated in Table 2.1. Several structural types of secondary metabolites are volatile as such (monoterpenes), while others serve as precursors that generate volatiles after enzymatic modification or degradation (glucosinolates). Despite this diversity, the number of corresponding biosynthetic pathways is surprisingly rather restricted. Figure 2.1 gives an overview on the metabolic pathways, leading to the biosynthesis of the various groups of volatile compounds that are shown in gray-shaded ovals and that are discussed in this chapter.

In the following presented reaction schemes, nonionized acids are usually depicted to simplify structures and to eliminate the need for counter ions. Likewise, amino acids are shown in unionized form, and not as zwitterions. The abbreviations **P** and **PP** represent the unionized phosphate and diphosphate group, respectively. Further common abbreviations for cofactors and certain substrates in enzymatic reactions that are used throughout the text are shown in Table 2.2.

2.2 Constitutive Biosynthetic Pathways

2.2.1 Carbohydrate-Derived Odorants

Carbohydrate-derived odorants, where the carbohydrate molecule is a direct precursor and is incorporated with the retention of the carbon chain without prior degradation, are very rare. The most prominent examples are furanones like furaneol (4-hydroxy-2,5-dimethyl-3(2*H*)-furanone – HDMF) and methoxyfuraneol (2,5-dimethyl-4-methoxy-3(2*H*)-furanone – DMMF) that are key aroma compounds in strawberry and pineapple fruits. HDMF and DMMF occur as natural racemic mixtures due to their oxo-enol structure and the resulting keto-enol tautomerism. The enantiomers smell different with either caramel-like to fruity-sweet odor notes ((*R*)-(+) enantiomers) or extremely weak to lactone-, coumarin-like ((*S*)-(−) enantiomers) [2.8]. D-Fructose-1,6-diphosphate is the biogenetic precursor of these substances and delivers the carbon chain of HDMF and DMMF [2.9]. The biosynthesis of both molecules is outlined in Fig. 2.2. The reaction sequence includes the intermediate 4-hydroxy-5-methyl-2-methylen-3(2*H*)-furanone (HMMF), and the products 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) and 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (DMMF). The enzymes so far characterized are Fragaria x ananassa enon oxidoreductase (FaEO) and Fragaria x ananassa *O*-methyltransferase (FaOMT).

Hexose diphosphate is converted by an as-yet unknown enzyme to 4-hydroxy-5-methyl-2-methylen-3(2*H*)-furanone (HMMF), which serves as a substrate for an enone oxidoreductase (FaEO), yielding furaneol [2.11, 12]. An *O*-methyltransferase converts furaneol to finally yield methoxyfuraneol [2.13]. It is interesting to note that HDMF is also generated in heated foods by a purely chemical reaction in the course of the Maillard reaction, and it has been suggested that this results in these foods appearing particularly attractive [2.14].

2.2.2 Terpenoids

Many of the compounds in plant-derived essential oils are biosynthesized from the *active isopren* C_5 units dimethylallyl diphosphate (DMAPP) and isopentenyl

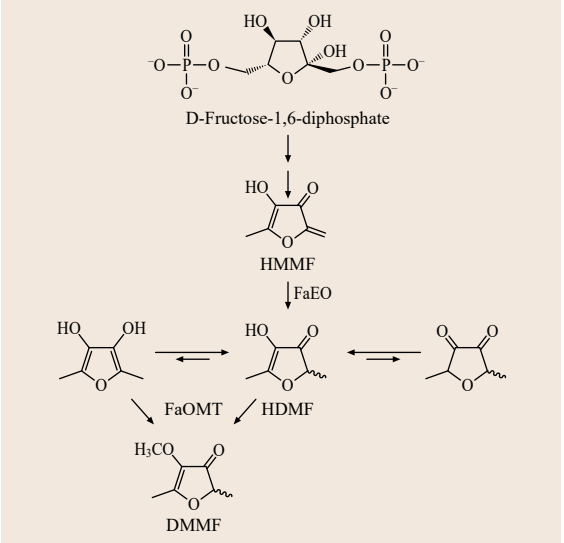

Fig. 2.2 Biosynthesis of the 3(2*H*)-furanones in strawberry (after [2.10])

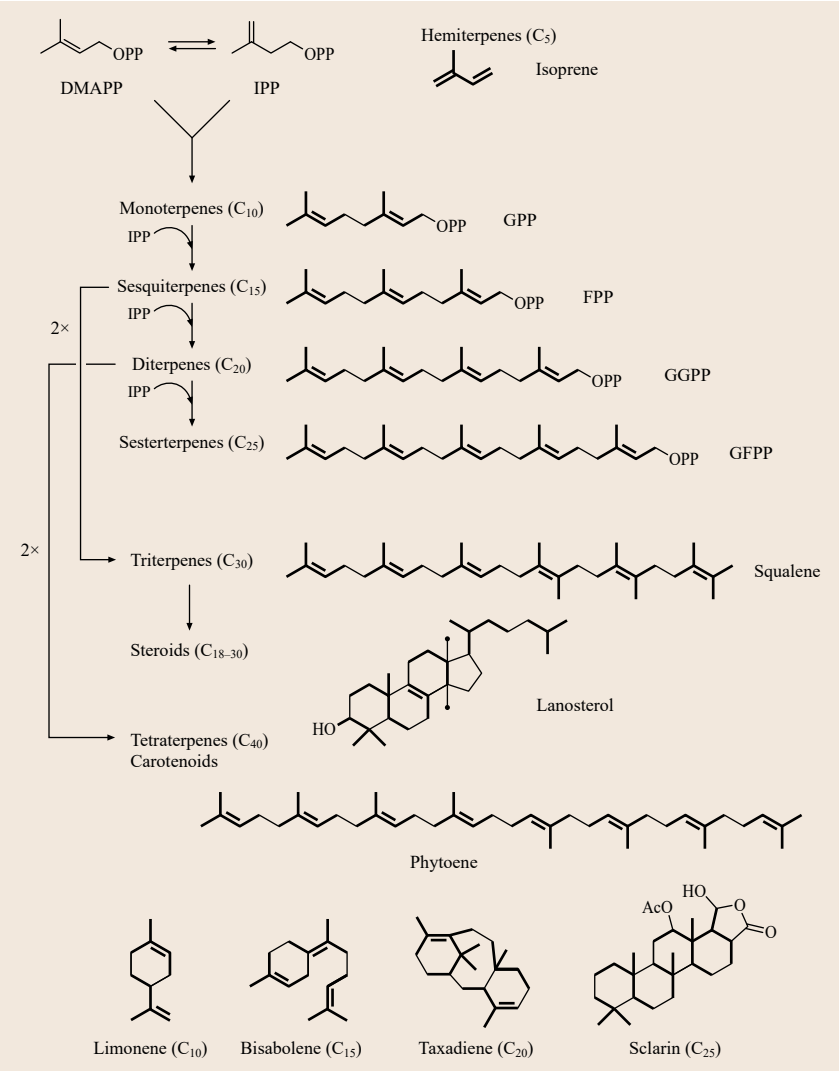

Fig. 2.3 Overview of the different terpenoid skeletons generated by head to tail addition of isoprene units. For each group the structure of a typical member is given. During the biosynthesis of lanosterol two Wagner–Meerwein rearrangements are observed so that two isoprene units are disassembled

diphosphate (IPP) and, thus, belong to the most diverse families of natural products – the terpenoids [2.15], also referred to as isoprenoids (Table 2.1). Essential oil-producing plants, among them are many herbs and citrus fruits, accumulate terpenoids in specialized tissues, such as glandular trichomes, oil ducts, or secretory cavities [2.16]. Terpenoids in low concentrations are also found in fruits and vegetables that do not accumulate essential oils. Nevertheless, they often contribute to the specific fruit and vegetable aroma due to their low-odor threshold values [2.17]. Terpenoids are classified according to the number of incorporated C_5-units and are distinguished into hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}, triterpenes (C_{30}), and tertraterpenes (C_{40}) (Fig. 2.3). Most of the volatile terpenoids are mono- and sesquiterpenes with only few diterpenes. The biogenetic precursor for all monoterpenes is geranyl diphosphate and for all sesquiterpenes (*E*, *E*)-farnesyl diphosphate, although exceptions from these rules have been described recently for the biosynthesis of terpenes in glandular trichomes of tomato [2.20]. Formally, all terpenoids are obtained by a head to tail addition of isoprene units (biogenetic isoprene roule by Ružička; 1939 Noble Prize winner in Chemistry). The biogenetic precursors of these isoprene units are IPP and DMAPP that are substrates for short-chain prenyltransferases, which produce geranyl diphosphate (GPP), farnesyl diphosphate (FPP), geranyl geranyl diphosphate (GGPP), and geranyl farnesyl diphosphate (GFPP) (Fig. 2.3) [2.21]. In plants, two independent routes are employed for the biosynthesis of IPP and DMAPP: the well-known

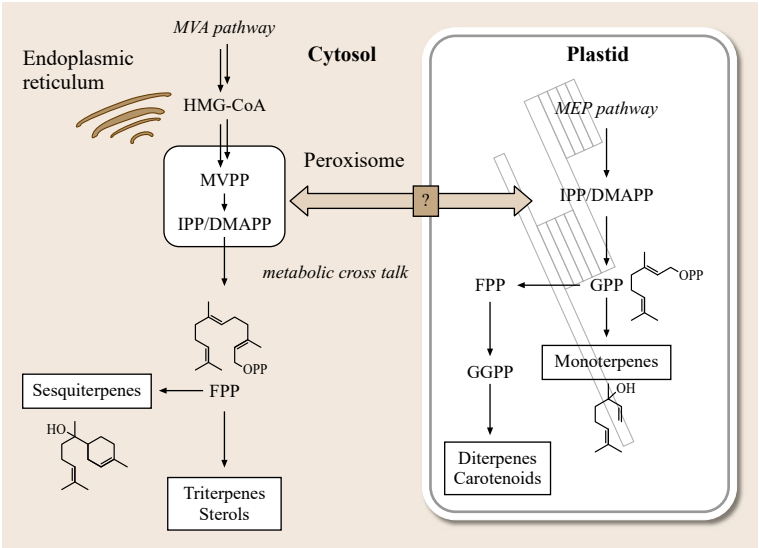

Fig. 2.4 Simplified scheme for the compartmentalization of terpene biosynthesis in higher plants. Metabolic cross talk is mediated by a yet unidentified metabolite transporter (after [2.18, 19]). IPP: isopentenyl diphosphate; DMAPP: dimethylallyl diphosphate; FPP: farnesyl diphosphate; GGPP: geranylgeranyl diphosphate; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; MVPP: 5-diphosphomevalonat

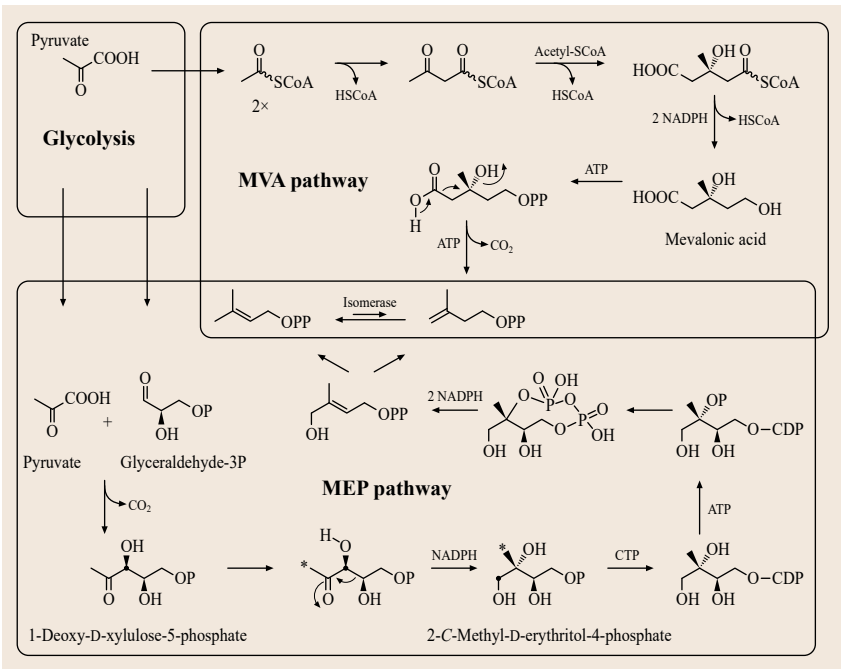

Fig. 2.5 Biosynthetic pathways to IPP/DMAPP in higher plants

cytosolic/peroxisomal mevalonic acid (MVA) pathway and the newly discovered plastidial methylerythritol phosphate (MEP) pathway [2.22].

In plant cells, both pathways are localized in different compartments. However, this compartmentalization is not absolute and some intermediates can be exchanged across the plastid membrane – a phenomenon that has been termed *metabolic cross talk* (Fig. 2.4). Insightful experiments with isotope labeled pathway-specific precursors and green fluorescent protein (GFP) labeled terpene synthases (TPS) have shown that the biosynthesis of monoterpenes is located in plastids and fueled by the MEP pathway, whereas the biosynthesis of sesquiterpenes is located in the cyctosol and is fueled by the MVA pathway and under certain conditions also by the MEP pathway via the above-mentioned metabolic crosstalk [2.23]. Exceptions from these rules could be demonstrated by feeding experiments using deuterium-labeled precursors and/or enzyme localization experiments using GFP fusion techniques for the

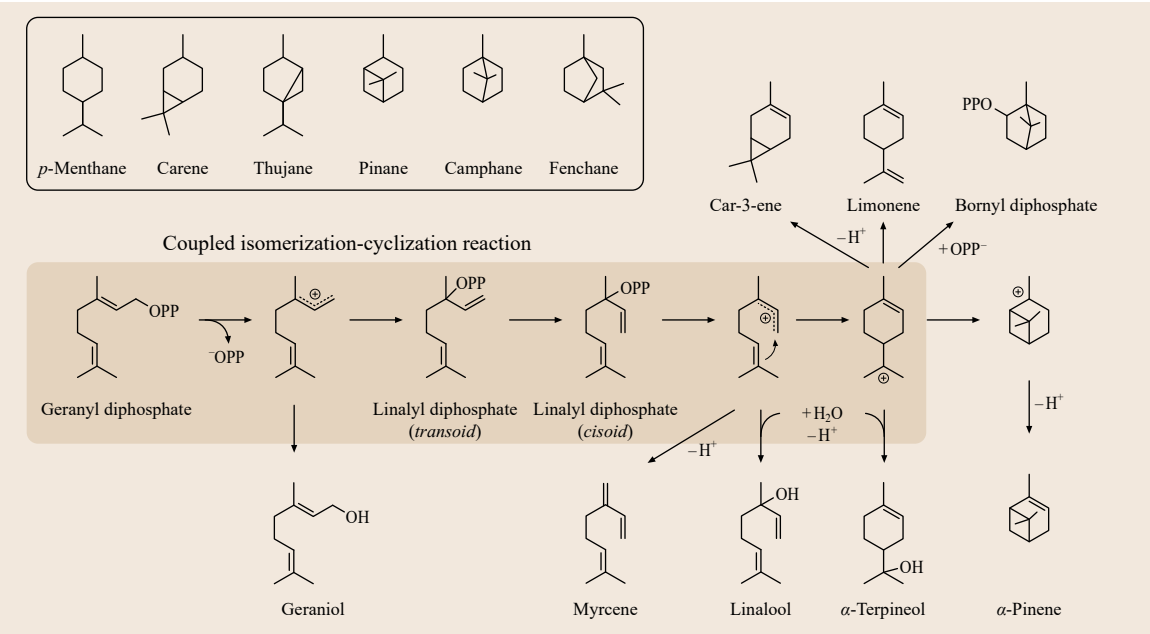

Fig. 2.6 Biosynthesis of acyclic (geraniol, linalool, myrcene), monocyclic (limonene, α-terpineol) and bicyclic (car-3-ene, α-pinene, bornyl diphosphate) monoterpenes from geranyl diphosphate. Cyclic monoterpenes are generated by TPS that share a coupled isomerization–cyclization reaction sequence. Carbon skeletons that are often found in plant-derived monoterpenes are shown in a box in the *upper left corner* of the figure

biosynthesis of monoterpenes in raspberry and strawberry fruits [2.24–26].

Figure 2.5 shows the reaction sequences of both pathways. The reduction of HMG-CoA to MVA has the highest degree of control over the metabolic flux through the MVA pathway. For the MEP pathway, it is now generally accepted that 1-deoxy-D-xylulose-5-phosphate synthase and 1-hydroxy-2-methyl-2-butenyl-4-diphosphate reductase, and to some extent 1-deoxy-D-xylulose-5-phosphate reductoisomerase, are the key enzymes that control flux [2.27].

Monoterpenes

Monoterpenes are generated by terpene synthases (TPS) that are capable of generating acyclic, monocyclic, and bicyclic products. The TPS gene family is a mid-size family, with gene numbers ranging from approximately 20 to 150 in sequenced plant genomes [2.28]. X-ray crystal structures of a limonene synthase from *Mentha spicata* [2.29] and a bornyl diphosphate synthase from *Salvia officinalis* [2.30] are now available and deliver a detailed picture of their reaction mechanisms. The reaction of all TPS starts with the stereoselective binding of GPP (or in some cases of NPP) at the active site followed by metal–ion-dependent ionization of the diphosphate ester to generate linalyl diphosphate (Fig. 2.6). A second ionization yields finally the universal monocyclic intermediate, the α-terpinyl cation, which can further react by deprotonation or nucleophilic capture of water following hydride shifts and other rearrangements. The formation of the acyclic monoterpenes geraniol, linalool, and myrcene might proceed either via the geranyl cation or via the linalyl cation.

The generated monoterpenes can be further modified by, for example, hydroxylations that are often mediated by cytochrome P450 monooxygenases [2.31]. The biosynthesis of the *p*-menthane-type monoterpenes menthone and carvone in glandular trichomes of peppermint and spearmint, respectively, is exceptionally well investigated and all enzymes of the respective pathways have been cloned and characterized (Fig. 2.7), which has facilitated the development of metabolic engineering strategies [2.32]. The regio-selectivity of the different cyctochrome P450 monooxygenases plays thereby a key role and determines which carbon atom at the limonene precursor is hydroxylated [2.33].

Sesquiterpenes

The diversity of sesquiterpene skeletal types is considerably greater than that of monoterpene types due to more double bonds and the longer, more flexi-

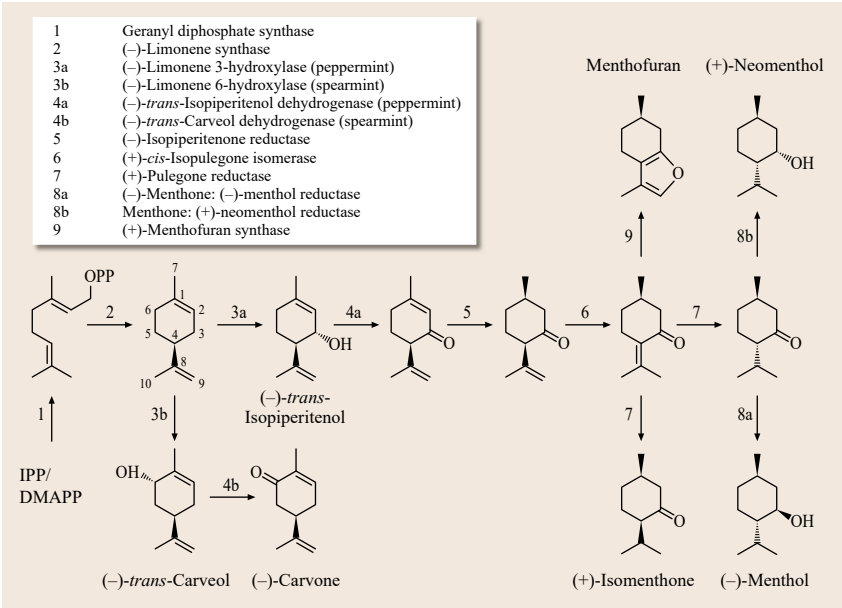

Fig. 2.7 Biosynthesis of (−)-carvone and and (−)-menthol in spearmint and peppermint, respectively

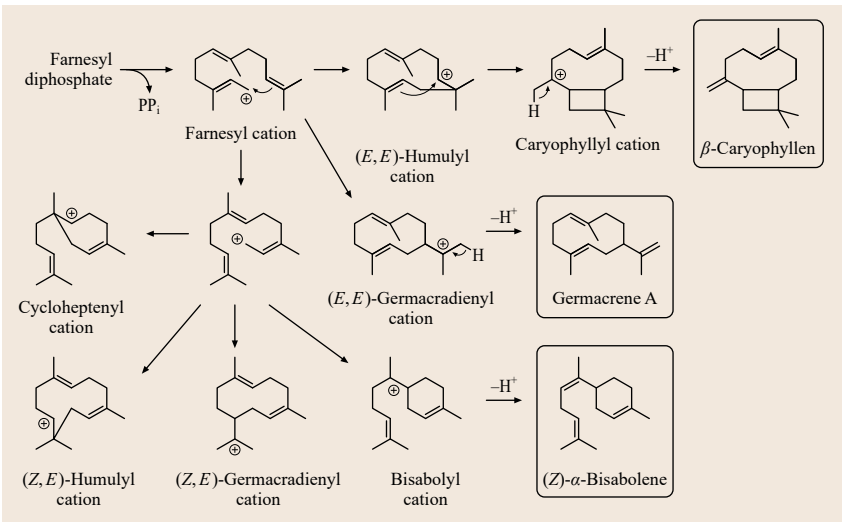

Fig. 2.8 A scheme of representative sesquiterpene cyclization reactions leading to volatile sesquiterpene hydrocarbons

ble chain in FPP. The cyclizations that are catalyzed by TPS as well may occur from the distal double bond to generate either the (*E*,*E*)-humulyl cation or the (*E*,*E*)-germacradienyl cation. Isomerization to the nerolidyl diphosphate permits the formation of the bisabolyl cation, the cycloheptanyl cation, the (*Z*,*E*)-germacradienyl cation, and the (*Z*,*E*)-humulyl-cation (Fig. 2.8) [2.34]. Further cyclizations, hydride shifts, methyl migrations, and/or Wagner–Meerwein rearrangements may occur prior to the termination of the reaction by deprotonation or nucleophile capture, thus producing a vast number of sesquiterpenoid carbon skeletons (over 80 skeletal types are currently known and used for structural sesquiterpene classification [2.35, 36]). One of the most unique traits of this enzyme class is their ability to convert FPP to diverse products during different reaction cycles. This property is found in nearly half of all characterized monoterpene and sesquiterpene synthases and may be attributed to the fact that the various reactive carbocationic intermediates can be stabilized in more than one way [2.37]. For example, the humulene synthase of grand fir (Abies grandis) generates 52 different sesquiterpenes [2.38]. Feeding experiments with deuterium-labeled precursors have shown that sesquiterpene biosynthesis in carrot roots and grape berry exocarp is supplied with IPP

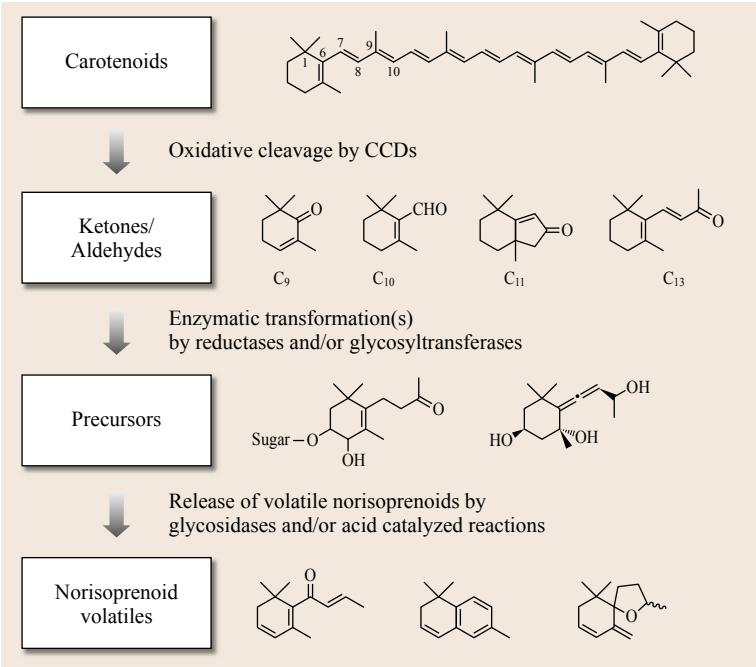

Fig. 2.9 Essential steps for the formation of carotenoid-derived volatiles (after [2.39])

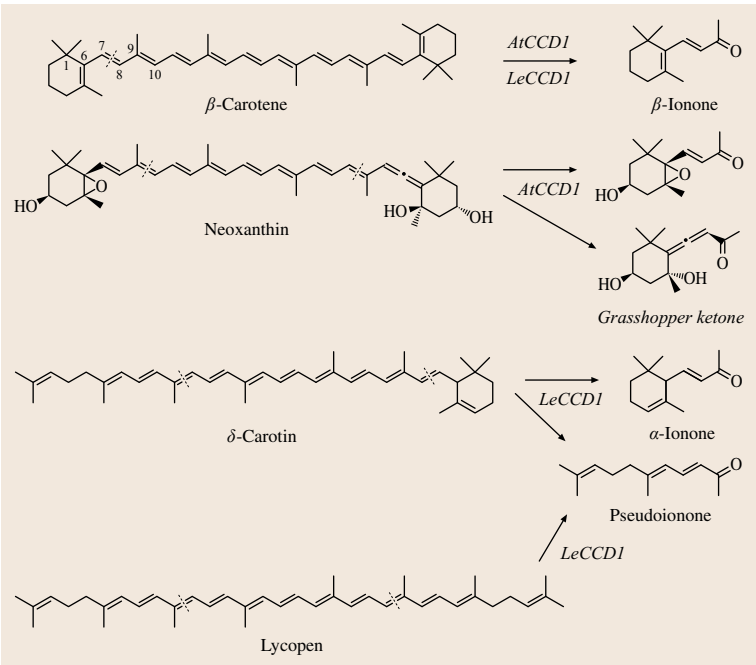

Fig. 2.10 Generation of the primary degradation products from different carotenoids by the action of carotenoid cleavage dioxygenases (CCDs) from tomato (*Solanum lycopersicum* – LeCCD1) and *Arabidopsis thaliana* (AtCCD1)

and DMAPP from both the MEP and the MVA pathway [2.40].

Norisoprenoids

The so-called norisoprenoids are volatiles that are generated from carotenoids by an oxidative cleavage that is followed by further enzymatic and nonenzymatic transformations. In most cases, the generation pathway consists of three essential steps [2.39] (Fig. 2.9). In the first step, the carotenoid is cleaved by the action of a so-called carotenoid cleavage dioxygenase (CCD). In the second step, the cleavage products

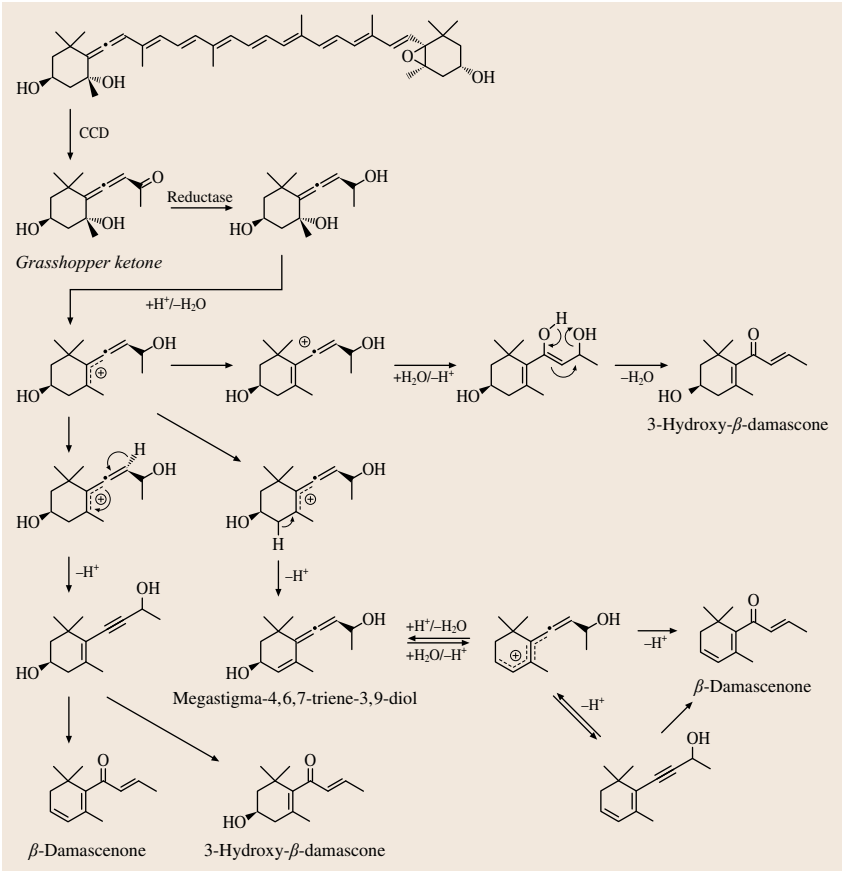

Fig. 2.11 Formation pathways of β-damascenone in grapes and wine starting from neoxanthin (after [2.39])

are further metabolized mainly by reductases and/or glucosyltransferases. In the third and last step, the volatile norisoprenoid is finally released by the action of a glycosidase and/or simply by acid catalyzed liberation.

In some cases, volatile norisoprenoids are directly obtained by the oxidative cleavage of carotenoids, α- and β-ionone (Fig. 2.10). In plants, CCDs are expressed in different tissues, such as roots, shoots, leaves, flowers, and fruits. Some of these CCDs show a rather broad spectrum of substrates and are capable of metabolizing structurally quite different carotenoids. CCD1 and CCD4 are the major families of carotenases found in fruits (so far functionally characterized: tomato, melon, grape, citrus, strawberry: CCD1; apple, peach: CCD4) [2.41]. Some reactions catalyzed by the CCD1 from tomato in comparison with the CCD1 from Arabidopsis are shown in Fig. 2.10.

Some of the norisoprenoids are powerful odorants with threshold values in the ppt-range and are, thus, key compounds for the aroma of several fruits and vegetables like tomato and watermelon [2.42]. Especially, C_{13}-norisoprenoids are important aroma contributors in both red and white wines [2.43], and their formation in grapes and wines has been extensively studied in the recent years [2.44]. The quite complex formation pathway of β-damascenone has been recently reviewed [2.45] and is illustrated in Fig. 2.11.

2.2.3 Fatty Acid Derived and Other Lipid-Derived Odorants

Fatty acids are biosynthesized from a plastidic pool of acetyl-CoA generated from pyruvate, the final product of the glycolysis. Fatty acids are stored in plants as triacylglycerides and are liberated by lipases before they act as direct precursors for various volatiles. In particular, the C_{18}-unsaturated fatty acids, linoleic and linolenic acid, are precursors for volatile straight chain alcohols, aldehydes, ketones, acids, esters, and lactones. They are found ubiquitously in the plant kingdom and are formed predominately by three processes: α-oxidaton, β-oxidation, and the lipoxygenase pathway [2.46].

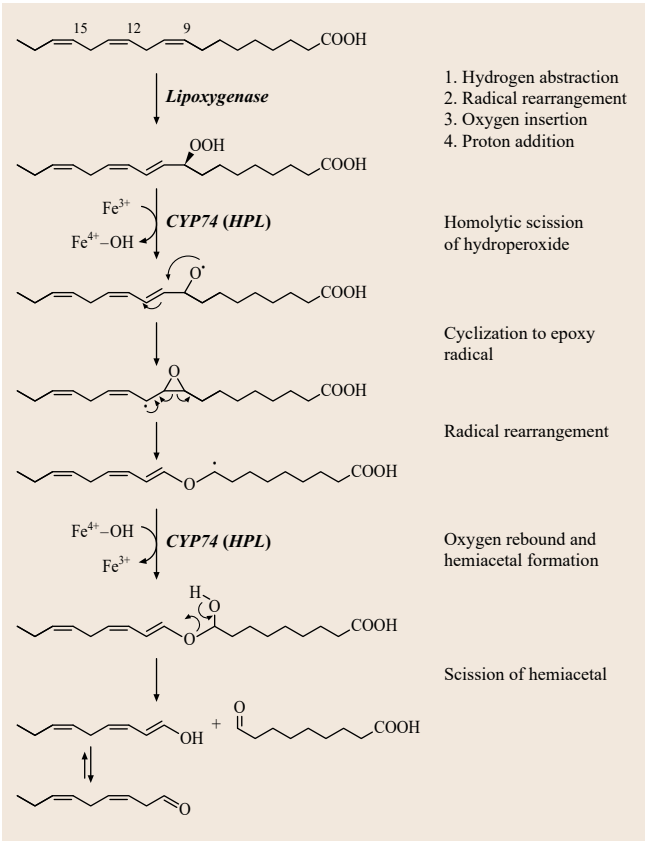

Fig. 2.12 Generation of an aldehyde and a ω-oxo acid from linolenic acid by the consecutive action of a type-1 9-lipoxygenase and a hydroperoxide lyase (HPL) ►

Lipoxygenase Pathway (In-Chain Oxidation)

The lipoxygenase pathyway (LOX pathway) is predominately active in green organs of plants in response to wounding, and it also gives rise to the formation of the so-called *green leaf volatiles* in fruits and vegetables, which are perceived as the characteristic *fresh green* aroma upon preparation in the kitchen or upon mastication. By the action of a nonheme, iron containing dioxygenase, the so-called lipoxygenase (LOX), unsaturated fatty acids are oxygenated regio- and enantioselectively to yield hydroperoxides [2.47]. Prerequisite is the presence of one or more (1*Z*,4*Z*)-pentadienoic moieties to yield the corresponding (1*S*,2*E*,4*Z*)-hydroperoxides that carry the hydroperoxy group either at position 9 or position 13 of the hydrocarbon backbone (9-LOX or 13-LOX) (Fig. 2.13). LOXs can be classified according to their subcellular localization: extraplastidial enzymes are designated as type 1-LOXs (ubiquitous cytosolic type-1 9-LOX and vacuole/lipid body type-1 13-LOX), and plastidial enzymes that harbor a chloroplast transit peptide are designated as type 2-LOXs (type 2-LOXs all belong to date to the subfamily of 13-LOXs) [2.48]. The generated hydroperoxides are substrates for hydroperoxide lyases (HPLs) that are enzymes of the cytochrome P450 family [2.49]. They catalyze in a multistep reaction sequence the homolytic isomerization of fatty acid hydroperoxides into short-lived hemiacetals that yield ultimately short-chain aldehydes and ω-oxo acids as scission products (Fig. 2.12) [2.51].

The short-chain aldehydes are further metabolized by isomerases and/or alcohol dehydrogenases to finally yield the so-called *green leaf volatiles* like (3E)-hexenol in olives [2.52]. Acyltransferases catalyze the formation

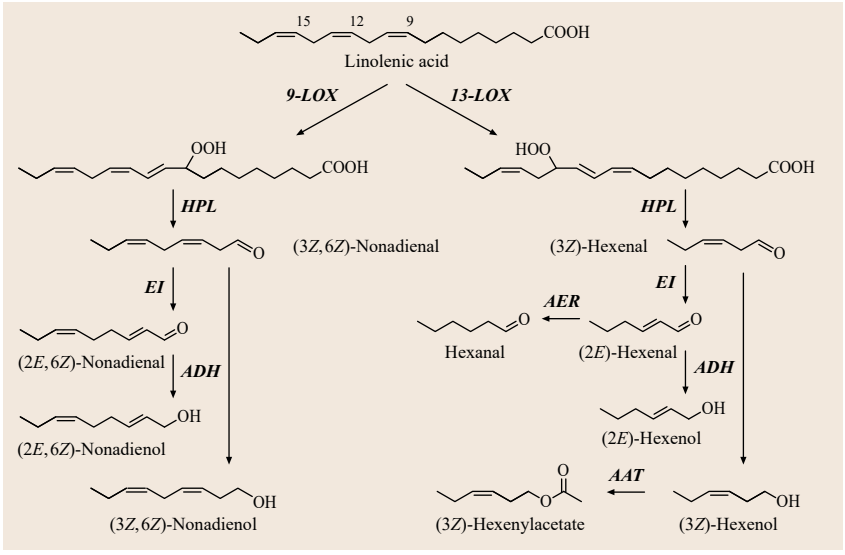

Fig. 2.13 Generation of linolenic acid-derived odorants (LOX: lipoxygenase; HPL: hydroperoxide lyase; EI: enal isomerase; AER: alkenal oxidoreductase; ADH: alcohol dehydrogenase; AAT: acetyl-CoA transferase) (after [2.50])

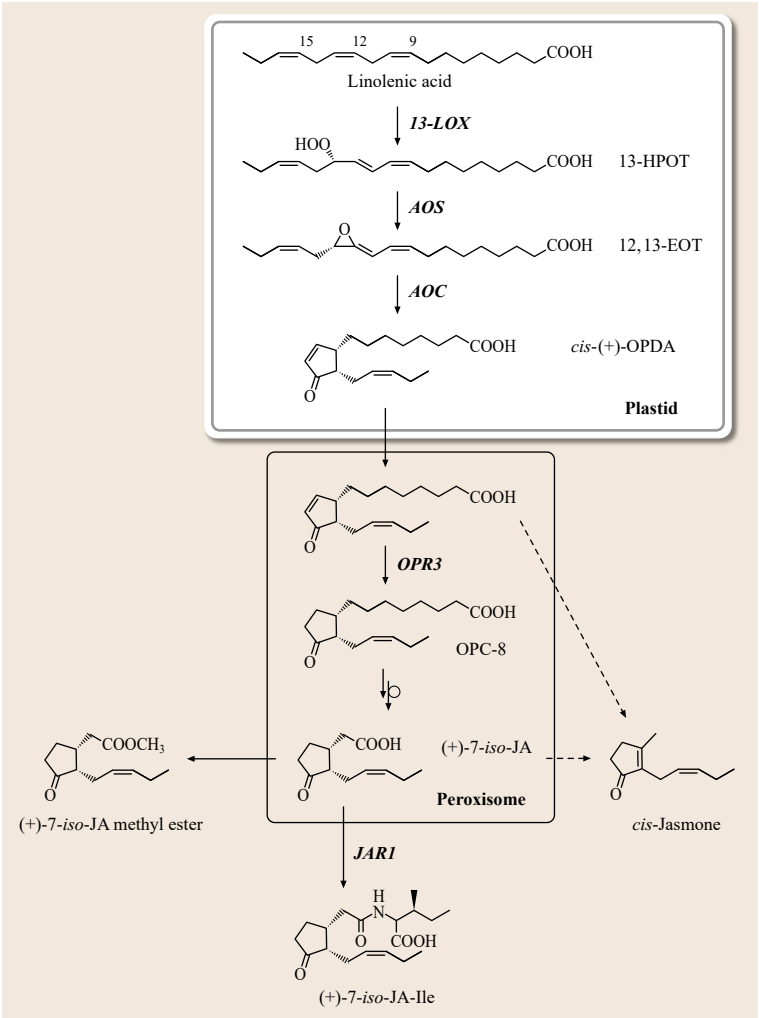

Fig. 2.14 Biosynthesis of jasmonic acid (JA)/ (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) and further metabolites from linolenic acid. The biosynthetic route leading to the formation of *cis*-jasmone is still unclear as indicated by the broken arrows. AOC: allene oxide cyclase; AOS: allene oxide synthase; 12,13-EOT: 12,13(*S*)-epoxy-octadecatrienoic acid; 13-HPOT: (13*S*)-hydroperoxyoctadecatrienoic acid; JAR1: JA-amino acid synthetase; 13-LOX: 13-lipoxygenase; OPR3: OPDA reductase3; OPC-8: 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid; *cis*-(+)-OPDA: *cis*-(+)-12-oxophytodienoic acid

of the corresponding esters and 2-alkenal reductases can reduce (2E)-hexenal to hexanal (Fig. 2.13).

Another branch of the LOX pathway gives rise to the formation of jasmonic acid (JA) and its derivatives [2.53] (Fig. 2.14). The key reaction is catalyzed by an allene oxide synthase that belongs to the CYP74 family and yields 12,13(S)-epoxy-octadecatrienoic acid [2.49]. β-Oxidation of the cyclization product 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid yields finally JA. Jasmonates are important regulators in plant responses to biotic and abiotic stresses as well as to development [2.54]. Cis-jasmone (CJ) is a volatile compound and represents the main constituent of the floral bouquet of different plants thereby attracting insect pollinators. It is emitted in response to herbivory, application of insect oral secretions, or JA treatment. However, the biosynthetic route leading to the formation of CJ is still unclear. JA methyl ester is the main component of the scent of jasmine flowers.

α- and β-Oxidation

Fatty acids of short- and intermediate-chain length are generated in the course of fatty acid degradation by α- or β-oxidation. β-Oxidation in plants occurs primarily in the peroxisomes [2.55] and the reaction sequence is now well established [2.56]. One reaction cycle results in the successive removal of C_2-units (acetyl-CoA) yielding the C_{n-2} carboxylic acid. However, volatile acids can also be generated by de novo synthesis and hydrolysis of the conjugate between the acid moiety and the acyl carrier protein, that is, the acyl acyl carrier protein (acyl ACP), during fatty acid biosynthesis. Alcohols, esters, and aldehydes can be generated as further volatile metabolites by the action of alcohol dehydrogenases, alcohol acyl transferases,

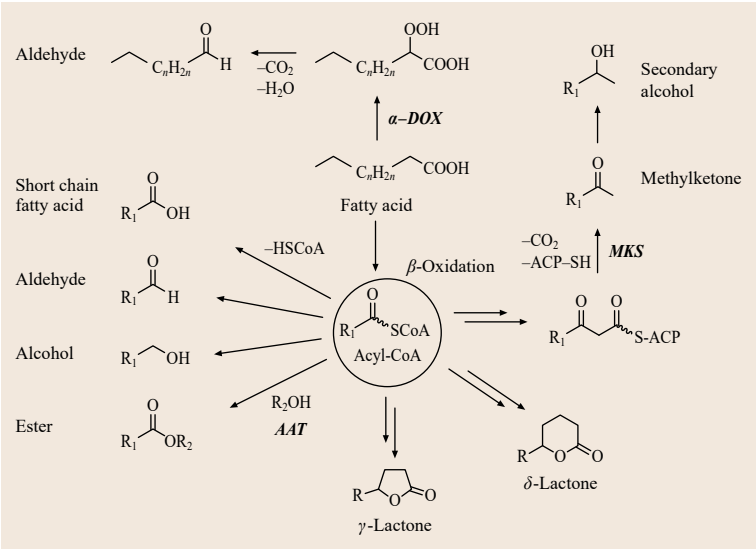

Fig. 2.15 Biosynthesis of volatiles from fatty acids. AAT, alcohol acyl transferase; MKS, methylketone synthase; ACP, acyl carrier protein; α-DOX, alpha-dioxygenase

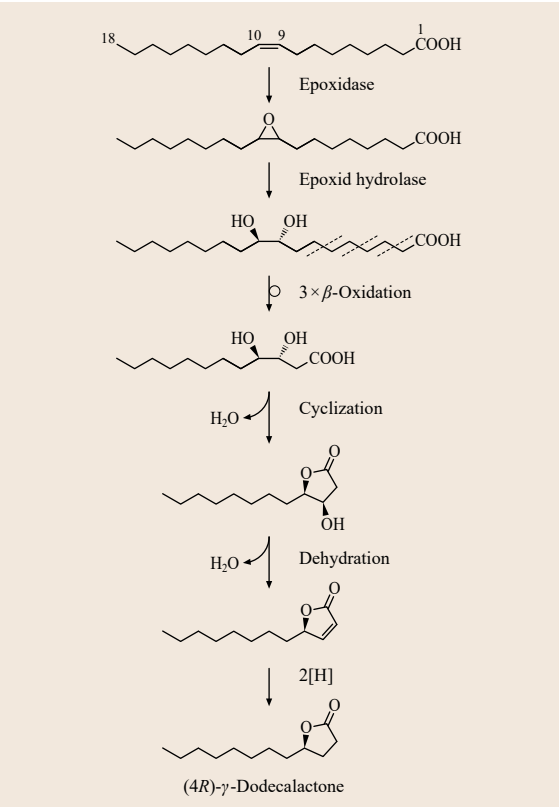

Fig. 2.16 Proposed biosynthesis of (4*R*)-γ-dodecalactone in ripening fruits (after [2.57]). The sequence was previously established by administration of deuterium labeled precursors to ripening strawberries and peaches (after [2.58])

or α-dioxygenases (α-DOXs) (Fig. 2.15). α-DOXs catalyze the formation of 2-hydroperoxy carboxylic acids that are unstable and yield the C_{n-1} aldehyde as product [2.59]. α-DOX from Arabidopsis resembles a *b*-type cytochrome, although with much more restricted access to the heme moiety. Methylketones are generated by hydrolysis and subsequent decarboxylation of β-ketoacyl ACPs [2.60] and are assumed to be precursors of aroma-active secondary alcohols like 2-pentanol and 2-heptanol in passion fruits [2.61].

Other important volatiles that are generated from fatty acids are alkanolides, which have 5- or 6-ring heterocyclic lactone structures, the so-called γ- and δ-lactones. Despite their importance for many fruit flavors, their biosynthesis in plants remained up to now largely obscure: no enzymes or genes associated with their biosynthesis have been characterized. However, it is generally accepted that all lactones originate from their corresponding 4- or 5-hydroxy carboxylic acids by nonenzymatic or AAT-catalyzed cyclization. Labeling studies with fatty acid epoxides and diols have shown that these precursors are efficiently incorporated into lactones in a stereoselective manner [2.57, 58] (Fig. 2.16). Expression profiling of genes and an integrative *omics* approach have recently identified new candidate genes that potentially impact aroma volatiles in peach fruit [2.62, 63].

2.2.4 Amino Acid-Derived Odorants

Branched chain and aromatic amino acids, cysteine and methionine, or intermediates in their biosynthesis, are very often precursors of odorants that are highly

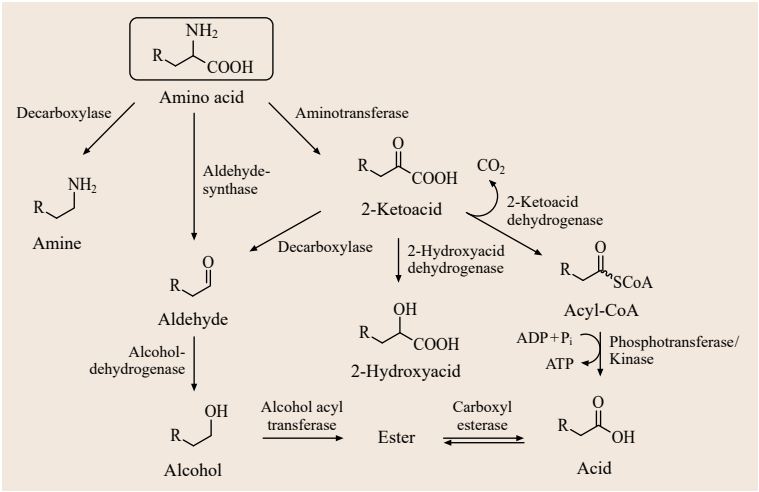

Fig. 2.17 Biosynthesis of amino acid-derived odor compounds (after [2.50])

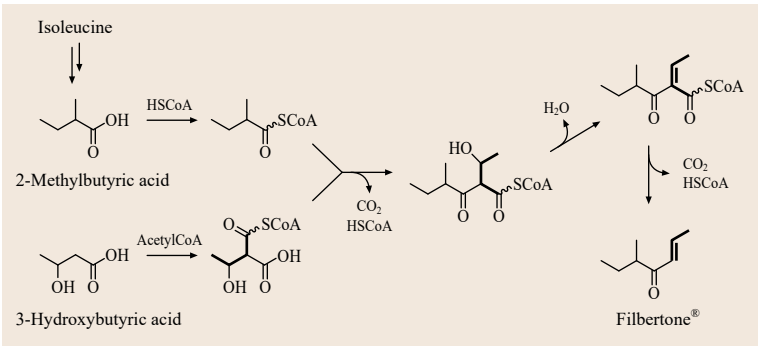

Fig. 2.18 Proposed biosynthesis of filbertone in hazelnuts

abundant in floral scents and fruit and vegetable aromas [2.17]. Especially important are branched-chain volatiles, derived from branched-chain amino acids, such as isoamyl acetate (banana), 2-methy-butyl acetate (apple) and methyl 2-methyl butanoate (prickly pear). The biosynthesis of these volatiles in plants is believed to proceed in a similar way to that found in bacteria and yeast, where these pathways have been studied more extensively [2.64].

Acids, Amines, Alcohols, Aldehydes, and Esters

The principal pathways to amino acid-derived odor compounds are shown in Fig. 2.17. The operation of these biosynthetic pathways is supported by numerous feeding experiments using intact plant tissues where the addition of intermediates enhanced the concentration of specific aroma compounds [2.65, 66]. An alcohol acyl transferase (AAT) from apple (cv. Royal Gala), MpAAT1, produces esters involved in an apple fruit flavor [2.67]. The recombinant enzyme can utilize a range of alcohol substrates from short-to-medium straight chain (C3–C10), branched chain, aromatic and terpene alcohols. A genomics approach has revealed that aroma production in an apple via the isoleucine degradation pathway is controlled by ethylene predominantly at the step in ester biosynthesis catalyzed by MpAAT1 [2.68]. In melon fruit tissues, the catabolism of amino acids into aroma volatiles can initiate through a transamination mechanism by branched chain amino transferases, rather than decarboxylation or direct aldehyde synthesis, as has been demonstrated in other plants [2.69]. A second route in melon apparently involves the action of an L-methionine-γ-lyase activity, releasing methanethiol, a backbone for the formation of thiol-derived aroma volatiles [2.70]. Exogenous L-methionine also generates nonsulfur volatiles by further metabolism of α-ketobutyrate, a product of L-methionine-γ-lyase activity. α-Ketobutyrate is further metabolized into L-isoleucine and subsequently other important melon volatiles, including nonsulfur-branched and straight-chain esters. In tomato fruits, the catabolism of branched chain amino acids supports respiration but not synthesis of volatiles, which are rather generated from keto acids as likely precursors [2.71].

One of the key aroma compounds in hazelnuts is 5-methyl-2-hepten-4-one (filbertone) whose biosynthesis

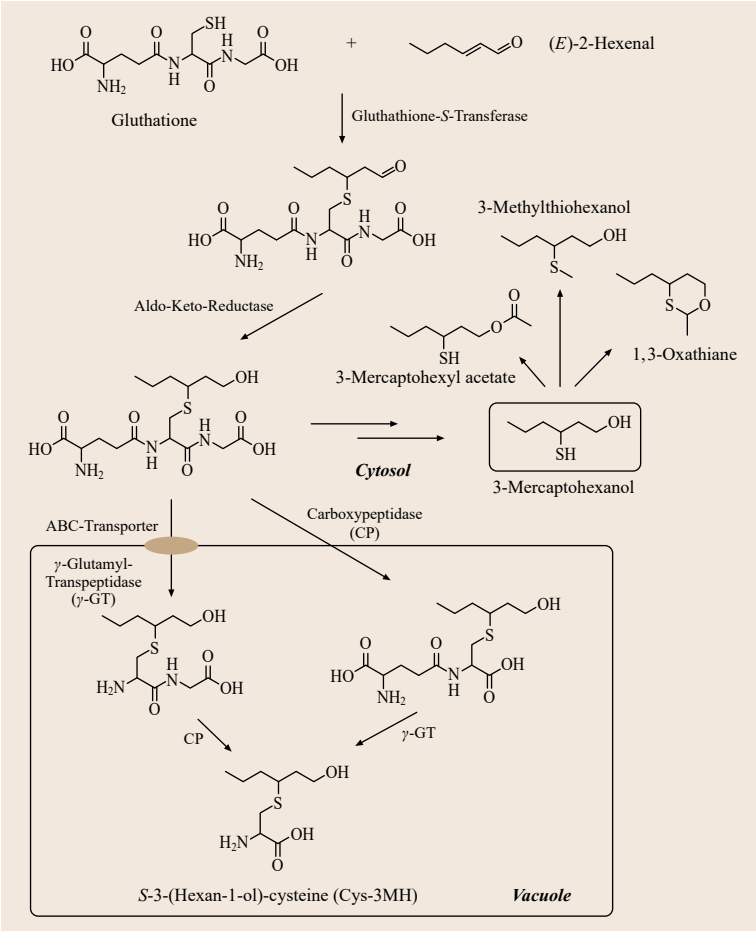

Fig. 2.19 Hypothetical pathway for the biosynthesis of 3-mercaptohexanol conjugates and their derivatives (after [2.73]). ABC-transporter, ATP binding cassette transporter

is believed to involve isoleucine as a precursor [2.72] (Fig. 2.18). Analysis of the volatile compounds during ripening and storage showed that the formation of methyl-branched ketones starts as soon as branched chain amino acids are catabolized. However, knowledge about genes and enzymes involved in this postulated pathway is still missing.

Aliphatic Thiols (Varietal Thiols)

Key compounds in explaining tropical fruit flavor like yellow passion fruit scent are volatile thiols. These molecules are generated from nonvolatile precursors like *S*-glutathionylated and *S*-cysteinylated conjugates, the latter being a degradation product of the former. A prominent example is 3-mercaptohexan-1-ol (3MH) that is a potent odorant in passion fruits [2.73] and certain grape varieties like Sauvignon Blanc and Petite Arvine [2.74]. It is believed that the first step of the precursor biosynthesis is the glutathionylation of (*E*)-2-hexenal, which is a product of the LOX-mediated fatty acid degradation [2.75]. After the reduction of the aldehyde moiety, the conjugate is gradually degraded by peptidases to yield the *S*-cysteinylated conjugate, which is stored in the vacuole or is further metabolized by a lyase that liberates 3MH (Fig. 2.19). The free thiol can undergo further transformations like esterification and formation of oxathians. However, no enzymes or genes associated with the biosynthesis of these metabolites have been characterized so far. Environmental stress enhances the biosynthesis of flavor precursors, *S*-3-(hexan-1-ol)-glutathione and *S*-3-(hexan-1-ol)-L-cysteine, in grapevine through glutathione *S*-transferase activation [2.76].

Phenylpropanoid/Benzoid Derivatives

Phenylpropanoid and benzoid compounds originate from the aromatic amino acid phenylalanine that is in turn generated by the shikimate/arogenate pathway [2.77]. The first committed step is the generation of cinnamic acid by the action of a phenylalanine ammonia lyase (Fig. 2.20). The formation of benzoids (C_6–C_1) requires shortening of the propyl side chain,

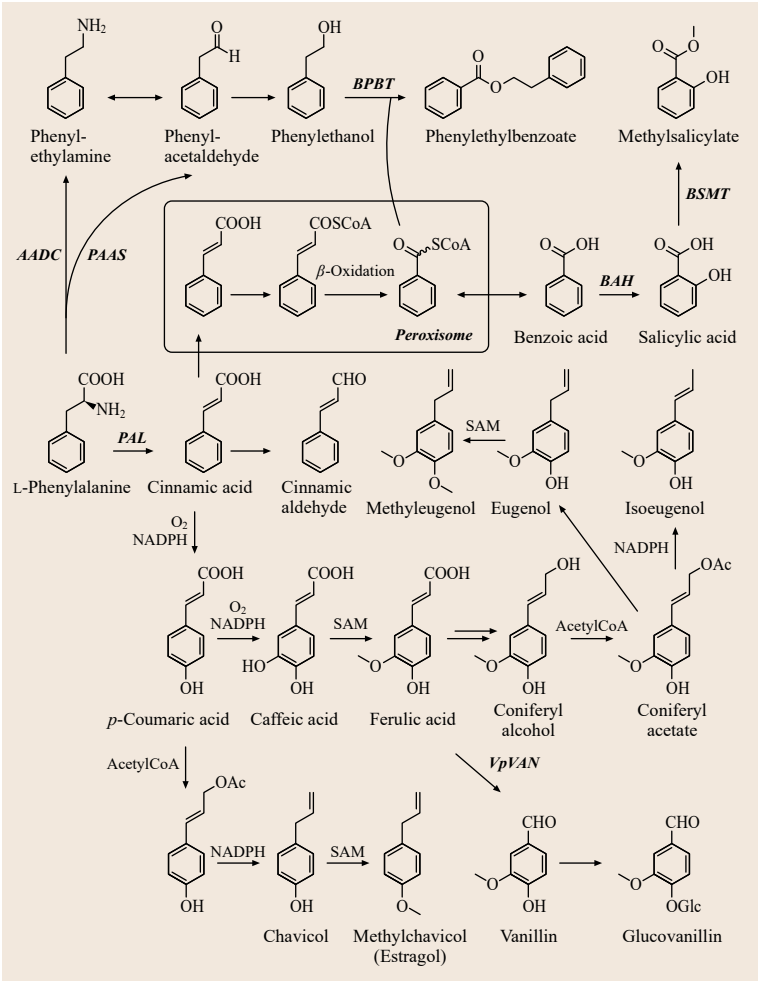

Fig. 2.20 Biosynthesis of benzoid and phenylpropanoid odorants (after [2.64]). AADC: aromatic amino acid decarboxylase; BAH: benzoic acid 2-hydroxlase; BPBT: benzoylCoA:benzylalcohol/2-phenylethanol benzoyltransferase; BSMT: benzoic acid/salicylic acid carboxyl methyltransferase; PAAS: phenylacetaldehyd synthase; PAL: phenylalanine ammonia lyase

which takes place in peroxysomes by the β-oxidation sequence [2.78]. Another non-β-oxidative reaction sequence to benzoic acid seems possible [2.79], but its biochemical steps are still in question. Volatile phenylpropenes (C_6–C_3) like eugenol and estragol require the elimination of the oxygen functionality at C-9 position, which is achieved by the reduction of the corresponding acetates (Fig. 2.20). Methyl anthranilate is generated in Concord grapes from anthranilic acid, which is an intermediate of tryptophan biosynthesis, by the action of an anthranilate-CoA ligase and a methanol acyltransferase [2.80]. However, herbivore-induced SABATH methyltransferases of maize that methylate anthranilic acid using *S*-adenosyl-L-methionine have been recently cloned and characterized [2.81]. The SABATH family is named after the first identified enzymes (SAMT, BAMT and Theobromine synthase) in this family.

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the most widely used flavor compound in the world. It is present as glucovanillin in the green pods of vanilla and is released only after fermentation, called *curing*, when the glucoside is hydrolyzed by an endogenous glucosidase in vanilla pods. Tracer experiments using ^{14}C-labeled precursors suggest that the biosynthetic pathway for vanillin is p-coumaric acid → feruclic acid → vanillin → glucovanillin (Fig. 2.20) [2.82]. A single enzyme designated vanillin synthase (VpVAN) catalyzes direct conversion of ferulic acid into vanillin.

Heterocyclic Odorants

Methoxypyrazines (MPs) are potent odorants and are responsible for the distinctive green and earthy aroma of some vegetables like 2-methoxy-3-isobutylpyrazine (IBMP) in bell pepper. 2-Methoxy-3-isopropylpyrazine (IPMB) is found in raw potatoes and peas [2.17]. The *vegetative* sensory attribute of Sauvignon Blanc and Cabernet Sauvignon wines is also due to the presence of MPs [2.84]. Little work has been done so far in de-

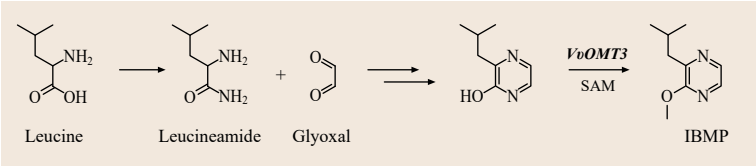

Fig. 2.21 Proposed pathway for 2-methoxyisobutylpyrazine (IBMP) biosynthesis (after [2.83])

termining the pathways of MP biosynthesis. A number of pathways have been proposed, which generally begin with an amino acid (leucine for IBMP or valine for IPMP) and an unknown 1,2-dicarbonyl compound leading to the formation of a 3-alkyl-2-hydroxypyrazine (HP) (Fig. 2.21). Recently, it could be shown that the final step of the methylation of HP is catalyzed by an *O*-methyltransferase that uses *S*-adenosyl methionin (SAM) as a methyl group donor [2.85, 86]. Interestingly, the corresponding gene is not expressed in the fruit of Pinot varieties, which lack IBMP, but is expressed in Cabernet Sauvignon at the time of accumulation of IBMP in the fruit [2.83, 88].

2-Acetyl-1-pyrroline (2AP) is a potent odorant that can be generated in the course of the Maillard reaction in heated foods like popcorn and white bread [2.90]. It

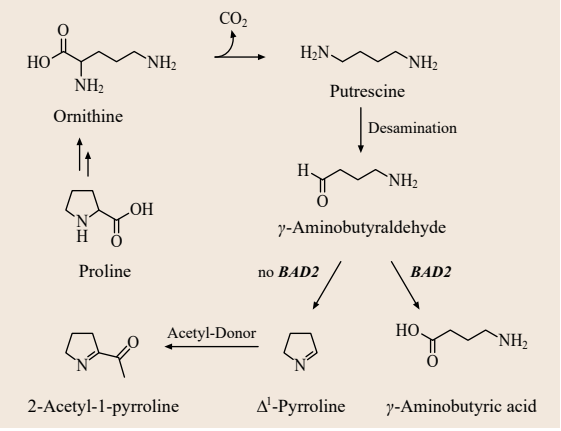

Fig. 2.22 Pathway from proline to 2-acetyl-1-pyrroline (2AP) (after [2.87])

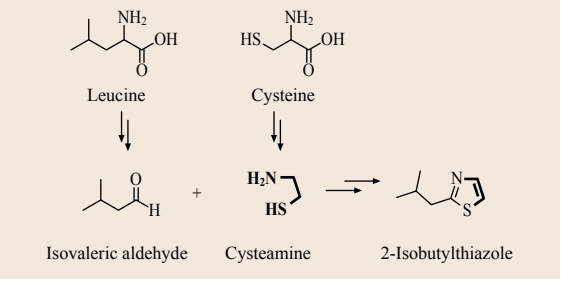

Fig. 2.23 Postulated biosynthesis of 2-isobutylthiazol in tomato (after [2.89])

can also be found in basmati and jasmine rice at higher concentrations and is biosynthesized there from proline [2.91]. Recent research has shown that a nonfunctional betain-aldehyde dehydrogenase (BAD2) leads to the enhanced generation of 2AP in these rice varieties due to the accumulation of pyrroline [2.87] (Fig. 2.22). The nature of the acetyl donor is still unknown.

2-Isobutylthiazole contributes to the aroma of tomato [2.92]. It is probably obtained as a product of the catabolism of leucine und cysteine (Fig. 2.23) [2.89].

S-Alk(en)yl Cysteine Sulfoxide-Derived Odorants

(+)-*S*-Alk(en)yl cysteine sulfoxides (CSOs) are nonprotein sulfur amino acids typically found in members of the family Alliaceae [2.93]. These molecules are precursors of volatile and reactive sulfur-containing odorants that cause the best known characteristic flavor of, *inter alia*, onion, garlic, and leek [2.94]. In onions, *S*-1-propenyl cystein sulfoxide is the main precursor of sulfur containing volatiles, whereas in garlic, *S*-allyl cysteine sulfoxide, better known as alliin, is most important. The biosynthesis of various CSOs from valine and glutathione is shown in Fig. 2.24. Alternative routes to CSOs imply the thioalk(en)ylation of *O*-acetylserine or the direct alk(en)ylation of cysteine. If both routes are active in all developmental stages of plant growth is not yet clear [2.95].

If onions or garlic are chopped, the typical aroma appears within seconds. Due to the disruption of the cells the enzyme alliinase comes into contact with CSOs and cleaves the C(β)-*S* bond, thus releasing ammonia, pyruvate and a series of unstable sulfenic acids (Fig. 2.25). These sulfenic acids yield, by purely chemical reactions, more stable volatiles like the well-known thiosulfinate allicin in garlic and further volatiles like disulfides [2.95].

Glucosinolate-Derived Odorants

Almost all vegetables that contain glucosinolates belong to the family of the Brassicaceae. Prominent examples are red and white cabbage, radish, mustard, and broccoli. Glucosinolates are thioglucosides whose basic structure is shown in Fig. 2.26. More than 100 different glucosinolates are currently known and can be grouped into aliphatic, indolic, and aromatic members [2.96].

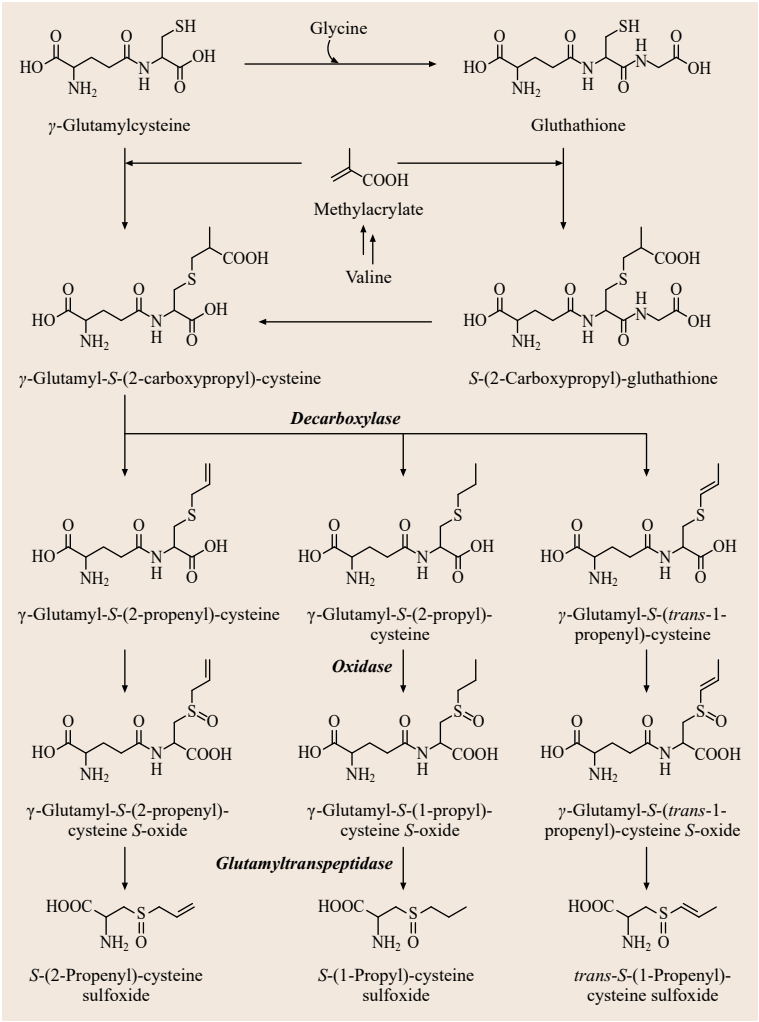

Fig. 2.24 Biosynthesis of flavor precursors in intact onion and garlic tissue (after [2.93])

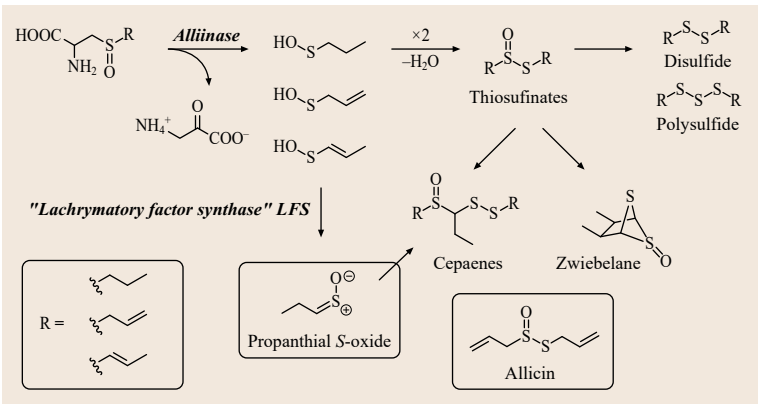

Fig. 2.25 The flavor volatiles resulting from the action of alliinase and lachrymatory factor synthase (in onions) (after [2.95])

Glucosinolate derivatives not only contribute greatly to the distinctive flavor and aroma of cruciferous vegetables and condiments, but also possess profound biological activities that range from their participation in plant defense and auxin homeostasis to cancer prevention in humans [2.97]. The biosyn-

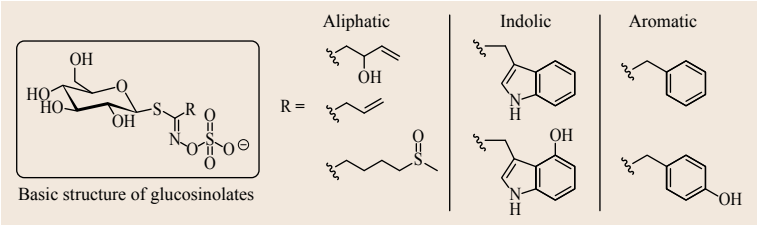

Fig. 2.26 Basic structure of glucosinolates and typical examples for members of the three groups

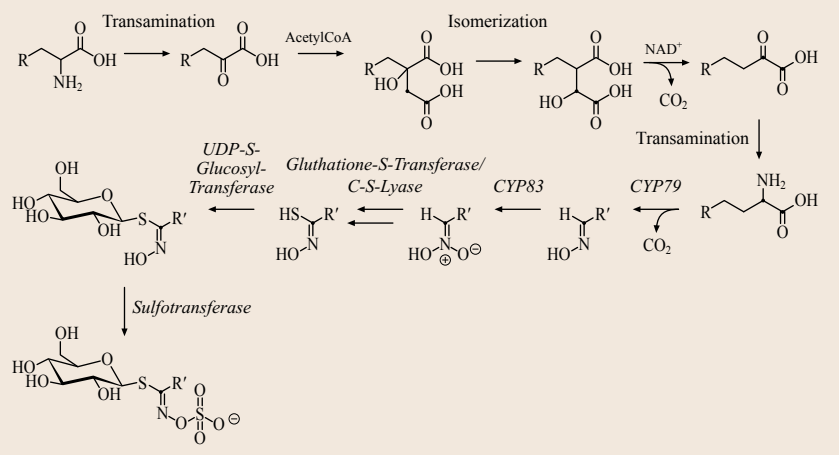

Fig. 2.27 Biosynthesis of glucosinolates (after [2.98]). Amino acid side chain elongation by one CH_2 group is shown and can be repeated up to 9 times. CYP83 and CYP79 are cytochrome P450 enzymes

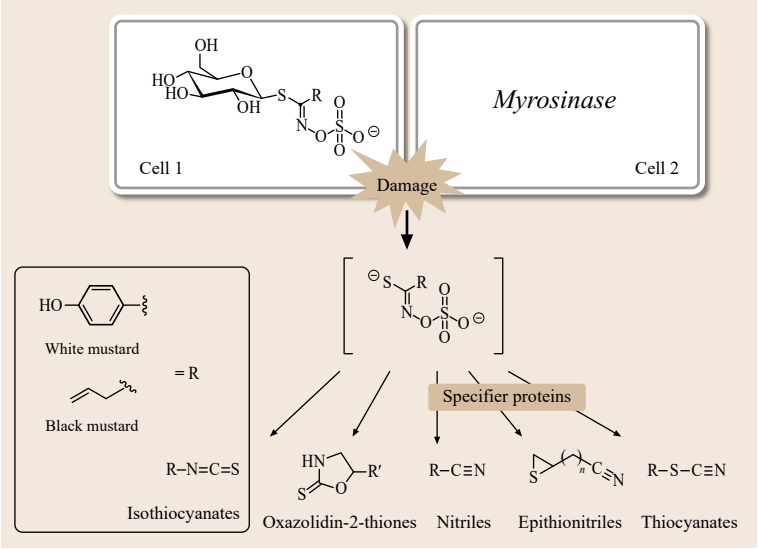

Fig. 2.28 Enzymatic degradation of glucosinolates by myrosinase and generation of further products. Allyl isothiocyanate (see lower left box) is a compound responsible for the pungent burning odor and taste of mustard. p-Hydroxybenzyl isothiocyanate is only slightly volatile and contributes significantly to the sharp pungent taste of mustard

thesis of glucosinolates can be divided into three steps:

1. Amino acid side-chain elongation
2. Glucone formation
3. Side-chain modification

and is shown in Fig. 2.27 [2.99].

If cells of cruciferous vegetables are disrupted glucosinolates come into contact with myrosinase, a thioglucosidase enzyme, which cleaves the thioglucosidic bond yielding glucose and an unstable aglycone that can rearrange into several final products (Fig. 2.28). The outcome is influenced by the structure of the side chain and the presence of so-called epithio-specifier

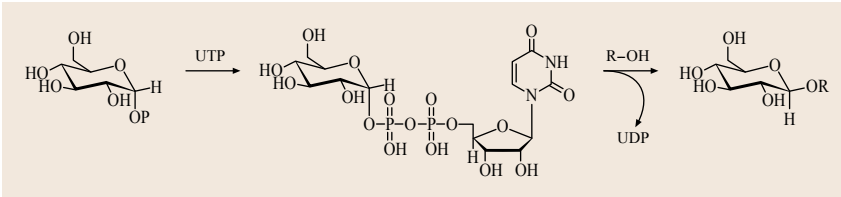

Fig. 2.29 Biosynthesis of β-glucosides by uridine diphosphate-glucosyltransferases (UGTs)

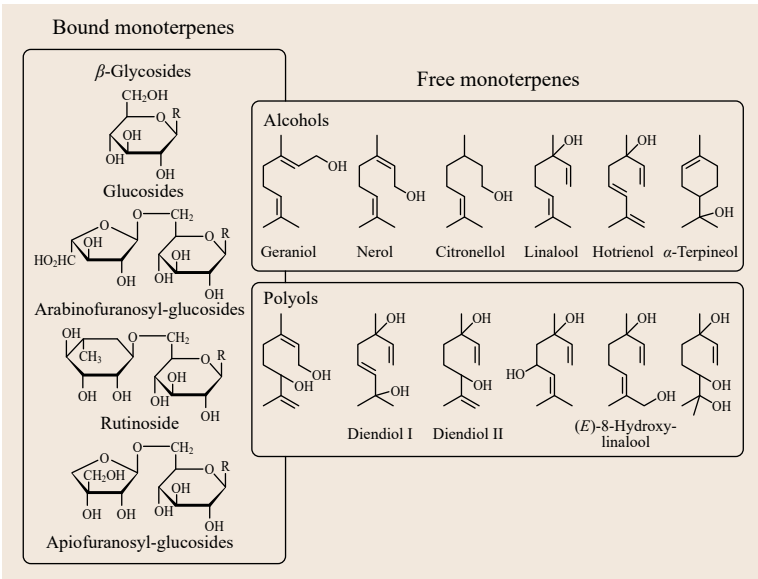

Fig. 2.30 Structures of typical monoterpene heterosides found in grapes

proteins (ESP) [2.95]. Isothiocyanates and nitriles are responsible for the pungent burning odor and taste and typical compounds in mustard oil.

Recent research has demonstrated that plant accumulation of glucosinolates and thus herbivore resistance are under circadian clock regulation. The simple stimulus of light is sufficient to entrain the clock not only of postharvest cabbage but also of diverse noncrucifer postharvest vegetables and fruits [2.100].

2.2.5 *O*-Glycosidically Bound Odorants

Plant volatiles are often *hidden* as glucosides whose glucose moiety is transferred from uridine diphosphoglucose by the so-called family 1 uridine diphosphate-glucosyltransferases (UGTs) [2.101]. Since the glucosylation process can be envisaged as a simple nucleophilic displacement reaction of S_N2 type, the product is a β-glucoside (Fig. 2.29).

By the sequential transfer of further activated sugar molecules, mixed disaccharides are generated (Fig. 2.30). A remarkably large array of different small molecules is glucosylated in fruits and vegetables and these include terpenoids, alkaloids, cyanogenic glucosides as well as flavonoids, isoflavonoids and other phenylpropanoids. It still remains an open question as to how this large number of structurally different flavor precursors is actually glucosylated in vivo because the in vitro activities of specific UGTs show large differences in the individual range of acceptors. Some UGTs are highly specific with respect to substrate-, regio-, and stereo-specificity, whereas others glucosylate a broad range of acceptors [2.102]. The latter phenomenon is called promiscuity and could decisively contribute to the immense structural variations of small plant secondary metabolites regarding their glucosylation pattern. Biochemical characterization of the substrate specificity of the UGTs is, therefore, a major challenge that scientists face when approaching the study of the actual biological function of these enzymes whose number of available sequences is rapidly increasing as a result of the expressed sequence tags (EST) and genome sequencing programs. In *Vitis vinifera* alone, more than 200 different glycosides have been identified, and there is a special interest in those glycoconjugates, which can contribute to wine flavor through the hydrolytic release of volatiles during the biotechnological vinification sequence leading from grape to aged wine [2.103]. These flavorless glycoconjugates accumulate in grape berries during maturation and can

be grouped into monoterpenes, C13-norisoprenoids, aliphatic alcohols, and shikimate-derived benzoids and phenylpropanoids [2.104]. Flavor enhancement in wine processing and in plant tissue products has nowadays become possible through the use of exogenous glycosidases [2.105].

2.3 Stress-Induced Biosynthesis of Plant Volatiles

Plants frequently emit volatiles upon feeding damage. The irregular acyclic homoterpenes 4,8-dimethylnona-1,3,7-triene (DMNT) and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) are among the most widespread volatiles produced by angiosperms with emission from vegetative tissues upon herbivore feeding. DMNT and TMTT have been implicated in attracting natural enemies of arthropod herbivores when released from damaged foliage [2.106]. This indirect defense strategy has been termed *plan's cry for help* and includes also the release of other volatiles like mono- and sesquiterpenes, aromatic compounds and fatty acid degradation products [2.7]. Today it is clear that jasmonates play a central role in signal transduction in plant stress response that leads ultimately to the emission of these volatiles by de novo biosynthesis [2.54]. The enzymatic steps in homoterpene biosynthesis are shown in Fig. 2.31. Key step is the oxidative C–C bond cleavage by cytochrome P450 enzymes from the so far uncharacterized plant CYP82 family [2.106].

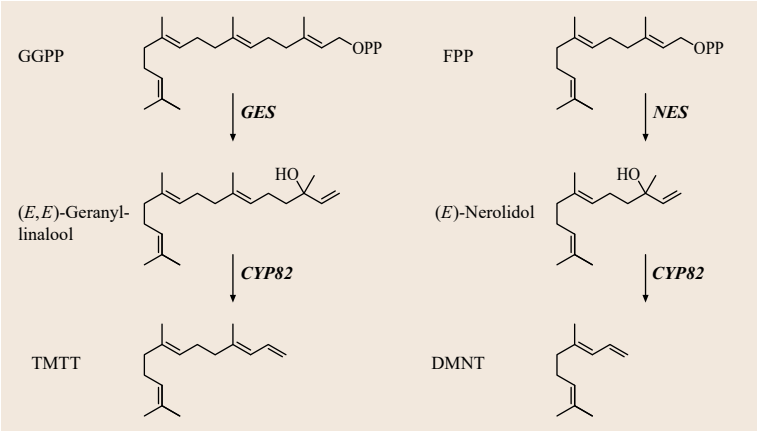

Fig. 2.31 Enzymatic steps in homoterpene biosynthesis (after [2.106]). DMNT: 4,8-dimethyl-1,3,7-nonatriene; TMTT: 4,8,12-trimethyltrideca-1,3,7,11-tetraene; NES: (*E*)-nerolidol synthase; GES: (*E*,*E*)-geranyllinalool synthase; CYP82: cytochrome P450 monooxygenase

2.4 Outlook

In the past decade, plant odorant research has witnessed a shift from studying odorant composition and identification toward the elucidation of their metabolic pathways [2.50, 64]. In particular, plant functional genomics that integrates genome sequencing, metabolomics, proteomics and high throughput biochemistry, will likely expedite the identification of genes and biochemical pathways of key odorants, whose formation in plant has been so far unknown. This will provide an expanded knowledge base for the genetic manipulation of biochemical pathways, which will ultimately lead to new crops with improved, altered or enhanced odor traits.

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3. Natural Fragrant Raw Materials

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The determination of the most important olfactory contributors of a fragrant natural raw material can be an extremely long and complex task which requires the combination of very efficient analytical techniques. Indeed, the characterization of these components is often difficult since the global odor of complex mixtures is not only due to the sum of the olfactory properties of each constituent, but also involves many synergies between each odorant constituents. In addition, the main contributors are often strongly potent odorants contained only in trace amounts, and therefore, their identification requires an exhaustive analysis of the whole mixture. Finally, since the olfactory sense is characterized by strong interindividual differences, a large number of panelists must be involved in such studies in order to bring generalizable data. Consequently, there is still lack of accurate knowledge about the main odoriferous constituents for many natural raw materials, and this situation is paradoxical when it concerns materials widely used for their odorant properties in the flavor and fragrance industry.

This chapter presents an overview of the published data about the main odor-active constituents of a selection of natural fragrant raw materials. It describes the chemical structures and olfactory properties of the main odorant components reported in the literature for 10 extracts and essential oils, after a brief description of the general analytical and sensorial issues concerning the determination of key odorants in a mixture.

The history of perfume is closely linked with that of natural raw materials. Indeed, before the birth of synthetic organic chemistry at the end of the nineteenth century, all of the materials used in perfume formulations were of natural origin, either vegetal or animal. Nowadays, even if modern perfumery is dominated by the use of synthetic substances, natural extracts are still considered prestigious ingredients. Moreover, fragrance chemists often use the vast pool of natural products as a source of starting materials for hemisynthesis [3.1] and the chemical structure of the most appreciated natural odorants also serves as inspiration for the creation of original synthetic fragrances. A large part of the artificial fragrant molecules used in modern perfumery has then a direct or indirect link with natural raw materials.

Therefore, the characterization of the odor-active constituents in natural raw materials is of crucial importance for the perfume industry. Paradoxically, the literature describing this type of analysis for a given natural odorant extract is generally limited compared to the number of publications focused only on its chemical composition. In the case of a raw material mainly used for its fragrant properties, such a situation is surprising, and is mainly due to the technical difficulty of giving some answers concerning the nature of the key

odorants of a complex mixture. Indeed, several issues will be encountered when trying to list the components which contribute to the odor of a fragrant mixture, especially in the determination of the relative importance of their contribution to the whole. However, this data is extremely useful, since it can help to formulate artificial reproductions of the raw material. In fact, many useful ingredients for the formulation of aroma and fragrances have been discovered in the frame of analytical studies devoted to the determination of impact odorants in natural raw materials [3.2]. A probably large part of the knowledge on the main odor contributors of the natural raw materials is not reported in the scientific literature for commercial reasons, but is rather stored in the archives of flavour and fragrance companies.

3.1 Identification of Odor Active Constituents in Natural Raw Materials

Two main types of natural raw materials are used in the flavor and fragrance industry for their odorant properties:

- The essential oils, obtained by hydrodistillation or steam distillation, are mixtures of volatile organic constituents with a low molecular weight (containing generally less than **20** carbon atoms). These components are usually phenylpropanoids, mono-, sesqui- and sometimes diterpenoids, but other types of constituents may also occur in these materials
- The solvent extracts (concretes, absolutes, resinoids, CO_2 extracts, etc.) usually contain the volatile constituents described earlier (with some differences), together with compounds of higher molecular weight which have dissolved in the solvent used during the extraction. These nonvolatile compounds generally have a weak impact on the olfactory characteristics of the mixture, but can modify significantly the evaporation rate of the volatile constituents. They act then as fixatives to increase the substantivity of their fragrance.

In both cases, the number of constituents of a raw material can be extremely important, with several hundreds of individual compounds present at a percentage higher than 0.1%. The odorants are contained in the volatile fraction of the extract, corresponding approximately to the essential oil; however, not all volatile compounds are odorants. Indeed, as detailed hereafter, distinct compounds can show huge differences in their olfactory properties, both in terms of quality and potency. The determination of key odorants in a natural raw material is a complex task which requires solving many classical issues of analytical chemistry, such as not only identifying accurately trace compounds, but also overcoming other difficulties related to the complexity of the human olfactory system. Some important points to consider are listed hereafter.

3.1.1 Physicochemical Issues

Sample Variability

The chemical composition of a given natural raw material is seldom uniform from one sample to another. As for any product obtained by transformation of living organisms such as foodstuff and beverages, several parameters can affect the chemical composition of an aromatic plant. The external issues (e.g., climate, nature of the soil) and the genetic factors of the species determine the nature of its secondary metabolites, which are also influenced by the harvesting and processing conditions (harvesting method, as well as storage, drying, cutting, cleaning conditions etc.). Eventually, the extraction process, which can also significantly differ from one producer to another, may also add another element of variability to the final composition of the extract. As a result, the odorant molecules present in two samples of a same essential oil or extract can strongly vary in terms of nature and content, and thus the overall olfactory properties of these samples will also differ. This situation is well known in the flavor and fragrance industry where a large part of the analytical activities is devoted to the quality control of natural oils and extracts. Therefore, any investigation on the determination of the odor-active constituents of a natural raw material should take into account that its results may not be generally valid to all samples of the same raw material.

Differential Volatility of the Constituents

When the odor of a natural extract is evaluated in a static mode (for instance on a smelling strip), the components of the mixture evaporate at a rate depending on their volatility. The most volatile constituents are perceived at the beginning of the olfaction, then quickly fade away to reveal the components of medium volatility, and eventually the less volatile odorants which can persist for a long time on the support (smelling strip, clothes, skin) [3.3]. The perfumers are familiar with

this phenomenon and commonly define the top, heart, and base notes of a composition or a natural extract. Hence, different components will contribute to different chronological parts of the general olfactory picture of the material, and accurate descriptions of the key odorants of a mixture sometimes mention if they are involved in the top, heart, or base notes.

3.1.2 Physiological Aspects

Sensitivity and Selectivity of the Olfactory System

The potency of an odorant molecule is usually expressed by its detection threshold, which is generally defined as the lowest concentration detected by at least 50% of a panel of evaluators. Large variations of detection thresholds exist between the known odorant substances, ranging from the ppt (parts per trillion) level (for the most potent odorants) to theoretical infinity (for a totally odorless compound like water). If we exclude such odorless substances, the human relative sensitivity ranges at least over 11 orders of magnitude [3.4]. At first sight, we may be tempted to use the detection threshold values to compare the contribution of different odorants of a mixture, and several analytical approaches detailed below are based on this intuitive principle. However, this reasoning would be valid only in the case of purely elemental processings, and is thus based on a false simplification (see below). Moreover, the published detection thresholds values should be considered carefully because a large number of different methods are used for their measurements; these methods often produce very different results for a same compound. Consequently, the comparison of threshold values compiled from different studies is not reliable [3.5, 6].

Interindividual Variability

It is well known that the detection threshold strongly depends on the person performing the evaluation [3.6, 7]. Individual sensitivity follows a log normal distribution in the population [3.6], and some cases of bimodal distribution are also known [3.8–11]. Hence, for some odorants, a significant proportion of the population can be either partially or totally anosmic, or on the contrary hyperosmic. For these reasons, the *typical odor* of a complex mixture can indeed be perceived differently from one individual to another. In extreme cases, two different individuals could then recognize different sets of odorant components among the main contributors to the global odor of the mixture. Finally, as in any sensory analysis, the most relevant data is obtained from averaged values collected from several evaluations realised by a panel as diverse as possible.

Synergies and Antagonisms in the Olfactory System

The perception of a mixture of odorants is the result of complex interactions at several levels in our olfactory system. In addition to the physical process of evaporation described above which delivers each constituent of the mixture at different times of the olfaction, a first set of synergistic and/or antagonist interactions might already occur when the odorants reach the olfactory mucus. Indeed, the odorant-binding proteins (OBP), a group of highly soluble proteins contained in high concentration in the nasal mucus, have been shown to bind odorant molecules with significant affinities. Although the function of the OBPs is still unclear, they may act as a primary filter which could modulate the perception of the odorant molecules [3.12], before the next step where they are delivered to the olfactory receptors (OR). Another complex interplay occurs then between all of these components and the different OR subtypes, and the discriminative power of the olfactory system is now considered to be mainly based on these interactions. Up to now, **396** genes coding for different functional human OR have been identified [3.13], but the mechanisms underlying the interactions of OR with the odorants are still not comprehensively understood. The same OR subtype can be activated by different odorants, and a single odorant can activate different ORs. Moreover, the binding of an odorant with an OR can occur with more or less high affinity [3.14], as is explained in detail in Chap. 27. Finally, another level of interaction appears when these combinatorial informations are processed by the brain to generate the olfactory perception. It has been demonstrated that a mixture of two components can be perceived either analytically with each component remaining identifiable, or as a single entity (*odor blending*). These processings are called elemental and configural, respectively [3.15]. *Zou* and *Buck* et al. have demonstrated the neuronal basis of the configural perception, by showing that a mixture of two components can activate cortical neurons that are not stimulated by any of these components taken alone [3.16]. For human naïve subjects, configural processing occurs with binary and tertiary mixtures, but trained subjects can perceive these mixtures in an elemental mode [3.17]. As a result, a mixture of several components can be perceived as something different than a simple arithmetical addition of each odorant. Complex mixtures such as natural raw materials often contain several hundreds of volatiles if one considers the trace constituents, but the number of odorants above their threshold level is much lower. Anyway, the level of perceptual odor blending between these active odorants is unknown, but is probably rather high.

3.1.3 Analytical Methods for the Characterization of Odor-Active Constituents in Mixtures

Direct Evaluation of Isolated Samples

The first answers concerning the odor-active constituents of natural raw materials were provided by analytical studies performed at the end of the nineteenth century, often by chemists working in the flavour and fragrance industry. Their methodology was based on the careful fractionation of the essential oils and extracts by physical and chemical methods, followed by the olfactory evaluation of the isolated substances. Despite the poor technical means of these times, several impressive characterizations could be achieved. Moreover, since no spectroscopic methods were yet available, all kinds of molecular characterization were exploited, and the olfactory properties of the purified substances were often reported in detail, as fragrance chemists were naturally open to olfactory evaluations.

The odor of a single compound can be determined with a sample obtained by fractionation of the raw material, but should then be considered with caution as it can be altered significantly by trace impurities [3.4, 18]. As mentioned above, the constituents of a natural extract can be totally odorless or on the contrary possess extremely low odor thresholds, because the human olfactory system is both extremely sensitive and selective. Even a few ppm of a strong odorant contaminant can completely modify the olfactory properties of another substance even if its purity is $> 99.9\%$. For this reason, the reliability of the evaluation depends on the purification method which provides the sample for evaluation. If it is based on the fractionation of the whole mixture, the efficiency of the separation is critical in order to avoid any remaining odorant contamination. The evaluation of the substance can also be confirmed by comparison with a sample of the same compound obtained from a different source, for instance a synthetic sample. In such a case, the probability of having the same odorant contamination in the sample is very low. However, for a complex mixture of many odorant compounds such as an essential oil or a solvent extract, it can be extremely time consuming to synthesize even a few of the most odorous components to prove their olfactory character, especially for some precious materials containing complex sesquiterpenic constituents.

Gas Chromatography–Olfactometry (GC-O)

Today, the most convenient method for the determination of odor impact constituents in a mixture is based on the use of gas chromatography-olfactometry (GC-O) (Fig. 3.1). This technique consists of a simple GC

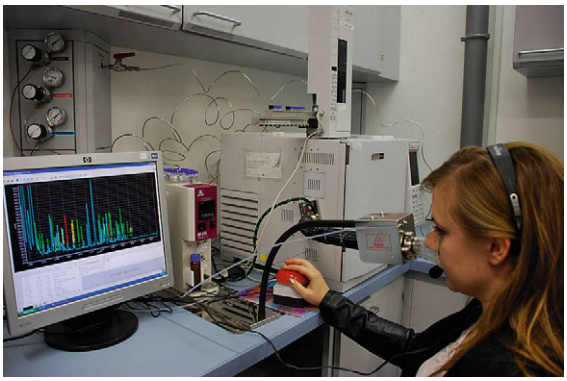

Fig. 3.1 A GC-O experiment. The panelist presses the *red button* when she perceives an odor at the olfactory port, and records the corresponding olfactory description via a voice recorder

experiment involving a human assessor who evaluates the effluent of the column, and describes the odor perceived in function of the retention time. The graphical result is named olfactogram (or aromagram). If a portion of the effluent is directed simultaneously to a conventional detector (mass spectrometer (MS), flame ionization detector (FID)), a correlation can be made between the olfactogram and the chromatogram, and for a given signal of the chromatogram, the odor of the corresponding compound(s) can be read on the olfactogram. GC-O is indeed as old as GC itself, because the first gas chromatographs equipped with a nondestructive thermal conductivity detector would release the effluent unchanged in the atmosphere, and it was then natural to perform olfactory assessments. Today, many technical evolutions have been proposed (multiple-port GC-O [3.19], GC×GC-O [3.20] etc.) and GC-O is the method of choice for the characterization of odor-active constituents, thanks to the high resolving power of modern capillary GC.

A single GC-O experiment can be used for a first overview of the odorant constituents in a mixture (GC-sniffing), but this technique is also used to determine the relative olfactory contribution of the odorants of a mixture. Several methodologies have been proposed for this purpose, and for the processing and interpretation of the data collected during the experiments [3.21–23]. They can be classified into three types:

1. Detection frequency
2. Dilution to threshold
3. Direct and posterior intensity.

Detection Frequency Methods

Detection frequency methods [3.24] are based on the comparison of the olfactograms realized by several dif-

ferent assessors. The constituents which are detected more frequently are concluded to have a greater relative contribution. This technique does not necessarily require trained panelists, but relevant results are obtained only with a large number of assessors (typically more than eight) and the determination of the relative olfactory contribution of the most potent components of a complex mixture can be problematic. Indeed, this method does not allow to rank the strongest odorants when they are detected by all the panelists [3.22]. *Pollien* et al. have proposed an improvement of the detection frequency method by taking into account the length of the stimulus [3.25]. However, many authors have reported that the end of the odor zone is often difficult to perceive accurately [3.26, 27] and that the importance of coeluting compounds is over-evaluated by this method [3.22, 28].

Dilution to Threshold Methodologies

In this approach, serial dilutions of an extract are prepared and analyzed by GC-O. The odorant constituents which are still perceived at the highest dilutions are considered to be the main odor contributors. The aroma extract dilution analysis (AEDA) takes into account the last dilution in which a constituent is still perceived, and attributes to this compound a flavor dilution (FD) factor corresponding to the dilution value [3.29]. In the CharmAnalysis, the duration of the stimulus is recorded, and peak areas can be calculated [3.30]. This last method has more discriminating power than AEDA, but presents also the limitations mentioned above for the measurement of the stimulus length. One of the drawbacks of the dilution to threshold method is the high number of experiments required per panelist, which precludes the possibility of handling a large population of evaluators. Consequently, many AEDA studies are based on the evaluations of only one or two assessors, which cannot then be considered as representative of the whole population since interindividual sensitivity differences are often important [3.7]. Moreover, the results of AEDA should be interpreted carefully, because such methods are based on the false assumption that the odor intensity increases linearly with the concentration. Indeed, the relation between odor intensity (I) and odorant concentration (C) follows the Stevens' psychophysical power law [3.31]. The sigmoid curve $I = f(C)$ is shown in Fig. 3.2. The saturation of the nose at high concentrations is visible, and the part of the curve before the saturation can be plotted in logarithmic scale ($\log(I) = f(\log(C))$, to give a line with a given slope. Different odorants are not necessarily characterized by the same slope [3.28, 32] and consequently, in a given mixture, a compound with a high FD factor may indeed induce a weaker olfactory stimulus than another

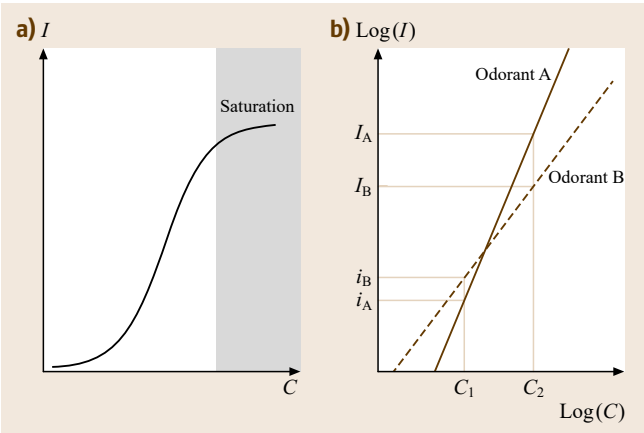

Fig. 3.2 Psychometric function. The intensity of the olfactory stimulus (I) is plotted against the concentration of the odorant (C), and gives either a *sigmoid curve* **(a)** or a *line* **(b)** if the scale is logarithmic

component showing a lower FD factor when this mixture is evaluated at a high concentration. In Fig. 3.2, an odorant A is perceived as stronger than an odorant B at a high concentration C_1 ($I_A > I_B$), but because of the differences in the slopes of their psychometric functions, the reverse is observed at lower concentrations C_2 ($I_A < I_B$).

Direct and Posterior Intensity Methods

Direct and posterior intensity methods [3.33–37] involve recording the stimulus with an evaluation of the intensity perceived by the assessor and the duration of the perception. An olfactogram similar to a classical chromatogram is then produced, and these methods give the most reliable results. However, they require a significant training of the assessors, and consequently, their application is not very frequent.

Some studies have demonstrated that for the same mixture of compounds evaluated by the same panel, each GC-O methodology gives different results in terms of odorant ranking [3.21, 28]. In consequence, any GC-O-based classification of the relative olfactory contributions of the constituents of a mixture should be considered with caution.

Odor Units

Once the concentration of an odorant in an mixture is determined, a calculation of its olfactory contribution has been proposed, by dividing its concentration by its odor threshold. This concept was first introduced by *Patton* and *Josephson* [3.38] and led to the definitions of aroma value [3.39], and odor unit (OU) [3.40]. The comparison of the OU of each odorant has been proposed to estimate their relative contribution to the over-

all odor, and was illustrated by Ohloff on the rose odorants [3.41] (Sect. 3.2.3). However, the same criticism as for AEDA can be applied to the OU concept, which assumes that there is a linear relationship between the perceived intensity of a compound and its concentration, with a same slope for all compounds. In addition, it does not take into account any synergistic/antagonistic effects that might occur between odorants [3.42, 43].

Reconstruction Experiments

The reliability of any kind of ranking of the main odor-active constituents of a mixture may be criticized, but despite this, it can be taken as a simple guide for a reconstruction of the mixture. Furthermore, it was the initial goal of the GC-O methods and the OU concept. If the odor of a mixture can be reproduced by blending a shortlist of some of its main odorant constituents in their original concentration, then it brings the ultimate confirmation of their role as key odorants. Omission experiments (in which a single constituent is withdrawn from the formula to evaluate if the overall odor is modified) are also very useful to determine if a given odorant has a real contribution [3.44]. Such an approach has been described for simple aromas [3.44], and wines [3.45] but is more tedious for complex essential oils or extracts, in which the key odorants are sesquiterpenic constituents that are not commercially available.

3.2 Odor-Active Constituents of Selected Natural Raw Materials

Several types of oils and extracts exist and can be classified regarding their odor-active constituents; thereby, *Petrzilka* and *Ehret* [3.46] have proposed to classify raw materials in three main categories, according to the following situations:

1. The typical odor of the material is due to one or a few major constituents and the minor constituents have almost no contribution.
2. The main components bring an essential olfactory base, but the typical and characteristic odor is created by their association with several key minor and trace constituents.
3. None of the main constituents has any olfactory importance, and the odor character is due to the minor and trace components.

Several important raw materials belong to the first category, and will be described hereafter. The last category is obviously the most problematic from the analytical point of view, and as an example, *Petrzilka* and *Ehret* mentioned the case of mimosa absolute (*Acacia dealbata*). The typical scent of this material is due to a complex interplay of constituents at a content below 1%, among which some trace compounds play a decisive role [3.46]. This observation was confirmed later by GC-O experiments which could not identify any single constituents possessing a typical mimosa odor [3.47]. In this review, a selection of some important natural raw materials is presented, with the data concerning their odor-active constituents, as reported in the scientific literature.

3.2.1 Jasmine

The extraction of jasmine flowers (Fig. 3.3) provides several types of extracts which were considered in the past as the most important natural perfume raw materials [3.48]. Still today, the jasmine absolutes and extracts are extremely precious natural floral ingredients for the preparation of high-grade perfumes. In the past, *Jasminum grandiflorum*, often grafted on *Jasminum officinale*, was cultivated mainly in the Grasse area in southern France, and contributed to the prosperity of the local fragrance industry [3.49]. Nowadays, most of the world production comes from India and North Africa.

Fig. 3.3 Jasmine (*Jasminum grandiflorum*) (courtesy of Céline Cerutti-Delasalle, *Albert Vieille*)

The first analytical investigations on the chemistry of jasmine fragrance appeared at the end of the nineteenth century, and were conducted by *Hesse* in Germany [3.50–54]. Because of the limited technical means, only few components could be characterized, with some inevitable errors. Among the substances which were confirmed by further studies, the most abundant constituents of jasmine absolute were characterized: (+)-linalool **1** and benzyl alcohol **2**, with their acetates **3**–**4** [3.54] (Fig. 3.4). Hesse also described the occurrence of some high impact odorant

constituents, and he pointed out the important contribution of indole **5** [3.53, 54] as well as those of jasmone **6**, a $C_{11}H_{16}O$ ketone with an *intense and pleasant jasmine odor* [3.54]. The structural determination of **6** was unattainable at that time, but in 1933, it was eventually elucidated simultaneously and independently by *Ruzicka* [3.55] and *Treff* [3.56]. Among the other jasmine constituents identified during this period, several important odorants were discovered: *p*-cresol **7** [3.57], eugenol **8** [3.58], benzaldehyde **9**, and creosol **10** [3.59]. *Naves* and *Grampoloff* also noticed the presence of lactones with a *tenacious potent fruity odor* but did not characterize their structures [3.59].

Nevertheless, the reconstitutions of jasmine odor with all these substances were still unsuccessful [3.58, 60], and it stimulated further analytical investigations. One of the missing links was discovered in 1962 by *Demole*, who identified methyl jasmonate **11**, a ketoester *playing an essential function in jasmine perfume* and structurally related to **6** [3.60]. Indeed, only the more potent **12** was detected in the headspace of jasmine flowers, but it epimerizes during the preparation of the absolute to give a 9 : 1 thermodynamic mixture of **11** and **12** [3.61]. Interestingly, (±)-**12** has an odor threshold **400** times lower than **11** [3.62]. *Demole* also described the occurrence of vanillin **13** and 6-methylhept-5-en-2-one **14** [3.63], and of (−)-(*Z*)-dec-7-en-5-olide **15**, a lactone with a *particularly sweet and fine, very floral odor* [3.64]. The dehydro, ethyl- and acetoxy-analogs of **11** (**16**–**18**) were also identified, as well as the peculiar bicyclic lactones **19**–**20** and other saturated and monocyclic ones (**21**–**26**) [3.49, 65]. **19** is odorless [3.65], and the olfactory contribution of **16**–**26** to the global odor of jasmine was not described, but is probably weaker than that of the much more abundant and strong odorant **11** and **12**.

Even if the presence of methyl anthranilate was already reported during *Hesse*'s pioneering investigations on jasmine oil [3.54], it proved to be indeed an artifact coming from the hydrolysis of methyl *N*-acetylanthranilate **27** [3.49] during hydrodistillation. Many other nitrogen compounds were also characterized, like methyl *N*-methylanthranilate **28** [3.66], and various pyridines and quinolines (**29**–**32**), as well as benzonitrile **33** and 2-phenylnitroethane **34** [3.67]. These compounds may participate in the olfactory character of jasmine, but the importance of their contribution is unclear. **29**–**32** possess pungent odors, **33** recalls benzaldehyde, and **34** has a cinnamic, phenylacetaldehyde-like odor. Their low amount might limit their olfactory importance, but **5** is probably the strongest nitrogenous contributor. Its amount in the headspace of living flowers is besides more substantial than in picked flowers [3.68]. Finally, maltol (**35**) should also be mentioned as an important odorant component of jasmine. This heterocyclic compound possesses a characteristic sweet caramel, fruity odor with baked bread undertones and is a well-known contributor to the aroma of many foodstuffs. It was isolated and identified in benzene extracts of Egyptian jasmine flowers [3.69], and was recently reported as a key odorant in an extract obtained by supercritical fluid extraction of an ethanolic infusion of French *Jasminum grandiflorum* flowers [3.70]. This latter study pointed out that the conventional hexane extraction produces an absolute which contains comparatively much lower amounts of **35**. These observations are consistent with the fact that **35** is significantly soluble in benzene and ethanol, but only poorly solubilized in hexane. **35** is indeed an actual constituent of jasmine, since it could also be detected in the headspace of living jasmine flowers [3.69]. In view of its significant percentage in the extracts, there is little doubt that its powerful odor certainly contributes substantially to the exquisite fragrance of natural jasmine.

Up to now, no published study has ever accurately described the relative contribution of the jasmine odorants, but the constituents unanimously [3.49, 61, 71] recognized as the most important elements of the typical beautiful jasmine fragrance are **15** and the two main jasmonoids: **6** and **11** [3.64]. Demole also reported that a significant portion of the absolute (more than one third) contained odorless heavy constituents acting as natural fixatives and synergists, and may then play a rather decisive role in the jasmine fragrance: phytol, isophytol, phytyl acetate, geranyl linalool, benzyl benzoate, fatty acids, and their esters [3.49, 72].

3.2.2 Tuberose

Tuberose (*Polyanthes tuberosa* L.) was one of the emblematic flowers (Fig. 3.5) cultivated in the Grasse area in the first half of the twentieth century, but the production of this delicate plant is now mainly located in India. By solvent extraction, the freshly harvested tuberose flowers furnish an absolute with a very typical sweet, heavy floral character [3.48]. Compared to jasmine extracts, tuberose absolute has a lower importance in perfumery, and consequently its composition has been much less investigated. Several constituents common to jasmine absolute have been reported. In the first investigations, methyl anthranilate **36**, eugenol **8**, geraniol **37**, nerol **38** and their acetates (**39**–**40**), benzyl benzoate **41**, farnesol **42**, methyl benzoate **43**, and methyl salicylate **44** have been characterized in tuberose solvent extract [3.73–75] (Fig. 3.6).

Later, *Kaiser* and *Lamparsky* identified coumarin **45** and several saturated δ- and γ-lactones **23**–**24**, **46**–**50** [3.76]. By preparative gas chromatography, they

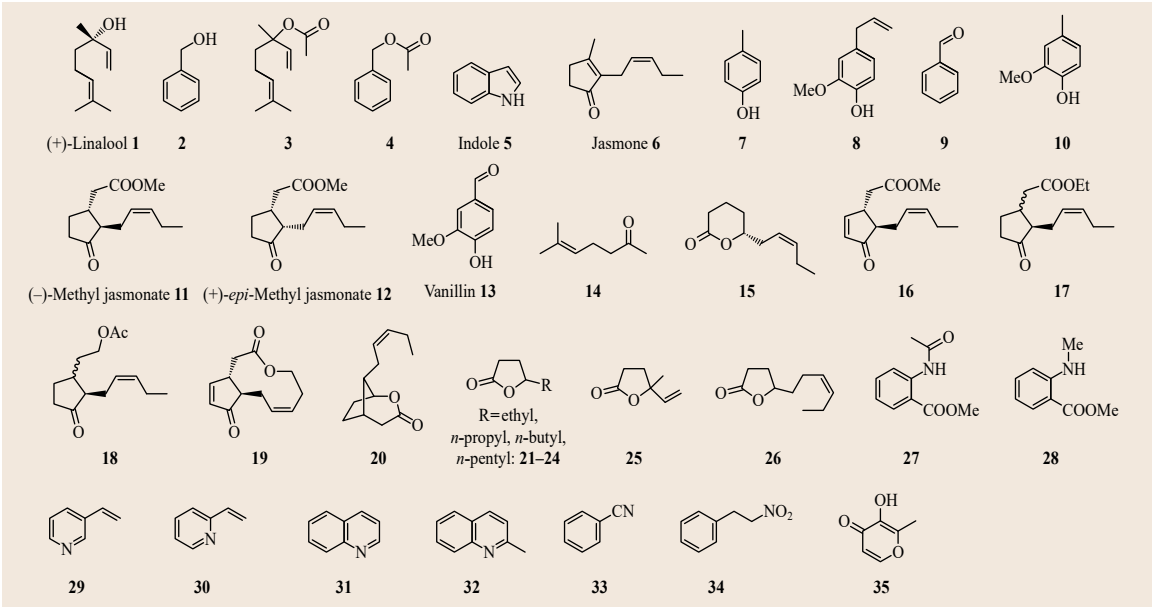

Fig. 3.4 Main constituents and key odorants of Jasmine absolute

Fig. 3.5 Tuberose (*Polyanthes tuberosa*) (courtesy of Céline Ceratti-Delasalle, Albert Vieille)

could also isolate some new δ-valerolactones **51**–**53** and *ent*-**15**, which is the optical antipode of one of the key odorants of jasmine, as mentioned above. *Maurer* and *Hauser* have subsequently identified **6** additional γ-butyrolactones **54**–**58** and the bicyclic lactone tuberolide **59** [3.77]. These components were detected in trace amounts (0.01−0.001%) but according to the authors, may contribute to the rich, heavy floral odor of tuberose absolute: **54**–**56** showed a strong smell reminiscent of the corresponding saturated γ-lactones, **57**–**58** displayed long lasting, slightly fatty aldehydic notes, and **59** had a tenacious lactonic odor.

Kaiser and *Lamparsky* also investigated the presence of nitrogen compounds [3.78], and identified *N*-formyl methyl anthranilate **60** and *N*-methyl methyl anthranilate **28** [3.79] in addition to **36**. Nitrogen heterocycles like **61** and the nicotinic acid esters **62**–**63** were also characterized, as well as 0.02% of indole **5** and traces of skatole **64**. The olfactory contribution of all these constituents is certainly quite significant: **62**–**63** bring a very special warm-tobacco like odor, and **5** also participates in the floral character. Besides, the presence of **5** has been measured in the concrete and absolutes of various strains of tuberose, and was shown to reach up to 2% [3.80]. Even if **64** is often designated as partly responsible for the odor of feces, it may bring, as a trace component, an important and interesting contribution to the pleasant odor of tuberose absolute [3.78].

3.2.3 Rose

With jasmine, rose is one of the major sources of natural ingredients for the flavor and fragrance industry. The *Queen of the flowers* is indeed an inescapable element in floral perfume compositions. Its beautiful shape and color makes it attractive also for horticultural purposes, and more than **13000** varieties have been created by crossings [3.81]. Many rose cultivars are odorous, with a large range of olfactory facets [3.82, 83]. Today, two main species are cultivated for the perfume industry: Damask rose (*Rosa damascena*) produced mainly in Bulgaria and Turkey, and *Rose de mai* (*Rosa centifolia*) (Fig. 3.7), grown in the past in the south of France, but cultivated now mostly in Morocco and Egypt.

The main aromatic extracts of rose are the concrete and absolute obtained by solvent extraction. By

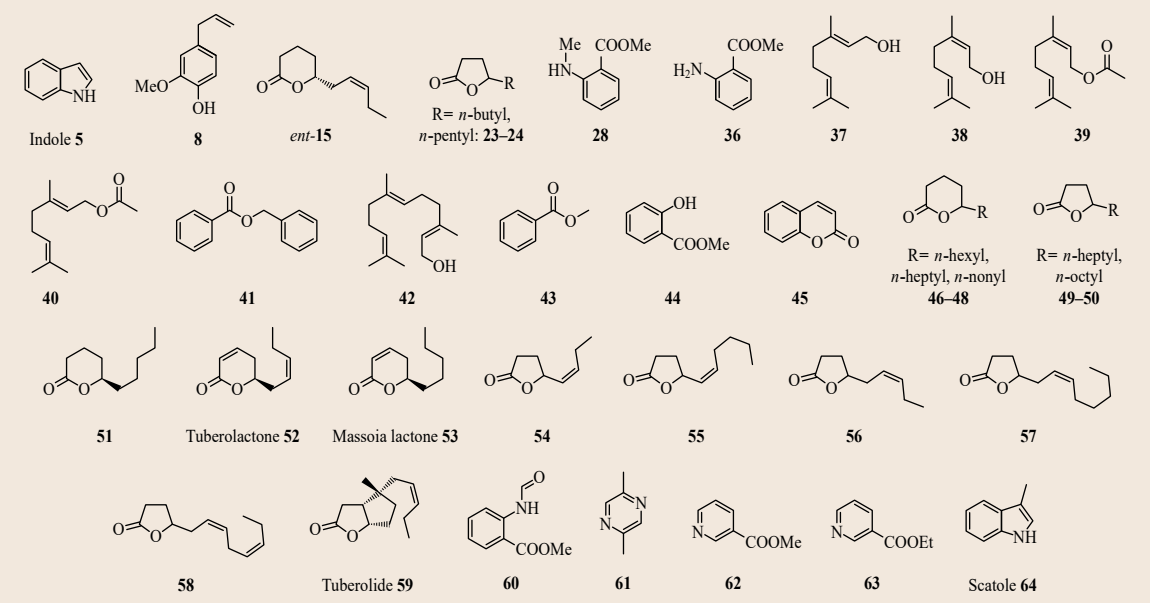

Fig. 3.6 Main constituents and key odorants of tuberose absolute

contrast, rose oil and rose water are produced by hydrodistillation of the flowers. Given the importance of these ingredients for the perfumers, the volatile part of these products has been extensively analyzed since the early times. Basically, the most abundant constituents of rose oil, which are also the main volatiles of concrete and absolute, are simple alcohols like phenylethanol **65**, (−)-citronellol **66**, geraniol **37**, and nerol **38** [3.84] (Fig. 3.8). Significant amounts of paraffins and olefins are often present as well, but their contribution to the overall odor is of little importance. A large variety of linear alcohols, aldehydes, acids, and esters can also be identified in rose solvent extracts [3.85].

Still today, the reproduction of the rose fragrance is a classical task for an apprentice perfumer. The determination of odor-active constituents of rose was thus a major concern for the fragrance chemists. **37**–**38** and **65**–**66** are often presented as necessary for the base note, and their odor is commonly described as *rose-like*, but their overall potency is low. Indeed, a simple mixture of these constituents is far from the typical rose fragrance. **65** is often presented as a key component, but rose reconstructions are possible without it [3.81]. Several key odorants contained at low percentages (< 1%) were discovered in extensive analytical investigations during the second half of the twentieth century. At first, *Seidel* and *Stoll* [3.86] identified the monoterpenic tetrahydropyranes **67**, which were named *rose oxides* and play a crucial role in the rose fragrance. The most abundant 2*S*,4*R* *cis*-isomer occurs at about 0.4% in the oil, twice more than its 2*R*,4*R* epimer [3.87]. **67** brings

Fig. 3.7 Rose de mai (*Rosa centifolia*) (courtesy of Céline Cerutti-Delasalle, Albert Vieille)

a powerful fruity odor essential for the floral green top-note [3.61].

An extensive analysis of Bulgarian rose oil was undertaken by Kováts at the ETH in Zürich between 1962 and 1967, in collaboration with the Firmenich company. This work remains a classic in the field of essential oil and fragrance chemistry, and its results started to be published only a decade later for commercial reasons [3.85]. Hence, **127** compounds representing 98.6% of the volatile part were isolated and identified, and the olfactory contribution of many minor constituents could be determined [3.88]. Kováts also gave an interesting account of a reconstruction of the rose oil. Hence, a rose base could be obtained by mixing **37**–**38** and **66** with some odorless paraffins and heavy natu-

ral constituents of rose oil acting here as fixatives. This base was described as possessing a pleasant rose odor, but it required to be strengthened by the addition of ethanol, hemiterpenic alcohols, hexenols, 2-alkanones, fatty alcohols, acids and aldehydes, and compounds **1**, **8**, **39**–**40**, **42**, **65**, and **69**–**72**. Finally, to obtain the sweet, powerful *honey note* of the oil, small amounts (0.05−0.2%) of **73**–**75** were added, but the full identity of rose was only revealed with the final adjunction of 0.1−0.2% of the key odorant **76** [3.89] which *changes the faint odor of this mixture to the well-known sweet odor of rose*. Several other constituents not included in this reconstruction (**2**, **9**, **77**–**78**) were nevertheless reported to be important for the organoleptic quality of the oil [3.88].

Ohloff exploited these results to illustrate the concept of odor unit (OU) [3.41] and proposed a quantitative estimation of the olfactory contribution of the main rose odorants [3.41, 61]. β-damascenone **76** was described as having *narcotic scent reminiscent of exotic flowers, and heavy fruity undertone* and showed the highest relative OU (70%) despite its low concentration in rose oil (0.14%) [3.61]. It was followed by β-ionone **79** (OU 19%, with a concentration of 0.03%), and the most abundant constituent (−)-citronellol **66** ranked only in the third position (OU 4%). The other contributors were, in decreasing order of OU: **67**, **1**, **37**, **42**, **8**, **38**, **80**, **65**, **68**, **81**, and several remaining odorants not included in this classification: nerol oxides **69** which, with **67**, contribute to the *geranium-like odor impression* and the hesperidin-like odorant *p*-menth-1-en-9-al **82** [3.90, 91]. Interestingly, β-damascone **83** was also reported to contribute to the overall character of rose oil with an odor resembling **76**, and even if its concentration in the oil was 0.0003%, it was still **10000** times higher than its threshold value [3.90].

Other experiments trying to reconstitute the rose fragrance reported that few additional natural components play an important role in the whole character: cis-3-hexenal (**84**) brought a *very lovely, fresh green and leafy aroma* whereas other C_5-C_9 aldehydes (especially mono- and di-unsaturated) which add fresh and spicy, or floral and fatty notes. Other facets sometimes recognized in the complex rose fragrance were attributed to specific components: minty (carvone **80** and methyl heptenone **14**), spicy (cinnamaldehyde **85**), woody (monoterpenes), and floral/fatty (alkanols) [3.81]. Başer has compared the compositions and odor properties of several Turkish *Rosa damascena* oils, and discussed the effect of **1**, **8**, **37**–**38**, **65**–**66** and **68** on the overall fragrance. He also pointed out that other compounds like **40**, **72**, and **86** create the typical fresh rosaceous character in the top note, which is boosted by nonanal **87** [3.92]. With the help of GC-sniffing experiments, a last class of highly potent odorant constituents has been discovered by *Omata* et al., who have reported the occurrence of **16** sulfur compounds such as mono-, di- and trisulfides, sulfur heterocycles and terpene sulfides like **88**–**94**. These trace constituents were described as important contributors to the characteristic odor of rose essential oil, with various notes like onion, green, smoky, and powdery [3.93, 94]. The actual natural occurrence of **93** in the plant is questionable, because this compound is a well-known constituent of crude oils and fuels [3.95] and might then be an artifact coming from the extraction process. Indeed, up to now, there are no established biosynthetic pathways related to the formation of such compounds in the plants, and attempts to identify traces of **93** in several rose extracts by other authors have been unsuccessful [3.69]. These last observations are obviously not a proof that **93** is not a natural constituent of rose. However, they justify a reinvestigation of the species where this compound was detected, using analytical procedures adapted to the detection of artifacts (involving instrument and procedural blanks).

To conclude this survey of the rose odorants, it should be considered that a great variety of garden roses have been created by multiple crossings during several centuries. Analytical studies on the chemistry of minor rose varieties have revealed a large number of constituents not common or even totally absent in *Rosa damascena* or *Rosa centifolia*, like for example 3,5-dimethoxytoluene **95**, 1,3,5-trimethoxybenzene **96**, dihydro-β-ionone **97**, theaspiranes **98** etc. [3.82, 83]. Many of these constituents are strong odorants, and therefore contribute to the rich palette of rose fragrances, which adds another layer of complexity to the aroma of the Queen of the flowers.

3.2.4 Cedars

The name cedar refers to several species rather distinct from the botanical and phytochemical point of view. In perfumery, the main types of cedars used for the production of fragrant raw materials are the Atlas cedar (*Cedrus atlantica*) and species of the *Juniperus* genus. The latter group is the most widely used, and is the source of the Texas and Virginian cedarwood oils, produced mainly in the United States by distillation of the woods of *Juniperus mexicana* and *Juniperus virginiana*, respectively [3.61].

The main constituents of *Juniperus virginiana* essential oil are (+)-cedrol **99**, (−)-α-cedrene **100**, and (−)-thujopsene **101** (Fig. 3.11). There seems to be a controversy about the key odorant constituents of *J. virginiana* essential oil, which recalls the case of patchouli. According to some sources, **99** is the principal odorant [3.61] but several authors have pointed

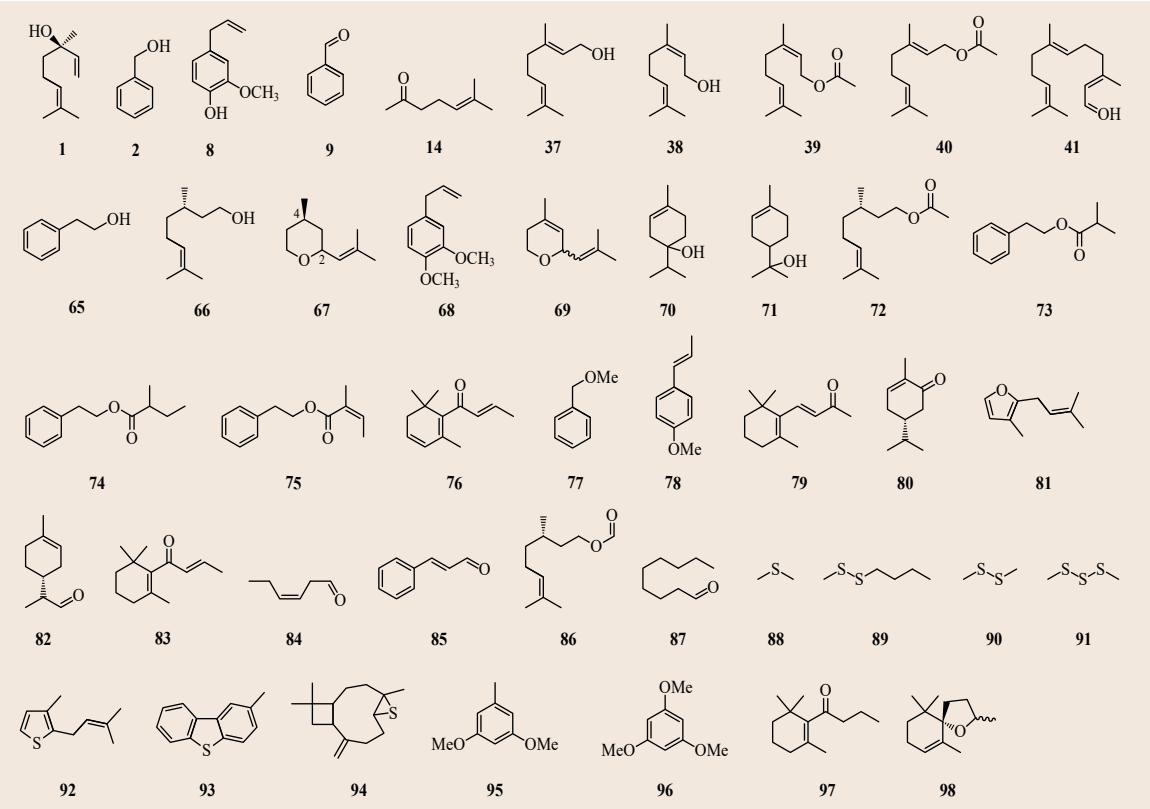

Fig. 3.8 Main constituents and key odorants of rose absolute and oil

out that crystalline **99** has a very weak woody odor, or is even odorless [3.48, 96, 97] so almost odorless, but small amounts of some odor-active components were reported in *J. virginiana* essential oils: cedrane oxide **102** (ambery) [3.61], (*E*)-betulenal **103** (sandalwood, acetylcedrene-like), funebrenal **104** (woody), 8-cedren-10-one **105** (musty, woody, mint myrrh-like), and nootkatone **106** (grapefruit) [3.96].

The second important cedar species is the Atlas cedar (*Cedrus atlantica*), native to the Atlas Mountains of Algeria and Morocco. It is botanically related to *Cedrus libani* (cedar of Lebanon) and also to its more distant Himalayan cousin *C. deodara* [3.98]. It is the main species of Moroccan forests used for timber production (Fig. 3.9), and the sawdust produced during the wood processing is often valorised by hydrodistillation to furnish an essential oil with a very particular sweet and tenacious woody odor, reminiscent of cassie and mimosa [3.48], and totally distinct from that of the above mentioned *Juniperus* oils. The main components of *Cedrus atlantica* essential oil are himachalane sesquiterpenoids **107**–**108** [3.99–106], and bisabolane sesquiterpenic ketones typical of this species, named atlantones **109**–**110** [3.99–107].

Fig. 3.9 Atlas cedarwood (Cedrus atlantica) trunks in a sawmill (courtesy of N. Baldovini)

Despite the large number of publications on the composition of Atlas cedarwood oil, the data concerning its odor-active constituents are scarce. As early as 1902, *Grimal* reported that the distillation of Atlas Cedarwood oil furnished a fraction *which possessed exactly the odor of the original essence* and which contained a ketone of the formula $C_9H_{14}O$ [3.108]. However, *Pfau* and *Plattner* [3.107] claimed later that

the mixture of **109** and **110** was *the true aromatic principle of the oils*. Recent sources confirmed this information [3.109] and also mentioned that deodarone **111** [3.110, 111] was an important contributor. Among the numerous other minor constituents identified in *C. atlantica*, vestitenone **112** was described as possessing an odor characteristic of the wood [3.112]. Recently, a GC-O investigation on Moroccan Atlas cedarwood essential oil was performed with eight panellists, following the AEDA methodology [3.113]. Interestingly, none of the panellists recognized **109** or **110** as strong and typical cedarwood odorants, and a purified sample of (*E*)-**109** was even very weak or odorless for most evaluators. The most potent odorant constituents were 4-acetyl-1-methylcyclohexene **113** (Atlas cedarwood-like), **112** (citrus-lemon), *p*-cresol **7** (phenolic, animal), 4-methylacetophenone **114** (almond-like), undecan-2-one **115** (aldehydic, coriander-like) and several non identified constituents. One of the strongest unknown components had a rather typical Atlas cedarwood note, but **113** showed the highest mean FD factor and possessed a very characteristic cedarwood odor for most of the panellists. **113** can then be reasonably considered as the principal key odorant of Atlas cedarwood essential oil, and probably corresponds to Grimal's ketone.

Fig. 3.10 Aerial parts of Vetiver (*Chrysopogon zizanoides*) (courtesy of Céline Cerutti-Delasalle, Albert Vieille)

3.2.5 Vetiver

Vetiver (*Chrysopogon zizanioides*) is a perennial plant of the Poaceae family (Fig. 3.10) that grows in tropical and subtropical countries. The hydrodistillation of vetiver roots produces an essential oil with a very characteristic and complex woody earthy, grapefruit odor, highly appreciated for the formulation of high grade perfumes. Vetiver essential oil has a very complex chemical composition, with a high number of constituents (mainly sesquiterpenoids) showing a huge structural diversity.

The first chemical studies of vetiver essential oil came up against the complexity of the oil, which proved particularly intractable for the poor analytical means available at the time. However, the precious scent of the oil stimulated the research on the nature of the odorants of vetiver, and as early as 1902, *Genvresse* and *Langlois* claimed that the typical vetiver odor was due to an ester: *vetivenyl vetivenate* [3.114], but it was only in 1939 that more solid analytical data permitted to *Pfau* and *Plattner* [3.115] (Givaudan) to report that the ketonic fraction was the most characteristic. The distillation of this fraction permitted to isolate two ketones, α- and β-vetivone, after purification by several successive recrystallizations of their semicarbazones. On the other hand, the alcoholic fraction was described as possessing a very weak odor. Because of the limited experimental methods for structural determination, the structures of these two ketones were wrongly described as isomers of **116** by deduction after tedious chemical transformations [3.116, 117] (Fig. 3.12). *Naves* and *Perrottet* (Givaudan) gave more details on their olfactory character: α-vetivone was described as possessing a characteristic potent and warm vetiver note, while β-vetivone was relatively weak, styrax, and vegetable like [3.116]. Their structures were definitely established as **117** and **118** by *Endo* [3.118] and *Marshall* [3.119], respectively (Fig. 3.12). Curiously, *Maurer* (Firmenich) described these ketones as ≪*relatively weak and uninteresting*≫ [3.120] but reported that in contrast, the norsesquiterpenic ketones **119**, **120**, and **121** possessed a characteristic vetiver note [3.121]. The important contribution of *Khusimone* **119** [3.122] to the characteristic scent of the essential oil was confirmed by *Büchi* [3.123] who also pointed out that another trinorsesquiterpenic ketone (**122**) *plays a significant role in the reconstitution of the essential oil* [3.124].

Jirovetz et al. applied GC-O to the study of vetiver essential oil [3.125]. A large number of olfactory zones were detected, but the correlation with the corresponding constituents was not clearly established, nor any kind of ranking of their relative contribution. However,

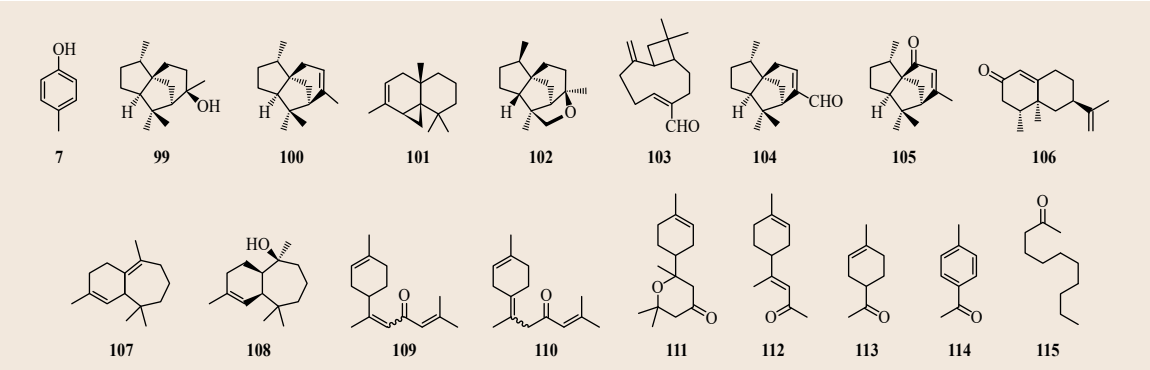

Fig. 3.11 Main constituents and key odorants of cedarwood oils (*Cedrus atlantica* and *Juniperus virginiana*)

some of the panelists involved in the study described as *typically vetiver* the elution zones of several constituents like **123–124** and **117–118**. The same team identified even more volatile compounds (monoterpenes and various light constituents), together with several known sesquiterpenic constituents, by purge and trap on the headspace of vetiver essential oil. They also analyzed these components by GC-O. The characteristic vetiver scent could not be associated with any of these constituents, but the authors affirmed that they were important for the top note of the oil, and *may have valuable synergistic effects with the other typical vetiver odor compounds* [3.126].

Mookherjee (International Flavors & Fragrances (IFF)) reported that **118** *undoubtedly plays the major role in contributing to the true odor of vetiver*, with several other carbonyl compounds (especially **117** and **119**) for which he gave an individual description of the olfactory character, and concluded that these other compounds *must also play some role to give the total precious woody note of vetiver oil* [3.127].

The controversies concerning the olfactory contribution of the vetivones continued, since *Spreitzer* et al. synthesized both enantiomers of **118** and described that the levorotatory form had a *quinoline-like, fruity (cassis, grapefruit) aroma with a woody by-note* but without *any odor reminiscent to the pleasant vetiver aroma*. Its antipode was characterized by an *unpleasant cresolic, medicinal note*. They concluded their article by hypothesizing that if most of the previous studies have attributed a typical vetiver scent to **118**, it was because of *traces of highly intensive odorous contaminations* [3.128].

The discrepancies of the literature on the odor-active components of vetiver led several authors to conclude in the mid-nineties that the constituents responsible for the true vetiver fragrance were still largely unknown [3.129, 130], and this statement probably stimulated some investigations like the outstanding work of *Weyerstahl*, published in a series of papers [3.131–133] which can up to now be considered as one of the most exhaustive analytical studies on a single essential oil. By a combination of distillations, chromatographic separations, chemical transformations, and structural analysis, he identified **155** constituents in a Haitian vetiver essential oil, and described numerous new structures. He also gave a detailed account on the olfactory properties of many isolated constituents and confirmed these data by synthesis for some of them [3.134, 135]. His contribution concerning the vetiver odorants is summarized at the end of his last publication [3.131] on the subject, and concludes that a large number of constituents possess strong odors with many different facets. Consequently, Weyerstahl claimed that the vetiver scent resulted from a complex sum of all these contributions, and he classified the constituents according to their olfactory notes. Three of the main alcohols were retained as the main contributors to the woody base note: khusimol **124**, (*E*)-isovalencenol **125**, and vetiselinenol **126**. Other olfactory notes related to the woody descriptor were mentioned for several compounds: sandalwood (**127–130**), ambery (**123**, **126–127**, **130–136**), and patchouli (**136–140**). One of the facets of the vetiver odor is often described as *aldehydic, very typical grapefruit-rhubarb*. Several carbonyl compounds (**135**, **139**, **141–146**) possessed this character, with **119** being the main representative, and **117** and **118** also falling within this category, with additional respective bitter and woody nuances. Many other constituents with various olfactory notes were also described: camphoraceous (**147–148**), fruity (**149–151**), musky (**152**), leather (**153**), floor-polish like (**154**), woody-peppery (**155**). *Weyerstahl* also wisely concluded that these olfactory evaluations had to be considered carefully, since even with the respectable GC purity of most of his samples, the presence of a strongly odorous minor constituent was still possible [3.4, 131]. Indeed, when he synthesized **145** for comparison with the natural

sample, he noticed slight olfactory differences that he attributed to the presence of odorous contaminants in the natural compound [3.133, 135].

In the work carried out by Weyerstahl, the relative contribution of the odorous components was not accurately established, but recently, Belhassen et al. performed a detailed analysis of Haitian vetiver oil using a combination of GC-O (AEDA), comprehensive two-dimensional gas chromatography (GC × GC)-MS, and chemical transformations. The AEDA conducted with five panelists underlined the importance of **142** and **144**, which showed the highest mean FD factor, accompanied by a typical vetiver character often mentioned for other zizaane derivatives such as **119** and **124**. Among the other constituents providing a high FD factor, **117** and **118** brought respectively rosy, lime-like and bitter grapefruit tonalities. β-vetivol (**156**) showed rosy-citrus aspects and **125** was perceived as strongly sandalwood-like. The other identified compounds with a lower FD are listed below in descending order of mean FD: dihydroeugenol **157** (dill-like, coconut, sweet), vanillin **13** (vanilla-like), 2-methylfurane-3-thiol **158** (meaty, nutty), β-damascenone **76** (fruity, sweet, plum-like), β-elemol **159** (carrot-like, pelargonium-like, green), *p*-vinylphenol **160** (metallic, phenolic), ziza-6(13)-en-3-α-ol **123** (Vetiver-like, cedarwood), nootkatone **106** (grapefruit-like, bitter, sour), cyclocopacamphanal **139** (marine, aqueous, calone-like), (*E*)-isoeugenol **161** (eugenol-like, smoky), guaiacol **162** (earthy, leek-like), *p*-vinylguaiacol **163** (smoky, warm, baked rice), spiroveta-3,7(11)-dien-12-ol **164** (baked apple, soft-fruit), khusimol **124** and khusimone **119** (both typically vetiver), 4-ethylphenol **165** (leather-like, smoky, burnt), khusian-2-ol **150** (fruity, sweet, woody), geosmine **166** (earthy-muddy), and β-vetivenene **167** (carrot-like, flowery). Strong variations were found between the five panelists for some constituents such as **118**, **117**, **150**, **156**, and **159** which showed significant deviations among their individual FD values. In contrast, the typical vetiver odorants **119**, and especially **142** and **144** displayed much more homogeneous FD values among the panelists. Since these two constituents possess a very characteristic woody vetiver note and were almost unanimously considered as the most potent odorants by the panel in this AEDA, they can then be recognized as the main vetiver key odorants [3.136].

3.2.6 Patchouli

Patchouli essential oil is produced by hydrodistillation of the dried leaves of *Pogostemon cablin*, a small plant (Fig. 3.13) cultivated mainly in Indonesia, Malaysia, and Philippines. Today, patchouli oil is the most important natural raw material in term of market size [3.61].

Fig. 3.13 Patchouli (*Pogostemon cablin*) leaves (courtesy of Sophie Lavoine, Charabot)

It possesses a very typical rich and strong odor, with woody, camphoraceous, spicy-balsamic and earthy tonalities [3.48, 61] (Fig. 3.14).

The main oil constituents are α- and δ-guaiene (**168–169**), and a specific sesquiterpenic alcohol, patchoulol **170**, which is almost systematically the major constituent. **170** is known since Gal's first analytical investigations on patchouli oil in 1869, since it can be easily obtained in crystalline form from fractional distillation, and was thus named *patchouli camphor* for this reason. Its history is also a famous example of a controversy about the key odor constituents of a raw material. Indeed, several authors claimed that **170** was odorless [3.137, 138] while most others maintained that it was the main odorant of the oil. The argument of the former was that crystalline **170** still contained trace amounts of a strongly odorous constituent, and for *Teisseire* et al., this compound was undoubtedly norpatchoulenol **171** [3.137]. Other analytical studies denied this assertion and the controversy eventually ended in 1981 when *Näf* et al. synthesized both enantiomers of **170** and demonstrated that the odor of the synthetic nature-identical levorotatory isomer was *practically indistinguishable* from the natural (−)-**170**. On the contrary (+)-**170** had a much weaker and completely different odor [3.139].

170 is now considered as the main odor donating constituent of patchouli essential oil, and according to many references, **171** has an important contribution [3.127, 140]. However, **171** shows a detection threshold three times lower than **170** but on the other hand, its typical content in patchouli oil is 70 times lower [3.61]. Anyway, specific individual anosmia for some of the patchouli odorants may have played a role in the controversies on the odor-active constituents of patchouli [3.61].

In 1988, *Nikiforov* et al. determined by GC-sniffing that **168–174** were the main odor-donating constituents

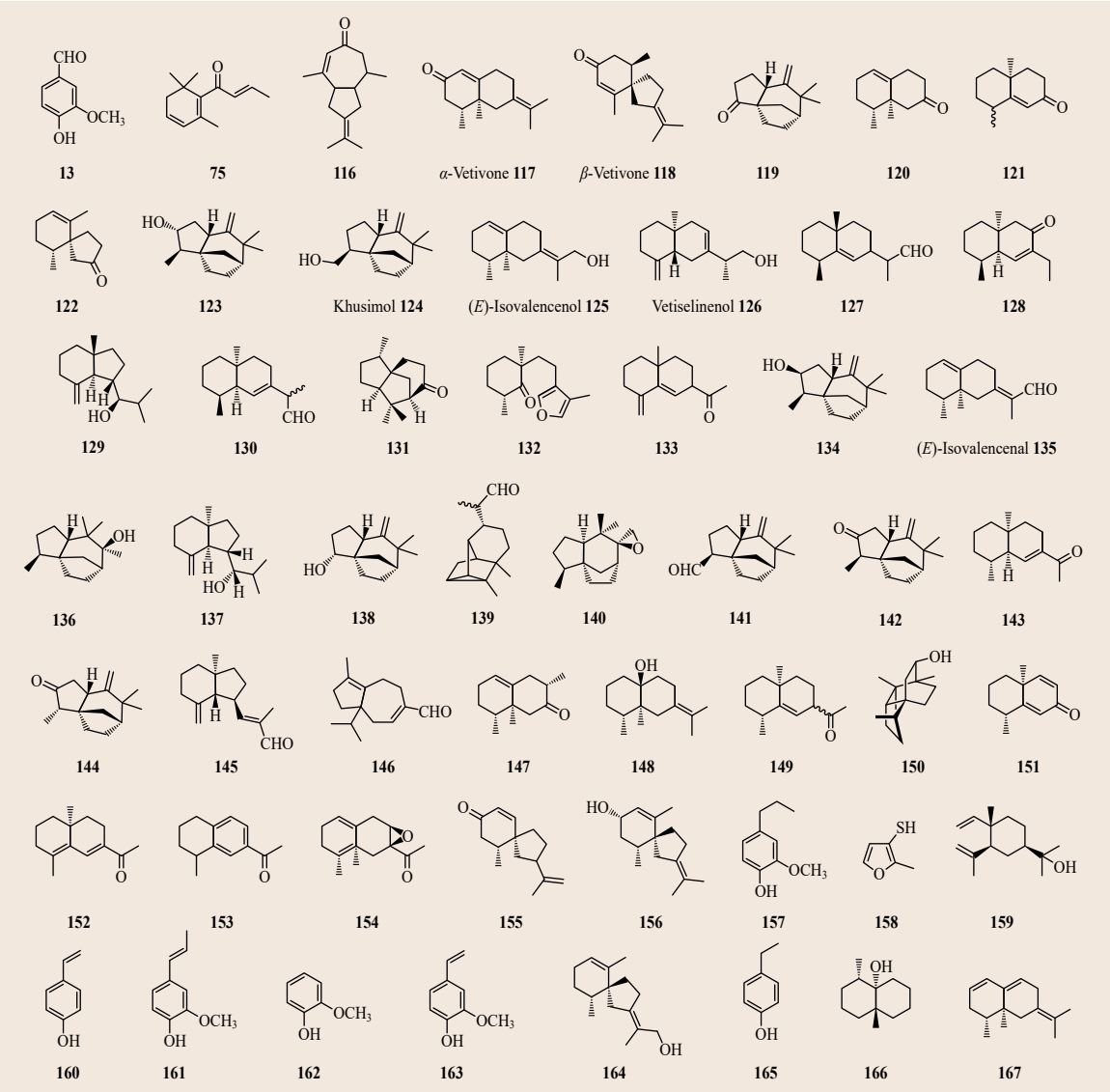

Fig. 3.12 Main constituents and key odorants of vetiver essential oil

of patchouli oil [3.141]. Thereafter, other minor constituents have been reported to contribute to the whole fragrance of patchouli, such as several nitrogen compounds: **175–176** [3.142], a series of pyrazines like **177–180** [3.142, 143] and some atypical sesquiterpene alkaloids **181–188** [3.127, 144]. Since these nitrogen compounds are contained in very low amounts, their olfactory contribution is probably limited, and rather negative [3.127]. *Mookherjee* performed a detailed analysis of patchouli oil and reported that it contained also acidic and phenolic fractions with respective *fatty, propionic* and *tarry, and phenolic* odors [3.127]. Several classical phenols and aliphatic acids had been previously isolated from patchouli oil [3.145], but their contribution to the odor of the whole oil was not determined. *Mookherjee* also isolated the following odorous constituents: cyclohexenones **189–191** and **192** (all possessing strong camphoraceous odors), **193** (strong woody, patchouli), **194** (ambergris), **195** (woody), **196** (celery), and concluded that these compounds contributed to the odor of patchouli oil, which was mainly due to the three sesquiterpenic alcohols **170–171** and the new tetracyclic potent patchouli odorant **197** [3.127, 146]. Researchers from Takasago recently reported the isolation of an isomeric structure **198** [3.147], which may be considered as the revised structure of **197**.

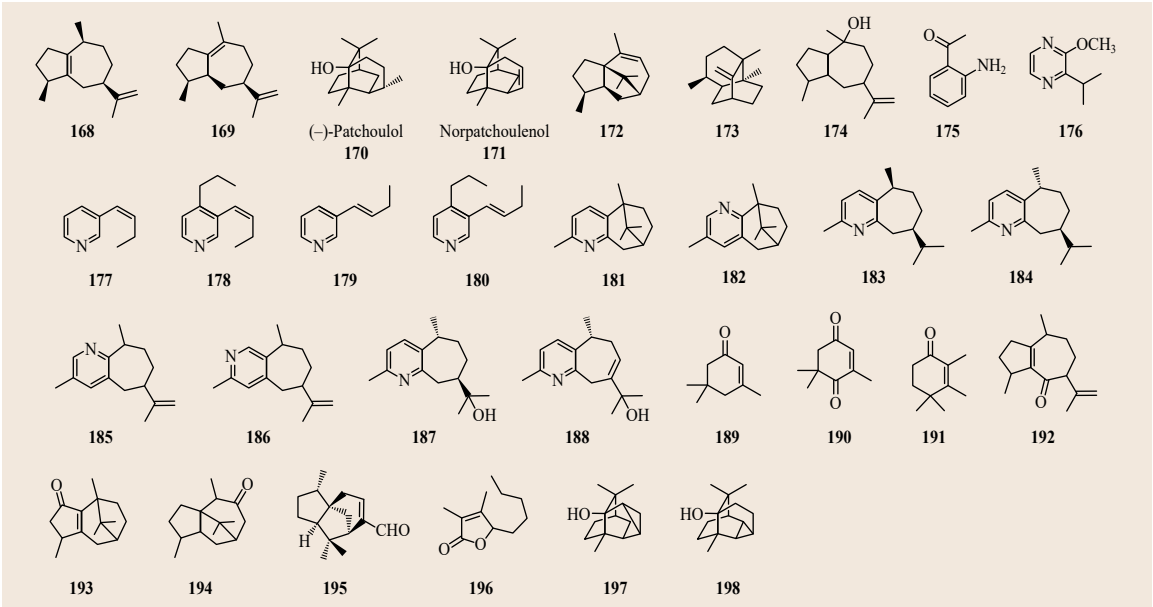

Fig. 3.14 Main constituents and key odorants of patchouli essential oil

3.2.7 Sandalwood

With its characteristic woody fragrance and its fixative properties, sandalwood essential oil is one of the most precious natural raw materials used in perfumery. This oil is produced by steam distillation of the heartwood and roots of various *Santalum* species. *S. album*, an evergreen hemiparasitic tree native from southern India [3.74, 148] is the most appreciated, and several other *Santalum* species are also cultivated for essential oil production, such as New Caledonian *S. austrocaledonicum* [3.149] or Australian *S. spicatum* (Fig. 3.15) [3.150–152].

The main constituents of sandalwood essential oil are (*Z*)-(+)-α-santalol **199** and (*Z*)-(−)-β-santalol **200** (Fig. 3.16), with typical amounts for *S. album* of about 40–50% and 20–30%, respectively. The structures of **199** and **200** were elucidated, respectively, by *Semmler* [3.153] and *Ruzicka* et al. [3.154] and these compounds were soon unanimously recognized as the most important contributors to the fragrance of *S. album* oil. (*Z*)-(+)-α-santalol **199** is described as slightly woody, reminiscent of cedarwood and α-cedrene [3.155], while its β-isomer **200**, usually contained with about half the amount of **199**, is more potent and is responsible for the highly prized typical warm-woody, milky, musky, urinous, animal aspects of sandalwood [3.127, 155–158]. Further minor constituents contributing to the odor of this material were then discovered in subsequent studies. *Demole* et al. analyzed distillation foreruns of *S. album* oil and identified many norsesquiterpenoids

Fig. 3.15 Australian sandalwood (*Santalum spicatum*) (courtesy of Céline Cerutti-Delasalle, Alvert Vieille)

and other constituents such as phenols **7**, **8**, **10**, **160**, **161–163**, and **201–203** which contribute to the smoky note of the sandalwood oil foreruns, as well the strong odorant **204** [3.159].

Nikiforov et al. [3.141, 160] performed GC-O experiments on *S. album* essential oil and described seven substances as the most intense odorous components in addition to **199** and **200**: α-santalene **205**, (*Z*)-α-santalal **206**, (*Z*)-β-santalal **207**, (*E*)-*epi*-β-santalal **208**, (*E*)-β-santalol **209**, α-bergamotol (isomer not specified) **210**, and spirosantalol **211**. The olfactory characterization of highly pure samples of some of these constituents was reported by *Brunke* et al. **209** (medium strength, woody to medicinal) and (−)-(*Z*)-α-*trans*-bergamotol **210** (bright, somewhat woody, waxy,

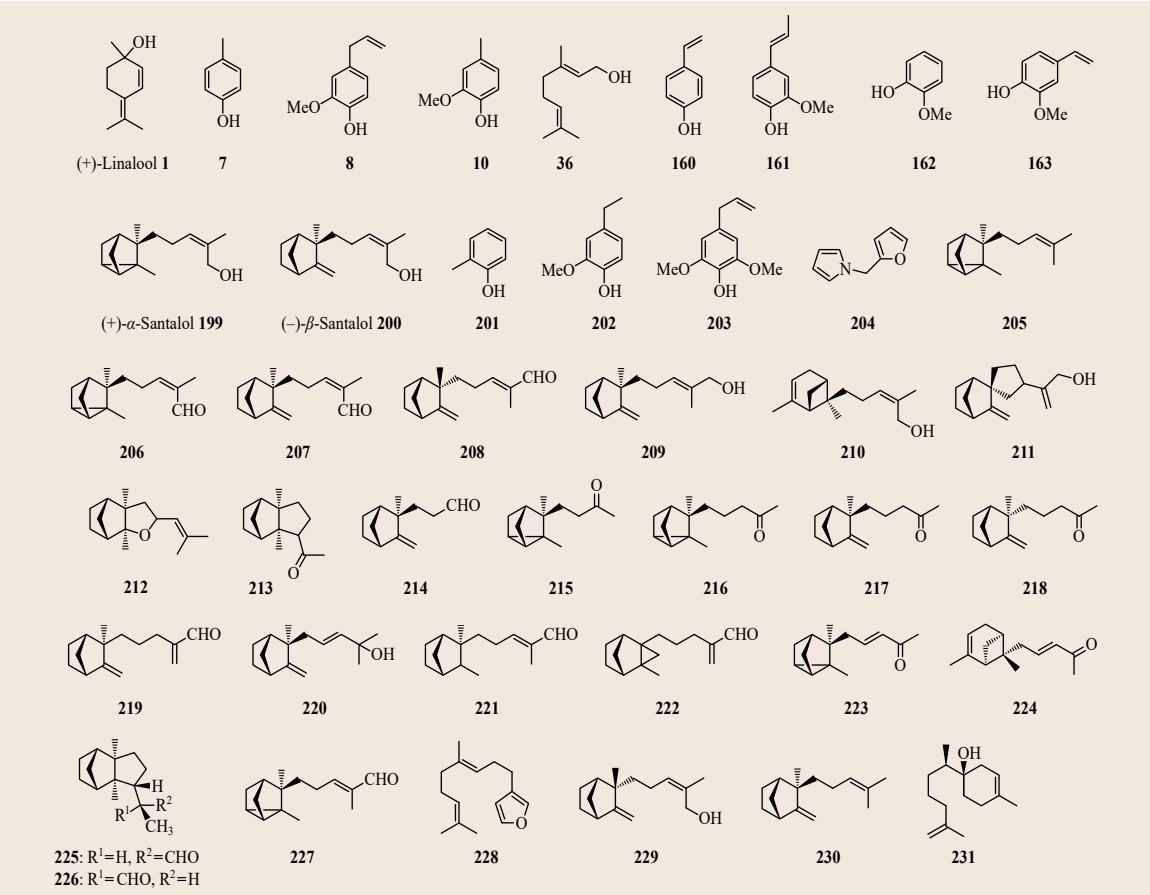

Fig. 3.16 Main constituents and key odorants of *Santalum album* essential oil

reminiscent of agrumen after-note, highly diffusive, and musky) [3.161].

Mookherjee later reported the olfactive properties of **19** additional constituents isolated after an extensive analysis of Javanese and Indian samples. Several of these components showed green, woody, melony and/or ambery tonalities (**212**–**219**) and five odorants were described as possessing very interesting woody odors: **213** (woody, ambery, ionone), **220** (fatty, sandalwood), **221** (sexy, sandalwood), **222** (woody, ambergris), and **223** (sweaty, sexy, woody) [3.127]. The most comprehensive study on the odorous components of sandalwood was published by *Brunke* and *Schmaus* who performed a GC-O (AEDA) of *Santalum album* essential oil [3.157, 158, 162]. Not surprisingly, the highest FD factor was attributed to **200**, followed by **199**. The third most important constituent was then identified as *nor*-α-*trans*-bergamotenone **224**, a trace constituent present in the essential oil at less than 0.01% [3.162]. This compound is responsible for the milky, nutty fatty tonalities perceived in the sandalwood fragrance. The fourth, fifth, and sixth contributors were respectively cyclosantalal **225**, *epi*-cyclosantalal **226** (both introducing green, aldehydic, aqueous, woody notes) and (−)-(*Z*)-α-*trans*-bergamotol **210** (sandalwood character, musky, with citrus tonalities). The next important constituents highlighted by this study were **1**, **7**, **8**, **37**, **204** (roasty, herbaceous), **205** (terpene-like), **220**, **221**, and **227** (all sandalwood), dendrolasine **228** (green, fatty, metallic), **229** (mild sandalwood) and **230** (terpene-like) [3.157, 158]. Finally, in a recent GC-O investigation on the odorous trace components of the *S. album* and *S. spicatum* essential oils, *Braun* et al. showed that **231**, present in less than 0.001% is also an important contributor to the fragrance of the oil, with a strong floral, muguet-like odor [3.163].

3.2.8 Myrrh and Frankincense

Myrrh and frankincense are among the oldest perfume materials known to mankind. They are oleo-gum-resins obtained from trees of the *Commiphora* species (for

Fig. 3.17 Myrrh gum-resin exuding from a *Commiphora myrrha* tree (courtesy of Céline Cerutti-Delasalle, Albert Vieille)

myrrh, Fig. 3.17) or the related genus *Boswellia* in the case of frankincense, which is also named olibanum. These trees grow in various parts of eastern Africa and southern Arabia [3.48]. Frankincense and myrrh gum-resins can be extracted or hydrodistilled to furnish an absolute or an essential oil.

Myrrh oils and extracts possess the characteristic warm-balsamic, sweet, and spicy odor of myrrh (Fig. 3.18). The main constituents of myrrh essential oil are furanosesquiterpenoids such as curzerene **232**, furanoeudesma-1,3-diene **233**, and lindestrene **234**. Compared to the other materials described before, the odorous compounds of myrrh have received much less attention. Wilson and Mookherjee have reported a detailed analysis on a sample of *Aden-Quality* myrrh essential oil. They noticed that the most volatile fraction of the oil contains common sesquiterpenes that have a low olfactory contribution, and that the typical odor of the oil was due to the components contained in the less volatile fraction. In order to characterize its key odorants, the sample was fractionated by column chromatography and preparative GC to furnish several furanosesquiterpenoids. The three main ones (**232**, **233**, **236**) were present at about 12% in the oil. **232** had a *green, woody, geranium odor of moderate intensity, but not characteristic of myrrh* but **234** was described as bearing a *deep rich leathery, incensey, warm, balsamic, sweet very typical myrrh character*. However, its olfactory evaluation was carried out on a fraction containing 25% of **234**. Together with these components, other minor (< 2%) furanosesquiterpenoids were also identified, and their olfactory properties were given. Several constituents showed an odor not particularly reminiscent of myrrh: **235** (relatively weak woody, balsamic odor), **236** (rose, tea, fruity-prunes), **237** (sweet, coumarin-like, tobacco-like), **238** (floral sweet, weak, hyacinth), **239** (weak, green, floral), and **240** (strong rose-tea, reminiscent of calamus). However, two components at 0.1% were reported to possess a strong resinous note: **241** (very heavy, subdued, resinous, compatible with myrrh, but not characteristic) and especially **242** (rich, sweet incense note, very characteristic of myrrh). The headspace of the myrrh gum was also investigated and did not contain these specific furanosesquiterpenes, which is consistent with the fact that the top notes are not characteristic of myrrh [3.164].

The olibanum resin is an uncommon example of a natural raw material in which each terpenic class is represented. Indeed, mono-, sesqui-, di-, and triterpenoids coexist in significant proportions in the resin, and its essential oil usually contains the three first classes. Two main types of compositions are known, distinguished by the main components: octanol **243**

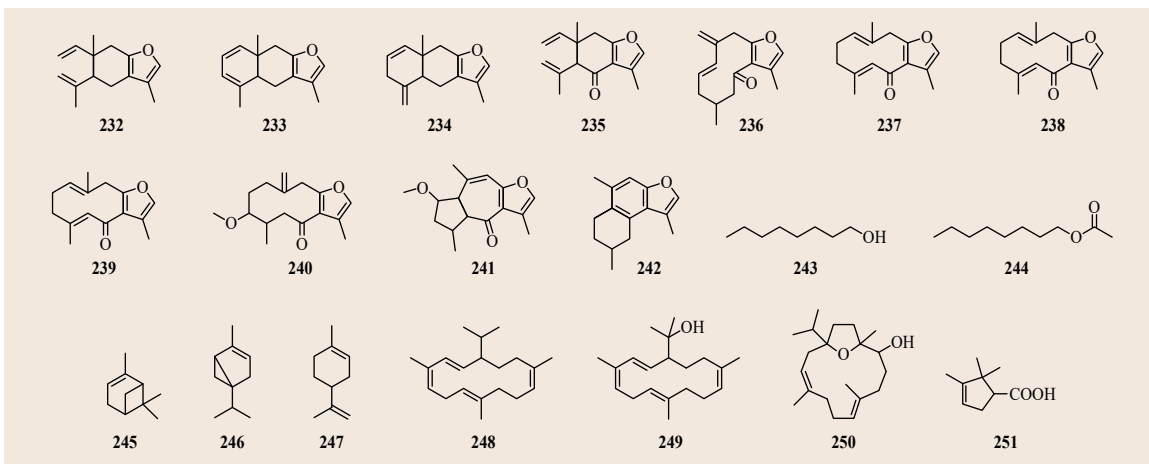

Fig. 3.18 Main constituents and key odorants of myrrh and frankincense essential oils

and its acetate **244** [3.165], or α-pinene **245**, α-thujene **246** and limonene **247** [3.166]. Specific diterpenic constituents are cembrane derivatives such as cembrene **248**, cembrenol **249**, and incensole **250** [3.167].

Paradoxically for a material which was often described as the oldest perfume known to mankind, very little is known about its main odor-active constituents [3.166]. The diterpenic components are odorless, and play no role in the characteristic old church-like base note, except maybe as fixative. However, the contribution of the carboxylic acids to this typical odor is certainly crucial [3.168, 169] and among these, α-campholytic acid **251** was reported to have *a rather strong odor reminiscent of the oil* [3.145].

3.3 Conclusion

For many natural raw materials used in perfumery, the published data concerning the most important odor impact constituents is often very scarce, even for extracts or oils which have been thoroughly analysed. *Weyerstahl* deplored that *synthetic chemists often do not smell their products* [3.131], and it is also regrettable that analysts who isolate pure constituents from fragrant raw materials seldom report their odorant characteristics. The characterization of the key odorants of natural raw materials is therefore still a vivid field of investigation, and as shown in this short overview, many discrepancies can still be found in the literature among the different studies, even on the most important raw materials. Anyway, the complexity of the olfactory system will certainly continue for a long time to supply new problems to the fragrance chemists working with natural extracts and oils, and many answers will be provided by the research studies trying to unravel the mechanisms of olfaction.

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4. Incense Materials

Johannes Niebler

Incense burning is probably the oldest perfuming method known to mankind. This chapter presents an overview of incense materials from different cultures and times, such as frankincense, myrrh, agarwood, palo santo, copal, and many more. Their botanical sources are given, and their chemical composition and odor properties are discussed. The methods of producing incense preparations are also briefly summarized. Incense use may pose certain health risks in the case of prolonged or repeated exposure, but may also have potential in medical applications. Incense use represents a special challenge to aroma research, as odorants can be newly formed during the process of burning.

The word *perfume* stems from the Latin *per fumum*, meaning *through smoke*. This indicates that incense burning was probably the first perfuming method available to mankind. Incense is, to put it simply, fragrant material meant for burning or smoldering, thereby releasing and/or generating the odorants and producing a pleasantly smelling smoke. In contrast to smoking and tobacco use, the smoke is usually not actively or directly inhaled. Such a simple procedure is easily discovered, and reports of incense rituals can be traced back as far as our written history can reach. They are found in varying extents in almost any culture and are still common even in modern times.

The use of incense materials is most often associated with rituals or religious ceremonies (Fig. 4.1). It adds an olfactory dimension to the experience and thus immerses the participants in a holistic manner. Nowadays, with perfumery, everyday scented products and flavored foodstuffs, incense has lost large parts of its attraction. Yet in former times, it can safely be assumed that everyday life was more often than not an olfactory nuisance. Incense use offered the simple (and only) way of scenting rooms, environments, and products, such as churches, ritual places and homes, including clothes, pillows, or hair.

Due to their ubiquitous importance, incense materials have been traded as precious commodities throughout the millennia. Perhaps the most prominent example is the Incense Route of antiquity, which refers to a main trade axis by land for camel caravans transporting frankincense, myrrh, and other goods from port towns in Hadhramaut (Yemen) up north along the Arabian Peninsula. Having passed numerous small kingdoms, tribe territories, and desert tracks, the road connected to Mediterranean trade routes in Petra, Gaza, and Alexandria. Trade by sea brought Arabian frankincense to India and Egypt [4.1, 2].

Even a brief account of cultures using incense and their rituals would easily fill a book on its own (e.g., *Fischer-Rizzi* [4.3]). A short description of its current or historical use in the world religions can perhaps give an impression of the variety of rites associated with incense.

Fig. 4.1 Woman praying with lit incense sticks (courtesy of szefei/iStock)

Fig. 4.2 An altar server with a typical censer (courtesy of Felipe Caparrós Cruz/digicomphoto/iStock)

In Christianity, mainly catholic and orthodox churches use incense for their services, whereas the protestant and other reformed churches have mostly abandoned it. Incense is seen as a symbol of prayers rising to God, and may have served to cover unpleasant smells in former times. Usually, a round metal censer hanging from chains (Fig. 4.2) is carried by altar servers

Fig. 4.3 Utensils for the Japanese Incense Ceremony (*left*), a traditional censer (*middle*) and two incense pieces wrapped in paper sheets (*right*). In the censer, a piece of charcoal is buried in ash and the incense wood is placed on a mica plate on the pile of ash (courtesy of Cristina Jaleru, www.LifeOfVenus.com)

Fig. 4.4 Depiction of the Japanese Incense Ceremony (courtesy of Nippon Kodo)

and the incense mixtures based on frankincense are placed on a glowing charcoal.

In Judaism, the priests performed extensive sacrificial offerings of a specially prepared incense mixture on the Altar of Incense in the Holy Temple in Jerusalem. The ingredients for the incense mixture, called ketoret, are given in the book of Exodus as stacte, onycha, galbanum, and pure frankincense. The Talmud lists several additional ones. However, the exact identity of the components is still a subject of discussion [4.4].

Islamic cultures use incense for pleasure and scenting rooms in their daily lives as well as for treating various medical conditions, but there is no ritualized, traditional use during worship. It is however mentioned as one of the smells that awaits people in Paradise.

In Buddhism, Chinese Taoism, and Hinduism incense offerings in temples are very common in the form of joss-sticks. A huge variety of scented and unscented incense raw materials is used in their production [4.5, 6]. In a nonreligious context, special incense sticks are also used as mosquito repellents in many tropical countries. Japanese high society in the Muromachi period (1336–1573 C.E.) developed Koh-do (香道, literally: Way of Incense, usually called Incense Ceremony, Figs. 4.3 and 4.4), a highly ritualized form of appreciating incense. *Listening* to incense was the central part in games during this Incense Ceremony and agarwood became the essential ingredient [4.7].

In almost all cases, incense use is closely interwoven with other effects, benefits, or interpretations apart from a profane scenting of the environment. Incense burning in the form of sticks or seals has even been used as a method of measuring time in ancient China and Japan [4.8]. Both the raw materials and the smoke could also be applied as a part of therapeutic treatments and medical procedures, for aphrodisiacal, cleansing, energetic effects, for inducing trance or hallucinations and connecting with ancestors, gods, or other spiritual entities [4.9, 10]. All these connotations make it hard to isolate the true potential for modern therapeutic applications, although some attempts have successfully been made. Frankincense, for example, shows promising anti-inflammatory properties due to its content in boswellic acids [4.11]. When looking at the odorant compounds, it is surprising that so few of these commonly available materials have been investigated by modern methods under an olfactory aspect. Quite probably, a large proportion of this knowledge has already been discovered but remains unpublished within the flavor and fragrance industry. Nonetheless, this chapter will try to gather basic information about common incense materials, preparations, and their olfactory properties.

4.1 Selected Incense Materials

All kinds of materials, from both plants and animals as well as occasionally even inorganic sources, have been used as incense in cultures around the world. This list can therefore only encompass a selection of the currently most common or renowned sources for incense materials. For an overview on incense in various culture areas, refer to *Fischer-Rizzi* [4.3], *Rätsch* [4.12], and *Mohagheghzadeh* et al. [4.10]. Common spices, like star anise, cinnamon, or cloves, are often part of incense mixtures. These will not be discussed here, but plenty of literature data is available on their composition and the relevant odorants in the general food chemistry literature.

Most chemical investigations on incense materials focus on the composition of the essential oil or the structural elucidation of new compounds. Investigations of odorant compounds and their specific contribution to the overall odor impression are rare, even for the essential oils. However, the established methodology in aroma research does not directly apply to the very special circumstances under which odorants are released during the burning of incense. High temperatures can lead to a variety of reactions and processes with potentially significant impacts on the odor, such as:

- Degradation or depolymerization of biopolymers (lignans, polysaccharides, proteins, etc.)
- Oxidation reactions, generation of pyrolysis products
- Vaporization of compounds of low volatility
- Release of odorants bound in precursors (glycosidic bonds, protein adducts, chromones).

Evidently, the study of these reactions involves many variables, such as the temperature (gradient), oxygen supply, grain size, or type of heat source. Furthermore, natural materials tend to vary in composition according to factors such as the climate, location, harvest, transportation, and storage conditions. Keeping track of all these influences is close to impossible.

A comprehensive investigation of any incense material would therefore have to involve reliably sourced raw materials and a realistic model of the burning process. A comparative, in-depth investigation of the smoke as well as the raw material would then (ideally) give trustworthy results. Unfortunately, such conditions are so far rarely achieved, and thus our knowledge on the aromas of many incense materials remains fragmentary.

Table 4.1 summarizes basic data on the various incense materials treated in the course of this chapter, and Table 4.2 gives additional odor descriptions of materials.

A study on tobacco additives [4.13] investigated several of the incense materials discussed in the following under pyrolytic conditions (temperature ramp from 300 to 900 °C). The authors only reported the five most abundant peaks in the chromatogram, often with uncertain identifications, but this is until now the only study comparing these materials or extracts

Table 4.1 Basic information on the incense materials treated in this chapter

Name	Alternative names	Botanical species or family	Main commercial source plants	Type of material	Countries of origin	Approx. price range[a] (US$/kg)	Main compound classes in the volatile fraction
Agarwood	Aloeswood, eaglewood, gaharu, oudh, jinkoh	*Aquilaria*	*Aquilaria sinensis, A. crassna*	Heartwood of infected trees	China, Vietnam, Cambodia, Indonesia, SE-Asia	100–100 000	Sesquiterpenoids, chromones
Asafoetida	Devil's dung	*Ferula*	*Ferula assa-foetida*	Gum resin	Iran, Afghanistan, Pakistan	30—100	Disulfides, monoterpenes
Benzoin	Benzoin Sumatra/ Siam, gum benjamin, benzoe, styrax	*Styrax*	*Styrax benzoin, Styrax tonkinensis*	Balsam	Thailand, Cambodia, Laos, Malaysia, Indonesia	20—200	Benzyl benzoate, cinnamic/benzoic acid derivatives
Bissabol myrrh	Scented/ perfumed myrrh, opopanax	*Commiphora*	*Commiphora guidotti, C. holtziana*	Gum resin	Somalia, Kenia	3—80	Monoterpenes, furanosesquiterpenes
Cedar-wood	–	*Cedrus, Juniperus, Cupressus*	*Cedrus atlanticus*	Wood	Morocco, Libanon, USA, China	1—50	Mono- and sesquiterpenoids
Copal manila	–	*Agathis dammara*	*Agathis dammara*	Gum resin	Philippines	3—80	
Copals	Copal blanco, santo Copal oro, amarillo Copal negro	*Bursera*[b] *Hymenaea courbaril*[b] *Protium*[b]	*B. bipinnata, jorullensis, microphylla* *Hymenaea courbaril* *Protium copal*	Gum resin	Mexico, Mesoamerica	–	Sesquiterpenes Monoterpenes Monoterpenes
Dammar	Copal dammar	*Shorea*	*S. wiesneri* (see text!), *S. javanica*	Gum resin	Indonesia	3—80	
Dammar	Black dammar	*Canarium*	*Canarium strictum*	Gum resin	India, South East Asia	3—80	
Dragon's blood	–	*Dracaena, Daemonorops, Croton*	*Daemonorops draco, Dracaena draco, D. cinnabari, Croton lechleri, C. draco*	Resin	Africa, India, Southeast Asia, South America	100—300	Flavonoids
Frankincense	Olibanum, Gum olibanum, Luban	*Boswellia* spp.	*Boswellia sacra, B. serrata, B. papyrifera*	Gum resin	Somalia, Oman, India, Ethiopia, Sudan, Eritrea	5—150	Monoterpenes, esters, diterpenoids
Galbanum	Mother resin	*Ferula gummosa (syn. F. galbaniflua)*	*Ferula gummosa (syn. F. galbaniflua)*	Gum resin	Iran, Turkey, Afghanistan	30—250	Monoterpenes
Guggul	Gugulu, false myrrh, bdellium	*Commiphora*	*Commiphora mukul, C. wightii*	Gum resin	India	5—100	Monoterpenes, furanosesquiterpenes
Juniper berries, leaves, wood	Red cedar, cedarwood	*Juniperus* spp.	*Juniperus virginiana*	Wood, berries, dried leaves	USA	–	

Table 4.1 (continued)

Name	Alternative names	Botanical species or family	Main commercial source plants	Type of material	Countries of origin	Approx. price range[a] (US$/kg)	Main compound classes in the volatile fraction
Labdanum	Ladanum, laudanum	*Cistus* spp.	*C. ladanifer, C. creticus*	Oleoresin	Mediterranean (Spain, Morroco, etc.)	40–400	Mono- and sesquiterpenoids
Mastic	Arabic/ Yemen gum, Chios Mastiha	*Pistacia lentiscus*	*Pistacia lentiscus (var chia)*	Gum	Greece, Mediterranean countries	100–500	Monoterpenes, sesquiterpenes
Myrrh	Heerabol myrrh	*Commiphora*	*Commiphora myrrha (var molmol)*	Gum resin	Somalia	5–100	Monoterpenes, furanosesquiterpenes
Opopanax	–	*Opopanax chironium*	*Opopanax chironium*	Gum resin	Mediterranean countries	–	
Palo santo	Holy wood	*Bursera graveolens*	*Bursera graveolens*	Wood	Mainly Peru, Middle and South America	10–150	Limonene, mono- and sesquiterpenoids
Palo santo	Holy wood, guaiac wood	*Bulnesia sarmienti*	*Bulnesia sarmienti*	Wood	Gran Chaco area (Argentina, Bolivia, Paraguay)	–	Sesquiterpenoids
Peru balsam	Balm of Peru	*Myroxylon balsamum*	*Myroxylon balsamum var pereirae*	Balsam	El Salvador, Central and South America	30–150	Benzoic/cinnamic acid and its esters
Sandalwood	–	*Santalum*	*Santalum album, S. austrocaledonicum, S. spicatum*	Heartwood	India, Sri Lanka, Australia, Indonesia	10–100	Santalols, sesquiterpene alcohols
Storax	Styrax, Levant storax, American storax, sweet gum	*Liquidambar*	*Liquidambar orientalis, L. styraciflora*	Balsam	Turkey, Honduras, Central America	30–100	Styrene, monoterpenes
Tolu balsam	Tolu balm	*Myroxylon balsamum*	*Myroxylon balsamum var balsamum*	Balsam	Columbia, Central and South America	30–150	Benzoic/cinnamic acid and its esters

[a] Determined as ranging from wholesale trading price to highest quality consumer price (approximate values, for orientation only).
[b] Attribution of common names to the species very tentative, multiple other species are traded as copal varieties.

thereof under pyrolytic conditions. Their main aim was identifying sources for harmful pyrolysis byproducts known as *Hofmann analytes*, for example, phenols and benzene. The reported compounds for incense materials treated in this chapter are given in Table 4.3. The data is therein compared to selected literature data, from which the five most abundant peaks were also listed.

It has to be noted that the study by *Baker* and *Bishop* [4.13] did not investigate their raw materials under nonpyrolytic conditions, so that no reliable conclusion can be drawn as to which compounds are newly formed by pyrolytic processes and which are just evaporated from the mixtures. For frankincense, for example, the data is in very good agreement with the literature data from a study by *Hamm* et al. [4.18], indicating that these major compounds evaporate unchanged from the olibanum oil. The same applies to galbanum and opoponax oil, whereas all other raw materials show distinct differences, which could either be attributed to changes occurring during the pyrolysis or simply be the result of natural variations in the raw materials or incorrect identification of compounds.

Many of the raw materials discussed in this section are plant exudations. A short note on their terminology [4.2] should help to clarify the expressions used in the following: Gums are water soluble, polysaccharide-based saps, whereas resins are

Table 4.2 Odor descriptions of a selection of incense raw materials, supplied by perfumers from Symrise AG (Germany)

Name	Source plant	Odor description
Agarwood	*Aquilaria*, species not stated	Rich sweet woody, wet wood-like, moist
Asafoetida	*Ferula assa-foetida*	Onion
Cedarwood	*Cedrus atlanticus*	Woody, dry, carrot, peppery
Dammar	Unknown	Smoky, birch tar-like
Frankincense	*Boswellia sacra*	Pine, woody, peppery
Galbanum	*Ferula gummosa*	Green, bitter, peas, earthy
Guggul	*Commiphora mukul*	Resineous, balsamic, spicy, olibanum
Gum benzoe Siam	*Styrax tonkinensis*	Balsam, powdery, cinnamic, ambra, slightly smoky, almond, prune
Gum benzoe Sumatra	*Styrax benzoin*	Balsamic, powdery, cinnamic, ambra
Labdanum	*Cistus ladanifer*	Waxy, incense, resin, ambra, animalic, fruity, prune
Myrrh	*Commiphora myrrha*	Liquorice, mushroom, hot and cold effect, wicker, undergrowth
Opopanax	*Opopanax chironium*	Mushroom, myrrh, nut, quinoline, tobacco, powdery
Palo santo	*Bulnesia sarmienti*	Greasy, pine, nut, tobacco, woody, powdery
Peru balsam	*Myroxylon balsamum var pereirae*	Balsam, vanilla, chocolate, powdery
Sandalwood	*Santalum album*	Woody, creamy, incense, powdery, smoky
Storax	*Liquidambar styraciflora*	Orange, animalic, almond, cinnamon, glycine, rose, peony
Tolu balsam	*Myroxylon balsamum var balsamum*	Tuberose, anise, balsam, vanilla, heliotrope, ciste, olibanum, tobacco, chocolate, powdery

lipid soluble and contain primarily terpenoid or phenolic compounds or a mixture thereof. The definition of balsams (balms) is not entirely specific, but it generally refers to soft, malleable exudations that are usually dominated by cinnamic and benzoic acid derivatives and are highly fragrant. Oleoresins are characterized by a high percentage of volatile terpenoids, and are therefore relatively fluid. It is evident that a clear distinction between them is not always possible, and many products are mixtures of these groups.

4.1.1 Agarwood

Agarwood, also called *gaharu* or *aloeswood* as well as *oud* in Arabic or *jinkoh* 沈香 in Japanese, is produced by tall, evergreen trees of the genus *Aquilaria* and, to a lesser extent also by *Gyrinops* and *Gonystylus*. They

Fig. 4.5 A large piece of agarwood presented during a Japanese Incense Ceremony. The dark color usually indicates a high oil content (courtesy of Cristina Jaleru, www.LifeOfVenus.com)

are typically found in southeast Asia, and most of the agarwood originates from *Aquilaria sinensis* Merr., *A. crassna* Pierre ex Lecomte, or *A. malaccensis* Lam. The trees respond to microbial infection by fungi or wounding with the secretion of a dark, resinous oil into the heartwood, roots, or branches; thus protecting it from further infection. The formerly lightweight and pale wood becomes dark brown to black and so dense that the best qualities of agarwood sink in water (Fig. 4.5). In nature, this process is rarely found, making agarwood an extremely luxurious commodity. In the recent decades plantations have been established, where artificial inoculation or wounding of the trees leads to the production of agarwood (an example can be found in *Liu* et al. [4.21]). In the wild, the trees have become rare due to overharvesting and exploitation [4.2]. Indeed, some species are now endangered, and their trade is regulated under CITES, the Convention on International Trade in Endangered Species of Wild Flora and Fauna. Agarwood is often reported as the most expensive wood in the world. Its price per gram for higher qualities often by far exceeds that of gold. The wood is highly valued as incense material, particularly in Arabic and Asian cultures. In Japan, agarwood is the essential ingredient for all incense rituals and ceremonies. Furthermore, steam distillation yields agarwood essential oil, which perfumers treasure for its unique *oud* character. In traditional medicine of various Asian countries, it is also a pharmaceutical drug for a large variety of purposes and generally considered an aphrodisiac.

Typical agarwood volatile constituents include a large variety of oxygenated sesquiterpenes, mostly based on the agarofuran, guaiane, selinane, and eu-

Table 4.3 Comparison of the top five constituents (by peak area %) for pyrolysate (data from [4.13]) and the volatile fraction (data from various literature sources, referenced in the table)

Ingredient	CAS number	Composition of pyrolysate	Area (%)	Composition volatile fraction	Area (%)	Reference
Peru balsam oil	8007-00-9	Benzyl benzoate Benzyl cinnamate Benzoic acid Cinnamic acid Vanillin	58.1 36.1 1.5 0.7 0.6			
Benzoin absolute (Sumatra)	84012-39-5	Cinnamic acid Benzoic acid Cinnamyl cinnamate Benzyl cinnamate Benzyl benzoate	50.4 10.5 3.5 3.0 2.2	Cinnamic acid Benzoic acid Cinnamyl cinnamate Benzyl cinnamate Benzyl benzoate Unknown Styrene	3.5 1.7 0.9 3.3 76.1 2.2 2.3	[4.14]
Galbanum extract (resinoid)	9000-24-2	α- and/or β-Pinene Valencene Unidentified compound Hydroxycoumarin α-Amorphene and/or α-muurolene	21.4 6.6 5.7 4.9 4.3			
Galbanum oil	8023-91-4	β-Pinene γ-Carene α-Pinene β-Phellandrene + limonene Cymene	48.6 14.5 8.4 3.6 3.1	β-Pinene Δ^3-carene α-Pinene Limonene endo-Fenchylacetate	59.0 0.2 36.6 0.3 2.7	[4.15]
Labdanum absolute	68917-77-1	Unidentified compound Aromadendrene and/or gurunene Acetamide Trimethylnaphthalene? Pentylfuran?	36.8 12.9 10.5 5.3 3.3			
Labdanum oil	8016-26-0	α-Pinene Ledene Valencene Cymene Pinocarveol	10.9 10.8 4.0 3.9 3.7	 Ledene p-Cymene *trans*-Pinocarveol Viridiflorol Cubeban-11-ol Borneol	 9.3 0.4 1.8 4.3 4.1 2.0	[4.16]
Labdanum oleoresin	977092-72-0	Methyl ionone Unidentified compound Ethyl hydrocinnamate and/or ethylphenylpropionate Ethyl heptadecanoate Hydrocinnamic acid and/or phenylpropionic acid	23.4 20.9 16.2 12.2 7.7			
Myrrh oil	8016-37-3	Unidentified compound Dimethyl(methylethenyl)-ethenyldihyrobenzofuran Unidentified compound β-Elemene Cadinol and/or β-cubebene	25.5 17.9 9.4 3.5 2.2	 β-Elemene τ-Cadinol Furanoeudesma-1,3-diene Furanodiene Lindestrene Germacrene B	[4.17] 8.7 1.6 34.0 19.7 12.0 4.3	

Table 4.3 (continued)

Ingredient	CAS number	Composition of pyrolysate	Area (%)	Composition volatile fraction	Area (%)	Reference
Olibanum oil (frankincense)	8016-36-2	α-Pinene	34.6	α-Pinene	23.2	[4.18]
		Limonene	15.0	Limonene	22.4	
		Caryophyllene	4.8	Caryophyllene	6.9	
		Sabinene	4.6	Sabinene	2.2	
		β-Myrcene	3.7	β-Myrcene	4.5	
Opoponax oil (*Commiphora*)	8021-36-1	Santalene	25.6	α-Santalene	15.8	[4.17]
		α-Bisabolene	21.8	*cis*-α-Bisabolene	22.2	
		Ocimene	16.1	*E*-β-Ocimene	33.0	
		α-Bergamotene + dimethyl(methylpentenyl)-bicycloheptene?	7.1	α-Bergamotene	3.0	
		β-Bisabolene	4.1		n.d.	
				trans-β-Bergamotene	6.6	
Sandalwood oil yellow	8006-87-9	α-Santalol	44.3	α-Santalol	48	[4.19]
		β-Santalol	23.9	β-Santalol	20	
		Cymene	7.1			
		Epi-Santalol	3.8			
		Curcumene?	2.7	*E*-β-Curcumen-12-ol	2	
				Z-α-trans-Bergamotol	6	
				Z-γ-Bisabolen-12-ol	2	
Styrax extract (resinoid)	8046-19-3	Methyl styrene	47.9			
		Cinnamyl cinnamate	36.5			
		Benzyl cinnamate	2.8			
		Cinnamic acid + β-caryophyllene	1.9			
		Cinnamyl alcohol	1.7			
Styrax oil	8024-01-9	Phenylpropene and/or methylstyrene	44.1			[4.20]
		Cinnamyl cinnamate	36.8			
		Benzyl cinnamate	2.6			
		Phenyl propanol	2.3	Benzenepropanol	3.4	
		Cinnamic acid + β-caryophyllene	2.1			
				Styrene	81.9	
				α-Pinene	3.5	
				Cinnamyl alcohol	6.9	
				4-Ethylphenol	1.9	

desmane skeleton. Notable is the complete absence of monoterpenes. Chromone derivatives are characteristic markers for agarwood, but have a low volatility and are not found in hydrodistillates [4.22].

A variety of sesquiterpenes in combination with smaller volatiles seem to be responsible for the typical, highly valued odor of agarwood [4.22], although this has not been satisfactorily investigated. Examples for potent sesquiterpenes are dihydrokaranone **1** and karanone **2** [4.23, 24] (Fig. 4.6). *Pripdeevech* et al. [4.25] identified many odor-active compounds in agarwood oils, but their study shows some irregularities and should therefore be considered with caution. For example, the presence of large amounts of isoamyl dodecanoate (13–55%) in all three investigated essential oils is rather surprising, as this compound has not been found in any other study on the essential oil of agarwood. Additionally, the authors reported a variety of monoterpenoids, whereas the review of the literature until 2010 by *Naef* states that *no monoterpenes at all have been detected* [4.22]. Moreover, the odorants detected were rated in intensity according to their relative peak area, which is not a valid conclusion to anyone familiar with flavor analysis. Nonetheless, this study showed a large variety of odor-active compounds being present in agarwood essential oils, their relative potency however needs further investigation.

In a different study by *Hashimoto* et al., chromones were shown to liberate aromatic compounds during burning (Fig. 4.7), a rare example of an investigation of the odorant generation in the process of incense burning [4.26]. As the review by *Naef* [4.22] summarizes, there are several other studies on the smoke of agarwood. Mostly, sesquiterpenes and a large vari-

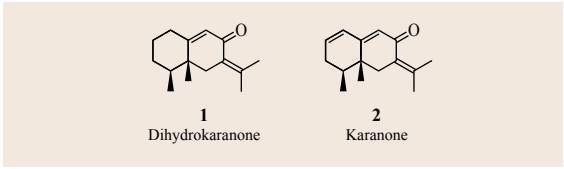

Fig. 4.6 Two odor-active sesquiterpenoids from agarwood

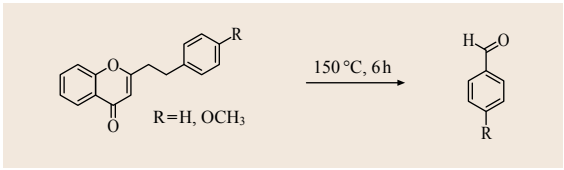

Fig. 4.7 Chromone derivatives were shown to release fragrant aldehydes, in this case benzaldehyde and 4-methoxybenzaldehyde during pyrolysis at 150 °C in the course of 6 h [4.26]

ety of aromatic compounds (e.g., phenol, anisole, or guaiacol) were detected, the former mostly unchanged except under high temperatures (500 °C), the latter presumed to be pyrolysis products from the chromones or from wood pulp (lignin). The pyrolytically generated compounds could be responsible for the change in the odor profile over a time period of 12 min from *floral*, *woody balsamic notes* to *heavy woody*, *phenolic*, *animalic notes* as observed by *Kaiser* [4.27].

4.1.2 Benzoin Siam/Sumatra

Firstly, some clarification on the naming of benzoin resins and storax (Sect. 4.1.13) is needed. Styrax is the resin derived from trees of the genus *Styrax*, whereas storax is obtained from *Liquidambar* species. However, some literature sources use the two names synonymously or interchangeably [4.2]. *Styrax* resins are also well-known under their other names: benzoin (resin), benzoe, or gum benjamin. For an easier reading experience, they will be treated here under the name *benzoin*, which is not to be confused with the chemical compound of the same name.

Two types of benzoin are traded on a larger scale: Siam benzoin from Thailand and Laos or Sumatra benzoin from the islands Sumatra, Java, and the Malay Peninsula. They are produced by repeated incisions made to the bark of *Styrax tonkinensis* Craib ex Hartwich for Siam benzoin and *Styrax benzoin* Dryand. for Sumatra benzoin. The latter product might also be obtained, though to a far lesser extent, from various other *Styrax* species occurring in the forests of Sumatra, such as *S. paralleloneurus* Perkins, *S. ridleyanus* Perkins and *S. subpaniculatus* Jungh. & de Vriese [4.2].

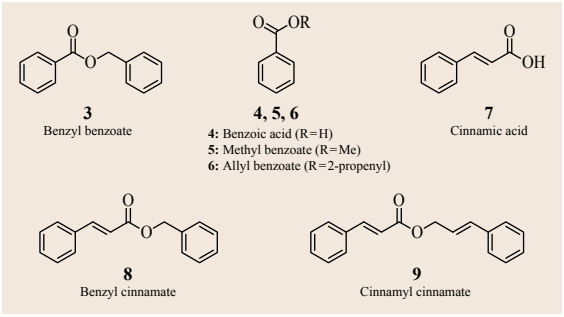

Fig. 4.8 Major constituents of *Styrax* resins

The volatile fraction of these two resins has been investigated in detail in a series of recent publications [4.14, 28, 29], in addition to several older studies summarized by *Langenheim* [4.2]. The two sorts can be well distinguished by their chemical composition: though both contain around 70–80% benzyl benzoate **3** in the volatile fraction, Siam benzoin contains benzoic acid **4** and its derivatives, like methyl or allyl benzoate **5**/**6** (Fig. 4.8). By contrast, Sumatra benzoin is, apart from **3**, dominated by cinnamic acid **7** and its derivatives like benzyl cinnamate **8** or cinnamyl cinnamate **9** [4.14]. Both balsams smell intensely balsamic [4.30, 31], and though their odorants have not been investigated in detail, it can safely be assumed that at least the aforementioned compounds contribute to the odor profile of benzoin. Siam benzoin is more valued in the flavors and fragrances industry due to its better aroma, whereas Sumatra benzoin is more commonly used in pharmaceutical applications [4.2].

4.1.3 Copal and Dammar

The designations *copal* and *dammar* (also spelled damar) are widely used for various resins, often with uncertain or unreliable botanical origin. Generally speaking, copal and dammar designate harvested as well as fossilized resins from trees of families Burseraceae, Dipterocarpaceae, Araucariaceae, and others. Historically, the distinction between dammars and copals was based on physical properties or local designations more than on botanical origin [4.32]. In colonial times, their export as nontimber forest products for the paint and varnish industries was introduced to many cultures in Southeast Asia [4.33].

Several sorts of copal, which are well-known incense materials, are commonly available on markets in Mesoamerica and have been used since precolonial times. They are frequently mentioned in literature and folklore as offerings to the gods in most Mesoamerican cultures in the form of incense or raw material. In Mayan cultures, copal is commonly known as *pom* and

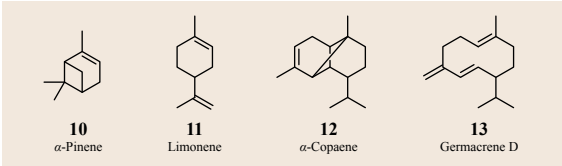

Fig. 4.9 Major compounds reported for various copals

transforms into *food for the gods* when it is burned as incense. Furthermore, the gums and resins from various trees were used as glue [4.34], medical ointments, and in modern times also for paint binders and varnishes.

Case et al. [4.35] investigated the three types of copal by performing an extensive literature search and GC-MS measurements and gives an excellent overview over the incense use of copals. They came to the tentative conclusion that copal blanco is commonly obtained from *Bursera bipinnata* Engl., copal oro from *Hymenaea courbaril* L. (from the family Fabaceae), and copal negro from *Protium copal* Engl. Considering the multitude of species from both *Bursera* and *Protium* growing in the area, it is highly unlikely that a solid attribution of trade names to a specific species will ever be possible. The authors also suggest that the color distinction between the copals comes from the tapping and processing techniques rather than from the source plants [4.35]. *Rätsch* [4.12], for example, lists 20 *Bursera* and 5 *Protium* species as sources of various copals. The samples depicted in this chapter (Sect. 4.1.15) were obtained from incense shops and claimed to be from *Protium copal* or *Bursera jorullense* for copal blanco and *Bursera microphylla* for Copal negro.

All three resins in the study by *Case* et al. [4.35] were dominated by mono- and sesquiterpenes. The main compounds in both copal oro and copal negro were α-pinene **10** and limonene **11** along with various other terpenes, whereas copal blanco was reported to contain mostly α-copaene **12** and germacrene D **13** and almost no monoterpenes at all (Fig. 4.9). *Stacey* et al. [4.34] used the triterpenic composition to categorize commercial and archaeological samples, and found that the commercial samples from local markets were probably *Bursera*-derived, but the inherent variability between the different *Bursera* species and lack of authentic reference material made it impossible to come to reliable conclusions.

Three additional products are also traded under the name copal or dammar. Firstly, copal manila is a translucent gum obtained from *Agathis dammara* (Lamb.) Rich. in the Philippines. Secondly, dammar usually comes from *Shorea wiesneri*, a species that does not exist in botanical literature and appears to be only a common trade name. Its exact botanical origin is therefore highly uncertain, but a potential major source of this dammar could be *Shorea javanica* Koord. & Valeton (Sumatra, Indonesia), reported in literature as economically important and tapped for its resin [4.2, 36]. Thirdly, black dammar from *Canarium strictum* Roxb. is, contrary to its name, a light yellowish to translucent, brittle resin. All three have multiple uses, as for instance paint binders and varnish, traditional medicine, medical glue components, or incense in the local cultures [4.37, 38].

4.1.4 Cedarwood

Cedarwood denominates a variety of woods from different trees and areas. Literature about its use as incense material is scarce. Cedarwood is mainly used as timber or for the distillation of essential oil for aromatherapy and perfumery (Chap. 3). In a strict sense, cedarwood comes from the genus *Cedrus*, with the most important species *Cedrus atlanticus* (Endl.) G. Manetti ex Carrière (Morocco, Algeria), *C. deodara* (Roxb. ex D. Don) G. Don (Himalaya) and *C. libani* A. Rich (Cedar of Lebanon). Commercial cedarwood (oils) often also originate from *Cupressus* spp. (Chinese cedarwood) and *Juniperus* spp. (Texan/Virginian cedarwood) [4.39]. Juniper berries, leaves, and wood, for example, from *Juniperus virginiana* L., were common incense materials in native American cultures [4.3].

4.1.5 Dragon's Blood

Dragon's blood is a bright to dark red resin, its name derived from medieval myths woven around it. However, the scholars of antiquity like the anonymous author of the *Periplus of the Erythrean Sea* already knew its true source to be a plant secretion from the island Socotra [4.40]. Today, three types of dragon's blood are known and traded: the resins from *Dracaena*, *Daemonorops*, and *Croton* species [4.2, 41].

Dracaena draco L. from the Canary Islands is closely related to *Dracaena cinnabari* Balf.f. from Socotra, both trees of extraordinary physical appearance. They exude a red oleoresin from small cracks in the trunk or from artificial woundings. *Dracaena* resins are characterized by a large variety of flavonoids and related polymers. The red color is caused by dracoflavylium **14** and other flavonoid colorants like dracorhodin **15** and dracorubin **16** [4.42] (Fig. 4.10).

Daemonorops draco Blume and other *Daemonorops* species are rattan palms native to parts of India and Southeast Asia (Sumatra, Borneo). The fruits of *D. draco* are covered in a red shell from which the resin is obtained; usually the harvested fruits are shaken to loosen the resin. This is the only type of

dragon's blood which enters world trade on a noteworthy scale [4.43].

Croton lechleri Müll.Arg., *C. draco* Schltdl. and several other *Croton* species are the source of South American dragon's blood, obtained from cuttings in the tree trunk. The main constituents of the essential oil from the bark of *C. lechleri* were reported to be sesquiterpenoids, namely sesquicineole **17**, α- **18** and β-calacorene **19**, 1,10-di-epi-cubenol **20** and epi-cedrol **21**, along with some monoterpenes like α-pinene **10**, p-cymene **22**, limonene **11** and borneol **23** [4.44, 45] (Fig. 4.11).

All types of dragon's blood have a large variety of medical uses in the local cultures, including treatment for open wounds and diarrhea. Like many other resins, it has also been used in some cases as lacquer in arts and crafts. As an incense material, it is particularly attractive for coloring mixtures, whereas its scent is neither particularly strong nor characteristic [4.12]. The attractive appearance as a dark red solid or bright red powder makes dragon's blood an interesting material for all kinds of scams. For example, it has been sold as *red rock opium* to drug users in the USA, although there is no evidence for any psychoactive substances in dragon's blood [4.46].

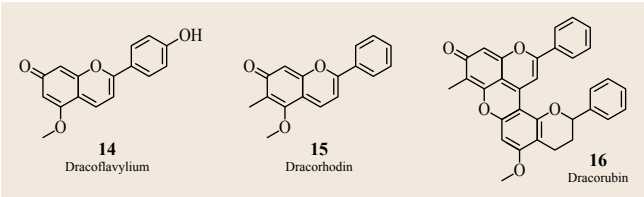

Fig. 4.10 Flavonoid compounds responsible for the intense red color of dragon's blood

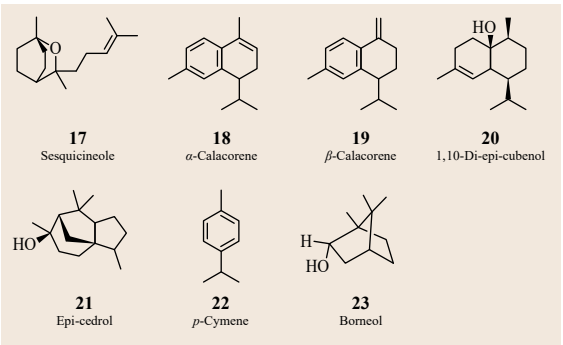

Fig. 4.11 Selected compounds from *Croton lechleri*

4.1.6 Frankincense

Frankincense is a central ingredient in many incense mixtures in Europe, Asia, India, and in Arabic countries. The frankincense trees of the genus *Boswellia* are tapped and their air-dried gum resin harvested, typically appearing as pea- to thumb-size grains of translucent, whitish-yellow to dark brown color. Four species are of economic relevance: *Boswellia sacra* Flueck. is native to Oman and Somalia, and *Boswellia carteri* Birdw. is generally considered to be the same species, differing only slightly in phenotype and habitat. Its gum resin (Arabian Frankincense) is said to be the highest quality available and was traded on the Incense Route since antiquity. *Boswellia serrata* Roxb. (Indian Frankincense) is found in parts of India and therefore deeply rooted in Ayurvedic medicine as well as in Hindu and Buddhist incense usage. *Boswellia papyrifera* Hochst. grows in coastal regions of Sudan, Eritrea, Ethiopia, and northern Somalia, and is often traded as Eritrea (or Ethiopia) type of incense. By contrast, *Boswellia frereana* Birdw. is of low commercial interest, typically found in Somalia and mostly used by locals [4.2, 47].

Applications typically range from direct use as incense, blending with other incense materials to therapeutic use in complementary and modern medicine. The so-called boswellic acids, pentacyclic triterpenic acids found in the resins, have been shown to possess anti-inflammatory properties [4.11]. The hydrodistillate of frankincense resins is a common perfumery raw material and often used in aromatherapy.

The volatile fractions of each species differ notably in composition and odor. Apart from *B. papyrifera*, which is dominated by *n*-octyl acetate **24** and *n*-octanol **25**, all other *Boswellia* resins mainly consist of mono-, sesqui- and diterpenes (Fig. 4.12). *Boswellia sacra* contains α-pinene **10** and limonene **11** and a variety of sesquiterpenes, whereas *Boswellia serrata* shows higher amounts of α-thujene **26** and a differing sesquiterpene profile. All three contain specific diterpenoid marker substances for frankincense, such as serratol **27**, incensole **28**, incensole acetate **29**, and others. Detailed compositions and a total of over 300 identified compounds in *Boswellia* resins are the result of a large number of chemo-analytical studies on the volatile constituents of frankincense, most of which have been summarized in the review by *Mertens* et al. [4.48]. Specific and comprehensive investigations concerning the odor-activity of these compounds are still missing. *Woolley* et al. [4.49] pointed out that differences in the enantiomeric ratios of α-pinene **10** and limonene **11** might allow to distinguish between *B. sacra* and *B. carteri* just by smelling the essentials oils, based on the olfactory differences for the enantiomers of both compounds. *Hasegawa* et al. [4.50] reported incensole **28** and several of its derivatives to be important contributors to the smell of *B. papyrifera* resins. Comprehensive investigations of the

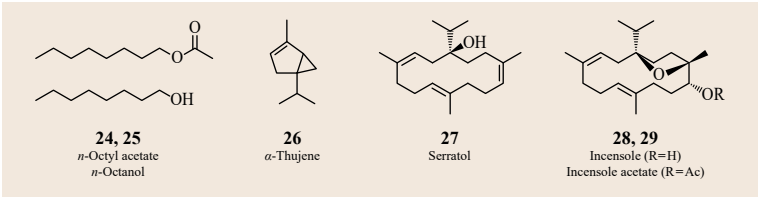

Fig. 4.12 Compounds found in *Boswellia* resins

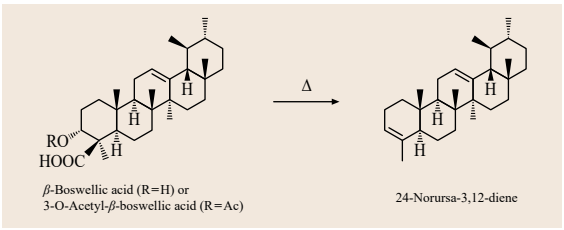

Fig. 4.13 Generation of 24-norursa-3,12-diene from boswellic acid precursors, an example of nortriterpenoids generated in the pyrolysis of frankincense (after [4.54])

odorant compounds in *Boswellia* resins that are currently conducted in our lab indicate that the different sorts of frankincense also differ extensively in their main odorants.

The smoke of frankincense has been investigated analytically with largely differing results. *Pailer* et al. analyzed pyrolysed fractions of the resins of *B. carteri* and found mostly newly generated compounds [4.51–53]. By contrast, *Basar* [4.54] observed mainly unchanged compounds, but was able to show the generation of nortriterpenic compounds in *B. carteri* pyrolysate by dehydration, deacetylation, and decarboxylation reactions of boswellic acids (Fig. 4.13). The apparent changes in the odor profile during pyrolysis have not yet been attributed to specific compounds or reactions.

4.1.7 *Ferula* Resins: Galbanum and Asafoetida

Some species of the giant fennels (*Ferula*) yield resins from their thick root when cut at the base. Two types have been known and traded for millennia: Asafoetida and Galbanum.

Asafoetida is the brown, sometimes greenish gum resin obtained primarily from *Ferula assa-foetida* L., a perennial plant of over 2 m in height with yellowish flowers and a large carrot-like root. Up to 17 other species like *F. foetida* Regel, *F. jaeschkeana* Vatke and *F. narthex* Boiss. yield a similar resin sold under the same name [4.55]. Typically, the plant is cut-off and incisions are made to the base of the root to collect the resin [4.2]. The essential oil of *F. assa-foetida* consists of over 40% but-2-yl-1-propenyldisulfide **30**, **31** (*E*-*Z*-ratio 7 : 3), accounting for its pungent, garlic-like, sulfurous smell. Its use is by far more common in traditional medicine applications and as a spice than as incense material [4.55].

Galbanum is a brown to yellowish, soft gum resin obtained from the lower stem or root of *Ferula gummosa* Boiss. (syn. *Ferula galbaniflua* Boiss. & Buhse). Some sources list *F. gummosa* and *F. galbaniflua* as distinct species based on chemotaxonomic evidence [4.15]. These plants grow in countries like Turkey, Afghanistan, and Iran and have been well-known since before biblical times. Galbanum is one of the ingredients in sacred incense from the Bible, has a multitude of reported uses for various medical conditions, and is also believed to be a contraceptive [4.2]. The chemical composition of the volatile oil, usually obtained by steam- or hydrodistillation, is dominated by α-**10** and β-pinene **32**, accounting for about 16–36% and 40–66%, respectively [4.15]. However, these are not major contributors to the characteristic odor of galbanum, which is generally described as powerful green, woody, fruity, and balsamic. Several authors investigated the odor of galbanum oil and found 1,3,5-undecatrienes (e.g., **33**) [4.56], alkoxyalkylpyrazines **34**, **35** [4.57], undeca-6,8,10-trienones **36**, **37** [4.58], and thioesters **38**, **39** [4.59] to be high-impact odorants [4.60] (Fig. 4.14).

Interesting to note is the occurrence of a large variety of sesquiterpene umbelliferone ethers [4.55, 61] in both gum resins, which could potentially serve as precursors for pyrolytically generated odorants; this has however not been investigated until now.

Another gum resin called silphium or silphion was highly sought after in antiquity and came from the Greek colony of Cyrene, located at the Mediterranean coast of modern Libya. It is generally believed to be a now-extinct *Ferula* species [4.2].

4.1.8 Labdanum

Labdanum is an oleoresin from *Cistus ladanifer* L. or *C. creticus* L. (common name: gum rock rose), a wild shrub growing in areas around the Mediterranean Sea, mostly in Morocco, Spain, France, and on islands like Cyprus and Malta. It exudes a dark brown to black, soft, and sticky oleoresin on its leaves and twigs. The lab-

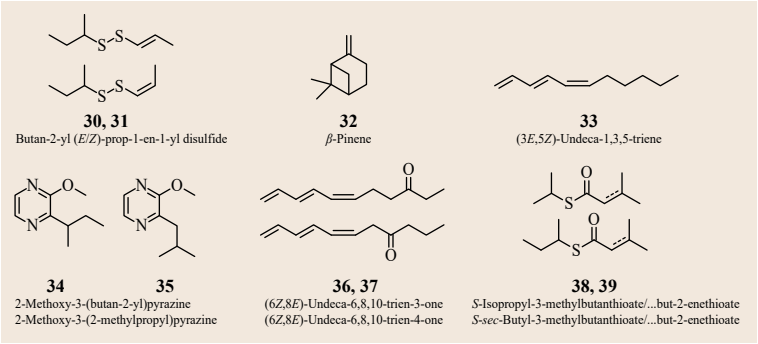

Fig. 4.14 Odorant compounds from asafoetida and galbanum

danum is obtained by extraction of plant materials with boiling water and the oil by steam distillation. Historically, shepherds scraped the resin off the pelt of their sheep or goats, who collected it by brushing through the shrubs of *Cistus* species. It used to be and still is a popular fragrance raw material, with documented use in ancient Egypt and discussed as the onycha of the ketoret incense mixture in Judaism [4.62]. Nowadays, it is prized for its ambergris-like tonalities and fixative properties [4.12, 63].

The essential oil is a highly complex mixture of over 300 compounds, mostly mono- and sesquiterpenoids, but also some diterpenoids. Among the main compounds reported in literature are α-pinene **10**, camphene **40**, bornyl acetate **41**, ledene **42**, viridiflorol **43** and cubeban-11-ol **44** (Fig. 4.15). Labdanum oil contains notable amounts (around 1%) of ambroxan **45** and is considered one of the very few natural sources of this important ambergris odorant [4.16, 64, 65].

4.1.9 Mastic

Mastic is the dried gum drops from *Pistacia lentiscus* L., gathered from the precleaned ground after incisions are made to the bark. The Greek island Chios with the variety *chia* is renowned for its long history of monopolistic mastic harvest and export. However, the shrub-like tree also grows in other areas around the Mediterranean Sea. It is reported to be an ingredient in the Egyptian incense mixture Kyphi and widely used in Orthodox churches in Eastern Europe. The predominant use, however, is as chewing gum and food additive in Greek recipes. The gum's oil consists of up to 90% α-pinene **10**, along with minor amounts of other terpenoids [4.2, 66].

4.1.10 Myrrh, Bissabol-Myrrh, Bdellium and Guggul (*Commiphora* Species)

The usually dark brown, resinous exudates from various *Commiphora* species are known since ancient times as myrrh, opopanax, bdellium, guggul, and locally also under many different names. They are small, shrub-like trees, usually with thorns, growing in countries in the Horn of Africa, Southern Arabia, and parts of India and Pakistan. With the number of different *Commiphora* species given in literature as ranging between 150 and over 200, it becomes evident that the exact terminology and botanical source of each resin is often inconsistent or contradictory in literature. Therefore, the descriptions given herein attempt to reflect the current state of consensus for the most important products.

Myrrh is the gum resin obtained by tapping *Commiphora myrrha* Engl. and *Commiphora molmol* Engl. ex Tschirch (sometimes used as synonyms). It is also known as heerabol myrrh, true, officinal, or bitter myrrh. The small, thorny tree grows in northern Somalia and Arabia; its resin is dark brown, sometimes reddish. Various authors report that the expensive true myrrh is often adulterated with other gums and resins, such as bissabol myrrh, gum arabic and bdellium. Myrrh typically contains a variety of furanosesquiterpenes, which serve as marker compounds for *Commiphora*. Most abundant in *C. myrrha* are furanoeudesma-1,3-diene **46**, furanodiene **47**, lindestrene **48**, and curzerene **49** (Fig. 4.16). Myrrh has a low content in monoterpenes, but is high in sesquiterpenes [4.47, 67].

The odor has not been intensively studied, or at least reported in the literature, but *Wilson* [4.68] isolated several furanosesquiterpenes and reported their olfactory properties. **46** and **48** as well as dihydropyrocurzerenone **50** resemble most closely the typical myrrh odor [4.69]. This publication is discussed in detail in Chap. 3.

There is plenty of literature on the therapeutic effects of myrrh in all forms – ingested, as extracts, lotions, mouthwashes, and so on. Most commonly known is the antimicrobial effect. The reviews by *El Ashry* et al. [4.67] and *Shen* et al. [4.70] present a good overview.

Bissabol myrrh, also named opopanax, habak haidi, or scented/perfumed/sweet myrrh (in contrast to the

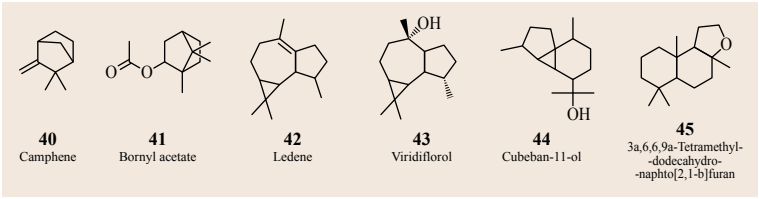

Fig. 4.15 Major compounds from labdanum

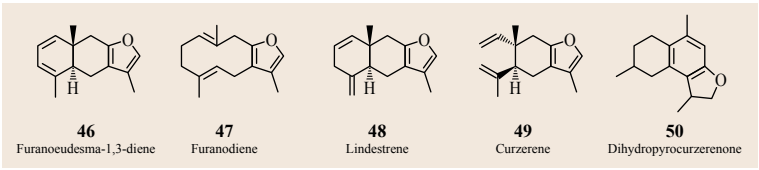

Fig. 4.16 Some furanosesquiterpenoids, characteristic compounds for *Commiphora* resins

true *bitter* myrrh), is the resin from the species *Commiphora guidottii* Chiov. Apparently, resins are sold under the same names from Kenia, but are derived from *C. holtziana*. Fomerly, *C. erythraea* Engl. (var *glabrescens*) and *C. kataf* Engl. were also given as source plants of bissabol myrrh, but *Thulin* and *Claeson* [4.71] convincingly showed that this was caused by historic misinterpretations of the herbarium specimens. Scented myrrh is generally considered inferior to heerabol myrrh, but is thought to be the myrrh in biblical times and Pliny's descriptions.

There is also a resin from *Opopanax chironium* (L.) Koch, a Mediterranean herb in the Apiaceae (carrot) family, called opopanax or sometimes falsely *sweet myrrh*. It is not to be confused with resins from *Commiphora* species and is in fact more closely related to asafoetida (Sect. 4.1.7). Nowadays, trade with *Opopanax chironium* material is almost nonexistent, so that it can safely be assumed that commercial opopanax is sourced from *Commiphora* species.

Indian Bdellium, guggul or guggulu is mainly obtained from *Commiphora mukul* Engl.; some authors additionally list *C. wightii* (Arn.) Bhandari, *C. caudata* Engl., or *C. roxburghii* Alston. *Commiphora africana* (A. Rich.) Endl. resin is called African bdellium and is harvested in Ethiopia, Eritrea, Sudan, and Kenya. Guggul is rich in plant sterols, the so-called *guggulsterols*, which were proposed to have positive effects on cholesterol levels, although more recent studies doubt these effects. The resin is still used as a traditional incense in India [4.72].

Commiphora opobalsamum (L.) Engl. is a small bush or tree without thorns, which yields the historic Balsam/Balm of Mecca (potentially identical to the Balm of Gilead). It grows along the coast of the Red Sea, yet it is nowadays insignificant as incense material. Its historical relevance as well as the botanical source of Balm of Mecca is, however, still a matter of discussion. Apparently, in ancient times, the tree's habitat extended even into Judea. The resin is still used in traditional Chinese Medicine [4.2, 73].

All sorts of *Commiphora* resins may be used as incense. It is not entirely clear which type of myrrh was more common in antiquity, although some claim that bissabol myrrh was more popular due to its sweeter, perfumed smell. Heerabol myrrh had a bigger significance in medical applications and for embalming the dead than as incense. Stacte, an ingredient of the incense mixture in Judaism, is often said to be an extract of myrrh or the spontaneous exudate of myrrh trees. In modern times, myrrh is still highly valued for its antimicrobial properties; for example, it is a common additive in toothpastes [4.2, 47, 70, 73].

4.1.11 Palo Santo

The indigenous people of Latin America have used fragrant woods as incense since precolonial times. Hence, the name Palo Santo (Spanish for *holy wood*) is ambiguous in its use. It commonly denominates either *Bursera graveolens* Triana & Planch. or *Bulnesia sarmienti* Lorentz ex Griseb. The name is sometimes also applied to wood from the genus *Guaiacum*, which is more commonly known as *lignum vitae* and lacks a reputation as incense.

Bursera graveolens yields a light, fragrant, and resinous wood typically found in tropical Middle and South America. Fallen branches can be collected from the ground, so that it is usually not necessary to fell the trees for their wood. It emits a pleasant odor when burnt in the form of shavings, but can also be distilled for its essential oil. The main constituents reported in the literature are limonene **11**, α-terpineol **51**, carvone **52**, as well as β-bisabolene **53** and other sesquiterpenoids, though in largely varying relative percentages (Fig. 4.17). GC-O analysis showed that **51**, **52**, mintlactone **54**, iso-mintlactone **55** and some sesquiterpenoids contribute to the characteristic odor, which

was described as woody, herbal, and minty [4.74, 75].

Bulnesia sarmienti is native to the Gran Chaco area in central South America (Argentina, Paraguay, Bolivia). Its fragrant wood is also burnt as incense, but it is better known for its essential oil, commonly named guaiac wood oil [4.63]. The wood is also often called guaiac wood, further complicating the distinction between true *Guaiacum* and *Bulnesia sarmienti* products. The main volatile compounds are sesquiterpene hydrocarbons and alcohols, including guaiol **56** and bulnesol **57** (Fig. 4.18). *Bulnesia sarmienti* is listed in CITES Appendix II.

4.1.12 Sandalwood

Sandalwood is the darker heartwood from *Santalum album* L., *S. austrocaledonicum* Vieill., and *S. spicatum* trees, which is rich in oil and highly fragrant. Native to India, Sri Lanka, Australia, New Caledonia and Indonesia, sandalwood has a long and rich history of use as incense, precious carving wood, and medicine. To harvest the wood, the tree is uprooted and the bark and sapwood separated from the heartwood. The essential oil, often used in perfumery, is typically extracted by steam distillation. Wood chips or powders are used as incense or in the preparation of joss sticks and incense mixtures. The volatile fraction is typically dominated by α- and β-santalol **58**, **59** (Fig. 4.19), along with many other sesquiterpene alcohols, aldehydes, and some phenolic compounds. The santalols are also the two key odorants, with other sesquiterpenoids modifying the odor (Chap. 3) [4.19].

4.1.13 Storax

Storax (often also styrax, Sect. 4.1.4) is a fragrant balsam from *Liquidambar* species, which exist as large deciduous trees in temperate zones. Two types are commercially relevant: the Levant/Oriental and the American variety, originating from *Liquidambar orientalis* Mill. in Turkey and from *L. styraciflua* L. in Honduras, Mexico, Guatemala, and the USA. Older descriptions of storax, for example, by Pliny and Dioscurides, give clear evidence that it was obtained from *Styrax officinalis* L. [4.4]. Nowadays, this species is still common in the eastern Mediterranean region, but of disputed relevance as a resin-producing species when compared to the *Liquidambar* balsams. *Hovaneissian* et al. [4.76], for example, state that solid storax comes from *Styrax officinalis* and the liquid storax from *Liquidambar* species. By contrast, *Zeybek* [4.77] tested wild-growing *Styrax officinalis* and was unable to induce any resin production, concluding that there must have been misconceptions in the botanical identification. To date, this question still remains unsettled. The lack of publications on the resin of *Styrax officinalis*, however, might favor Zeybeck's position.

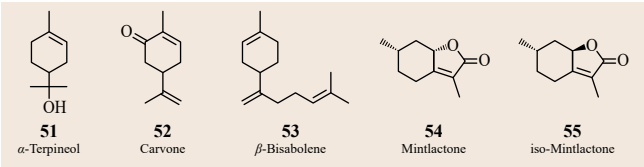

Fig. 4.17 Odorants from *Bursera graveolens*

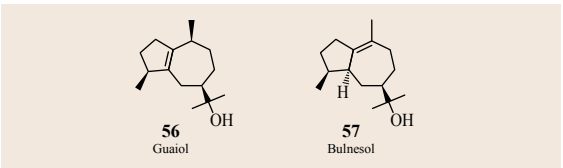

Fig. 4.18 Sesquiterpene alcohols from *Bulnesia sarmienti*

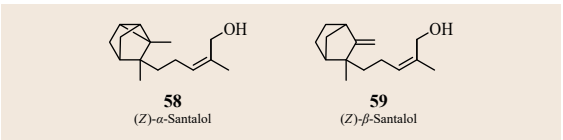

Fig. 4.19 Key odorants in sandalwood

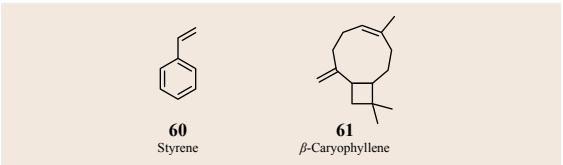

Fig. 4.20 Compounds from *Liquidambar* balsams

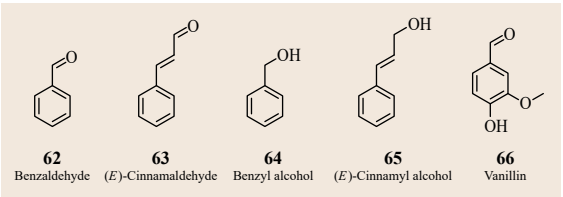

Fig. 4.21 Odor-active compounds found in Tolu and Peru balsam

The exudations of *Liquidambar* species were and to a lesser extent are still common in pharmacy, cosmetics, and perfumery [4.78]. The most prominent local, traditional use in Turkey is against ulcers [4.20, 79].

The balsams are usually grey-brown viscous liquids, and are most commonly traded either as charcoal chips or bark, both infused with the liquid resin. This allows easier handling and direct application for incense purposes. Essential oils and resinoids are also available.

Both balsams are characterized by a high content in styrene **60** (Fig. 4.20), with around 70–80% in the essential oil of *L. orientalis*. This compound was first isolated from storax and named accordingly [4.80]. Styrene is easily detectable by its distinctive sweet smell in the balsams. Additionally, the volatile fraction is mainly composed of α-**10** and β-pinene **32**, and in the case of *L. styraciflua* also β-caryophyllene **61** [4.20, 81]. Cinnamic acid **7**, its esters, and related compounds [4.82] might contribute to the balsamic, sweet smell, but this has not been properly investigated.

4.1.14 Tolu Balsam/Peru Balsam

Tolu balsam and Peru balsam are derived from *Myroxylon balsamum* varieties that are native to northern South America and Central America. Tolu balsam (*M. balsamum* Harms var *genuinum* Harms, some sources also: var *balsamum*) can either be a solid red-brown resin that melts at low temperatures or a thick yellow-brown fluid when fresh. Peru balsam (*M. balsamum* Harms var *pereirae* Harms) is a red-brown viscous fluid. Both smell strongly balsamic, with notes of vanilla and cinnamon. The organoleptic properties can be linked to high contents in benzoic acid **4** and cinnamic acid **7**, their esters (for example, **3**, **8**), aldehydes **62**, **63**, alcohols **64**, **65** but also vanillin **66** and related phenolic compounds (Fig. 4.21).

Tolu balsam is characterized by a lower content of the volatile fraction, historically named cinnamein, than the liquid Peru balsam. Both are known as components in incense mixtures, but are more commonly employed as fragrance raw material, for cosmetics, and medical applications. Due to the allergenic potential of the balsams, only the distilled essential oil is used in cosmetics and perfumes [4.63, 83, 84].

4.1.15 Overview of Incense Materials

These pictures are intended to give an overview of the physical appearance of the various incense materials mentioned in the text. The botanical species given below are based on suppliers' information and are not verified by chemical analysis.


Eritrean Frankincense
(*Boswellia papyrifera*)


Indian Frankincense
(*Boswellia serrata*)


Arabian Frankincense
(*Boswellia sacra*)


Labdanum
(*Cistus ladaniferus*)


Mastic
(*Pistacia lenticus*)


Myrrh
(*Commiphora myrrha*)


Bissabol/Sweet myrrh
(*Commiphora holtziana*)


Guggul
(*Commiphora mukul*)


Opopanax
(*Chironium opopanax*)


Palo Santo
(*Bursera graveolens*)


Guaiac resin
(*Guaiacum officinale*)


Sandalwood powder
(*Santalum album*)


Storax bark
(*Liquidambar orientalis*)


Tolu balsam
(*Myroxylon balsamum var genuinum*)


Peru balsam
(*Myroxylon balsamum var pereirae*)

4.2 Incense Preparations

Incense can be divided into two types: combustible and noncombustible. Combustible incense burns by itself after ignition, whereas noncombustible incense requires an external heat source, such as a glowing charcoal, although other hot surfaces can also be used. Examples are hot stones taken from or placed around a fire pit, or mica plates in the Japanese Incense Ceremony (Fig. 4.22), during which a thin sheet of this silicate mineral is heated by a glowing coal buried in ash [4.7, 85].

This chapter focuses on the preparation of combustible incense, as noncombustible incense, even in mixtures, does not require any special techniques. The components are simply mixed in whatever form preferred: ground to a fine powder, in granules, pieces, or chunks. Resin based mixtures can be melted and formed by gentle heating. Non-combustible incense encompasses the most famous mixtures from historical sources, such as Kyphi, an ancient Egyptian mixture and Ketoret, reported in Jewish tradition as the holy

Fig. 4.22 Mica plate placed on a glowing charcoal buried in a pile of ash, the typical form of heating incense materials during the Japanese Incense ceremony (courtesy of Nippon Kodo)

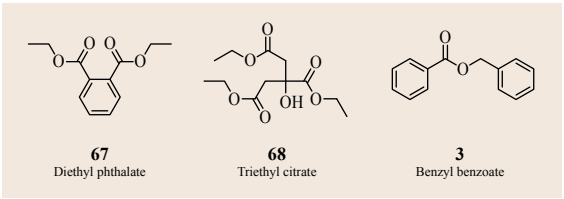

Fig. 4.23 Synthetic fixatives used in incense products

incense in the Jerusalem Temple. Many speculations circulate as to their exact compositions [4.12].

By contrast, combustible incense requires to be mixed with fuel, thereby making its preparation a lot more complicated. The mixtures typically contain the following types of ingredients, and the appropriate proportions need to be determined experimentally. The exact compositions are usually a closely guarded company secret, although examples for preparations can be found in the following sources, and many more are readily available on the Internet: *Fischer-Rizzi* [4.3], *Rätsch* [4.12], *Cunningham* [4.85], *Hsieh* [4.86], *Neumann* [4.87], *Newell* [4.88].

Firstly, a binding material is needed, which can be soaked in water to form a malleable paste, into which all other ingredients can be mixed. After forming and drying, this binder also serves as combustible material to keep the incense burning. Additional fuel can be included in the mixtures, such as powdered charcoal, incense woods, or barks. The most common binders in incense manufacture are gum tragacanth, gum arabic, makko powder (from the bark of *Machilus* spp.) or starch. Then, an oxidizing agent is usually introduced to keep the embers glowing more evenly. Most commonly, this is sodium or potassium nitrate. This dough is completed with pulverized incense ingredients to give the incense preparation the intended smell. Aside from

Fig. 4.24 Brightly colored incense cones from Thailand (courtesy of Johannes Niebler)

the aforementioned materials and many other natural products, essential oils can also be used to add fragrance notes otherwise unavailable. Care needs to be taken to ensure that the mixture still burns well, as too much essential oil or resin can impede an even combustion. In many industrial incense products, synthetic fragrances are used exclusively or additionally, for example, to include musk, vanilla, or patchouli notes. These would otherwise be unavailable or far too expensive, and can easily be included by adding synthetic musks, vanillin, or patchouli alcohol [4.89, 90]. Finally, most preparations include some form of fixative to avoid a strong loss of odor intensity during the drying and storage process. Traditionally, these fixatives were natural materials, including orris root (*Iris* spp.), ambergris, musk, resins (frankincense, myrrh, labdanum, etc.), balsams, and many others. Nowadays, synthetic fixatives are usually applied, including diethyl phthalate **67**, triethyl citrate **68**, benzyl benzoate **3** and others (Fig. 4.23). A study by *Tran* and *Marriott* [4.89] as well as similar studies performed in our laboratory (*Niebler* and *Buettner*, unpublished data) on the composition of incense sticks gave proof for this practice.

Combustible incense comes in many forms (Fig. 4.24 and 4.25). Powders can be flexibly used and allow exact dosage. Incense cones are popular around the world, and have a long tradition in the Ore Mountains in Germany. Incense sticks, also called joss sticks, come in various forms, depending on production method and intended use. They can be made from solid incense mixture or wrapped

Fig. 4.25 Various incense products. *Top row*: Incense cones from Germany (*left*) and Thailand (*middle*), a strip of folded Papier d'Arménie, ready to be lit (*right*). *Middle row*: reconstituted Kyphi, an ancient Egyptian incense mixture (*left*) and three different incense sticks (*right*). *Bottom row*: Incense mixtures used in Christian churches, rose scented incense (*left*), *Rhenish* mixture (*middle*) and *Original Arabic* mixture (*right*) (courtesy of Johannes Niebler)

around a wooden stick (mostly bamboo) in a layer. Methods of production include extrusion, dipping, pressing, coating, or rolling [4.5]. Additional forms of combustible incense include balls, coils, ropes and other specialized shapes [4.91]. It is also notable that in Asian countries particular cheaper, nonfragrant incense sticks are produced that are mainly used for offerings at the temples. These sticks only produce smoke and are not meant for scenting purposes [4.6]. Moreover, a special kind of combustible incense, the so-called Papier d'Arménie, is a French product that is essentially a strip of paper infused with an incense solution based on storax. Furthermore, many incense preparations are brilliantly colored by natural or synthetic dyes. The colorants can be included in the incense dough or applied externally on the finished product.

In conclusion, incense technology is not particularly sophisticated, even though some products require a certain experience and knowledge.

4.3 Benefits and Hazards of Incense Use

The use of incense products, in any form, does provide some benefits but it can also pose certain risks to human health. The next section includes a brief scientific review of documented positive and negative effects on humans.

The obvious benefits include a pleasant scenting of the room or environment, the aesthetical pleasure of watching incense smoke rise and curl in the air, as well as spiritual and psychological effects arising from such a ritualized experience. There are also numerous accounts of medicinal use of smokes in traditional medicine all over the world (for an overview, see *Mohagheghzadeh* et al. [4.10]). However, none of these have been investigated as medical incense treatments in clinical studies. This is probably caused by the fact that incense or smoke from plant materials is a virtually nonexistent form of application in modern medicine, and it is commonly associated with alternative treatments or drug abuse. Generally, inhalation of active compounds leads to a fast resorption and a sharp peak in the blood level. This can be beneficial in cases where a fast administration is intended, for example, in anesthetics or in the administration of anti-asthmatic drugs, but also in drug abuse (e.g., cannabis). Drawbacks are the lack of control over the exact dosage, side effects, and potentially harmful byproducts in the case of smokes. Another aspect to consider with inhalatory exposure is the metabolism of substances in the airways, which could generate a range of potentially bioactive derivatives [4.92]. A modern medical approach would therefore seek to isolate active substances from incense smoke and apply them in pure or enriched form through inhalator devices.

As an example, *Moussaieff* and *Mechoulam* [4.93] reviewed the scientific literature on the benefits of frankincense. Most of the trials were performed on extracts or isolated, nonvolatile compounds meant for oral administration. Only a few studies investigated the bioactivity of volatile compounds, which could also be found in the smoke of frankincense, and none were specifically targeted at an inhalative exposure. By contrast, several studies on adverse effects of frankincense smoke exposure are available (examples from the most recent literature: *Hussain* et al. [4.94], *Ahmed* et al. [4.95]). Furthermore, the nonvolatile fraction of incense materials can also be the source of promis-

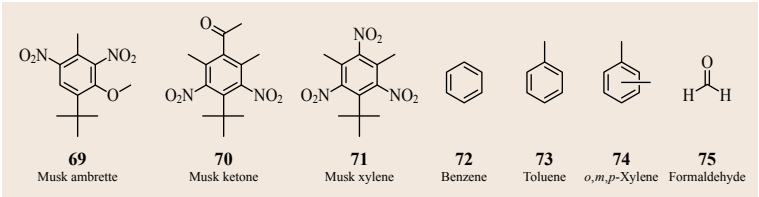

Fig. 4.26 Synthetic nitro-musks (**69**–**71**) and toxic byproducts from incense burning (**72**–**75**)

ing drugs, as exemplified by Crofelemer (SP-303), an oligomeric proanthocyanidine fraction from dragon's blood (*Croton lechleri*), that has recently been approved as an antidiarrheal drug by the Food and Drug Administration (FDA) [4.96].

Notably in the last decades, scientific investigations have increasingly raised awareness that incense burning should be considered as an activity with noteworthy risks to human health. Incense smoke is a complex mixture containing particulate matter, gases from the burning process, and organic compounds.

Particulate matter is a substantial health risk associated with burning of incense of all kinds. Highly elevated levels of particulate matter under 10 μm (PM_{10}) and under 2.5 μm ($PM_{2.5}$) in diameter from incense burning have been documented in numerous studies [4.97–104]. PM_{10} can enter and accumulate in the human respiratory system, whereas $PM_{2.5}$ can penetrate even further, as far as into the alveoli [4.105]. In a study comparing incense sticks and cigarettes, the former showed a 4.5-fold higher emission of particulate matter than a cigarette (45 mg/g compared to 10 mg/g, *Mannix* et al. [4.106]); another study stated that the indoor air pollution is comparable to cigarette smoking [4.101]. Particulate matter can carry mutagenic substances absorbed to the surface, and the mutagenic properties of incense smoke condensates have been documented by *Chen* and *Lee* [4.107] and *Löfroth* et al. [4.101]. Nevertheless, the distance from the source of particulate matter is surely quite different for both cases, cigarettes and incense; in any case, incense smoke is not as immediately and directly inhaled as is the case for cigarettes.

Polycyclic aromatic hydrocarbons (PAH) are generated during the burning process and quickly condense onto particulate matter [4.108]. Some PAHs are potent carcinogens and mutagens and have been repeatedly found in various kinds of incense smokes, in both field investigations and controlled laboratory measurements [4.97, 102, 109, 110].

Polychlorinated dibenzo-*p*-dioxins or dibenzofurans (PCDD/F) have also been found to be a notable risk factor associated with heavy incense use. Highly elevated levels were measured by *Hu* et al. [4.111] in a Taiwanese temple.

Additionally, volatile organic compounds (VOC) are heavily released. This is, obviously, part of the purpose of burning incense, as aromatic substances need to be vaporized or thermally generated. However, some compounds generated or released in the process can possess toxicological relevance. On the one hand, intentionally added substances like the nitro-musks (musk ambrette **69**, musk ketone **70**, musk xylene **71**, Fig. 4.26) were found in several incense preparations [4.90]. Musk ambrette **69** may cause photocontact dermatitis [4.112] and musk xylene **71** is listed as a substance of very high concern (SVHC) under REACH in the European Union (ED/67/2008), because it was rated as very persistent and very bioaccumulative (vPvB). Such risk factors can be eliminated by changing the formulation of the incense. On the other hand, in particular the slow and incomplete burning process releases toxic byproducts such as benzene **72**, toluene **73**, xylenes **74**, formaldehyde **75**, and many others (Fig. 4.26) [4.105, 113–115]. Furthermore, gaseous emissions from incense burning include toxic inorganic gases like carbon monoxide, sulfur dioxide and nitric oxides [4.106, 113].

Even though there is large variability between different samples, *Lee* and *Wang* [4.113] were able to show that incense use can lead to indoor air pollution at levels that are considered to have adverse effects on human health. *Lin* et al. [4.105] reviewed the available literature and stated that several epidemiological studies did not indicate any harmful effects of incense use, whereas other studies and reports showed that incense burning can be associated with negative effects on the respiratory tract, allergenic reactions, dermatological problems, cancer, neoplasms, and elevated IgE levels in cord blood. Overall, burning incense indoors or in insufficiently ventilated environments is, like cigarette smoking, a major cause of indoor air pollution and may pose adverse health effects, particularly in the case of prolonged and repeated exposure.

4.4 Conclusion and Outlook

The (aroma) chemistry of incense burning is a wide and complex topic of local and international relevance that cannot be adequately discussed in a few pages. The materials presented in this chapter were chosen to reflect aspects from several different culture areas. Nonetheless, it is obvious that some culture groups have barely been mentioned, even though they possess a rich history in incense use. For example, North American native people have used dried sage (*Salvia* spp.) as incense in their rituals.

Ethnobotanical studies like the one by *Staub* et al. [4.5] form the first step to understanding and investigating incense use in any culture. Unfortunately, these studies are rare, leading to a frequent uncertainty when talking about botanical sources of incense materials. In historical contexts, an exact identification of the plants described in the sources is for most cases impossible. Additionally, a considerable portion of the available literature is written by self-proclaimed experts in magic, rituals, and shamanism.

A better data basis is available on the chemical composition of many essential oils of various plant materials, even though the quality of the analysis varies considerably and several materials in this chapter would need a thorough reinvestigation. From the point of view of aroma research, this field still presents a largely undiscovered territory of new, potent odorants in complex mixtures. The highly dynamic and complex processes involved in incense burning represent an interesting challenge to established methodologies. The identification of thermolabile precursor substances might enable a better understanding of the apparent changes in the odor profiles of some incense materials during the burning process. To date, the aroma chemistry of incense remains a neglected subject; however it can offer promising and fascinating research opportunities for the future.

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5. Mechanistic Pathways of Non-Enzymatic Flavor Formation

Marcus A. Glomb

This chapter focusses on the formation of flavor active structures by mechanisms based on the degradation of reducing carbohydrates in the presence of amines. As model reactions have led to the elucidation of a confusing diversity of compounds, special attention is given to the understanding of the basic reaction pathways explaining the evolution of the most abundant odorants predominately shaping the aroma profile of most foods.

In 1912, the French chemist Louis Camille Maillard reported on chemical changes in amino acid sugar reaction mixtures with respect to browning and release of carbon dioxide. Since then the reaction of reducing sugars with amines is termed nonenzymatic browning or Maillard reaction [5.1, 2]. The colored high molecular weight products of the late reaction stages are called melanoidines. Although intermediates are similar in their basic reactive structure, the reaction has to be clearly differentiated from enzymatic browning processes. Here in contrast, the initial phase of the reaction of phenolic educts is catalyzed by enzymes, while nonenzymatic follow-up reactions then lead to late stage products like the browned high molecular weight melanines.

5.1 Maillard Reaction – General Considerations

Thus, in the historic sense the word Maillard reaction describes the reaction of two main constituents of foods. Not only reducing sugars glucose, fructose, maltose, and lactose, but also oligosaccharides are of importance. Pentoses are of interest for the processing of meat and meat products (Chap. 10), but can also be released from hemicelluloses, for example, under roasting conditions. Amino components are widely abundant in foods, most relevant are free amino acids, peptides, and proteins. The impact of the Maillard reaction on foods during processing, storage, and retail is of significant and very diverse importance. Other than browning, taste and aroma are changed, and not always just to yield better products but also to lead to off-flavors. However, these changes define our traditional perception of a specific food. Protein modifications not only lead to an irreversible loss of essential amino acids, but also to desired functional physical alterations. Not only some of the intermediates, but also late stage Maillard products are of significant antioxidative capacity and can prolong the shelf life of foods. On the other hand especially at very high temperatures, structures with strong carcinogenic and mutagenic properties are formed, for example, acrylamide and aromatic amines.

Reducing sugars in aqueous solutions are relatively stable within a pH range of 5–7. However, in the presence of amines this changes drastically. Amino components will degrade sugars by acid–base catalysis and by nucleophilic carbonyl reactions. For free amino acids the primary amine functions react, for peptides and proteins other than the N-termini the side chains of lysine and arginine are mainly targeted. Within the course of Maillard reaction α-hydroxycarbonyl and, most importantly, α-dicarbonyl structures are the key intermediates. Most, if not all follow-up structures can be mechanistically related to the latter group. Figure 5.1 shows established compounds relevant for the degradation of glucose [5.3, 4]. Exceptions are 1-amino-1,4-dideoxyglucosone and 1,4-dideoxyglucosone. Although these dicarbonyls show the typical C_6-carbon

backbone, they are formed in higher amounts specifically during the degradation of disaccharides. It is important to note that compared to the starting sugar, the dicarbonyls with the intact carbon backbone are of much higher reactivity. Other than these, the complete array of fragmentation from C_5- to C_2-products is found.

The understanding that α-dicarbonyl compounds are of central importance has changed the historic definition of the Maillard reaction. In the modern view this term is related to any amine-dicarbonyl reaction. This means that in addition to the classic degradation starting from intact sugars, the intermediate dicarbonyls can also stem from, for example, fat autoxidation, oxidation of phenolic structures, or from fermentative processes.

It is important to realize that the term Maillard reaction is not meant in the sense of a single reaction, for example, found in organic synthesis starting from educts A and B leading to products C and D. Instead, it is an extremely complex reaction cascade with multiple simultaneous processes. The main aspects can be described by cyclization, elimination, fragmentation, condensation, hydrolysis, rearrangement, and redox reactions, including both ionic and radical mechanisms. Owed to this complexity, today many Maillard products can still not be described by conclusive mechanistic pathways. The situation is even more difficult because many products are only formed in negligible amounts in the ppm range or even much lower. However, especially relevant for the aroma of foods, it is exactly these structures which due to their extremely low odor and taste threshold levels are of central interest to food industry.

In 2014, *Dunkel* et al. published a review on the chemical signatures of food odors and their relevance to human olfaction and to food biotechnology [5.5]. They screened literature from 1980 to 2013 for reports on aroma profiles of foods using very stringent inclusion/exclusion criteria. These were a bioactivity-directed approach based on coupled gas chromatography-olfactory analyses using the flavor dilution approach, unequivocal mass spectrometric, or retention index verification based on authentic reference standards, and a reliable quantitation by, for example, stable isotope dilution analyses. As a result, they selected 119 publications describing the key food odorants of 227 foods across the complete market basket with alcoholic beverages, meat products, fish and sea food, cereal and bakery products, dairy products, fats and oil seeds, fruits, vegetables, mushrooms, spices and herbs, cocoa and chocolate, coffee, tea, and many others. Surprisingly, the authors identified only 226 key odorants sufficient to describe the odor space of these foods. The authors even extrapolated from this observation that less than 230 main odorants, out of a total of about 10 000 volatiles assumed to be present in foods, might be enough to describe the total odorant space present in our foods and beverages. Out of the 226 odorants, the authors categorized three classes, namely 16 generalists with an abundance of more than 25% in the 227 food samples, 57 intermediaries with less than 25% but more than 5% abundance, and 151 individualists with less than 5% abundance. However most interestingly, many representatives of the generalist and also of the intermediaries can be related to the degradation of carbohydrates and amino acids, based on sole or alternative nonenzymatic pathways or a combination of both. This is especially true for the class of the generalists, where 10 out of 16 structures can be explained by Maillard reaction pathways. Table 5.1 lists these structures, and their abundances. Some of the compounds can evolve from both metabolic–enzymatic and nonenzymatic pathways. However, this chapter focuses

Table 5.1 Key odor structures in foods and beverages are formed by nonenzymatic pathways

Odorant *generalists*	**Abundance (%)**
3-(Methylsulfanyl)-propanal (methional)	54
2-/3-Methylbutanal	51
Butan-2,3-dione (diacetyl)	42
4-Hydroxy-2,5-dimethyl-3(*2H*)-furanone (furaneol)	41
3-Hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolone)	36
Acetic acid	29
Acetaldehyde	29
Ethyl-2/3-methylbutanoate	28
2-Acetyl-1-pyrroline	26
2-/3-Methylbutanoic acid	26
Odorant *intermediaries*	**Abundance (%)**
2-Methylpropanal	24
Phenylacetaldehyde	23
Ethyl-2-methylpropanoate	23
2-Ethyl-3,5-dimethylpyrazine	19
Dimethyltrisulfide	19
Phenylacetic acid	18
2,3-Diethyl-5-methylpyrazine	17
Furan-2-ylmethanethiol (2-furfurylthiol)	15
2-Methoxy-3-(propan-2-yl)pyrazine	14
Dimethylsulfide	14
Methanethiol	13
2-Acetyl-2-thiazoline	9
Propanal	7
2-Methylfuran-3-thiol	6
2-Ethyl-4-hydroxy-5-methyl-3(*2H*)-furanone (homofuraneol)	6
1,1-Diethoxyethane	6
Methyl-2/3-methylbutanoate	5

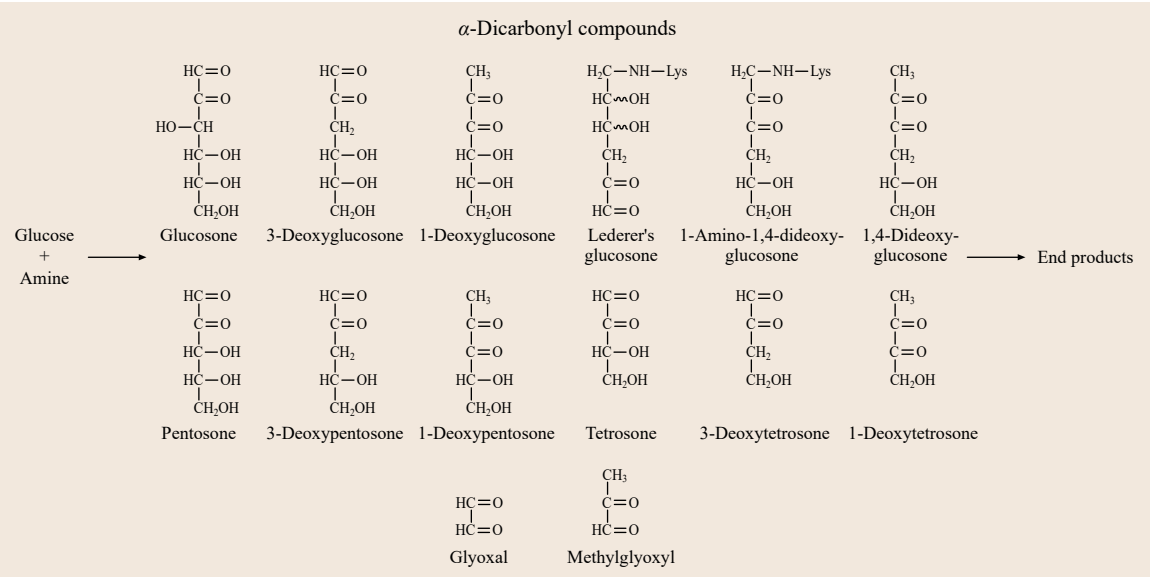

Fig. 5.1 Dicarbonyl compounds are the central Maillard intermediates

on the mechanisms explaining explicitly their nonenzymatic formation. Wherever possible, the following discussions are based on publications using isotopic labeling as the carbon module labeling (CAMOLA) approach [5.6] or using authentic intermediates for verification.

In general, both sugars and amino acids are too polar to contribute directly to the odor profile. This means that polar functionalities must get lost during the degradation process, which in most cases can be related to dehydration, elimination, fragmentation, and decarboxylation reactions.

5.2 α-Dicarbonyl Compounds

The early stage of the Maillard reaction is determined by the reactivity of the carbonyl function of the original sugar. In Fig. 5.2, glucose was chosen as a model compound. The structure is drawn in the open-chained form showing the native carbonyl function for simplification, although in aqueous systems carbonyl compounds exist mainly as hydrates or as half-acetalic structures. However, it is the availability of the free carbonyl function which determines the reactivity of the respective sugar. Reaction with the amine and dehydration leads to an imine, also called Schiff base or aldoimine. Keto-enol-tautomerism then gives an aminoketose via an 1,2-enaminol. This isomerization is also called Amadori rearrangement, the aminoketose termed Amadori product. The acid–base catalysis of the amino compounds basically allows the enolization to proceed along the complete carbon backbone of the sugar, which is prerequisite for the elimination of water or the amine. Starting from the 1,2-enaminol 3-deoxy-2-glucosulose (3-deoxyglucosone) is formed, the 2,3-enediol gives 1-deoxy-2,3-glucodiulose (1-deoxyglucosone). Substantial amounts of Lederer's glucosone verify that the enolization indeed proceeds to a 5,6-enediol intermediate [5.7]. In contrast, the formation of 2-glucosulose (glucosone) can only be explained by oxidation, that is, the amine catalyzed autoxidation of glucose, but mainly of the intermediate imine and of the Amadori product.

After enolization, 1-deoxyglucosone and glucosone show an α-oxoenediol motive. This means that these compounds are reductone structures, which are highly redox active and are easily degraded to give lower molecular weight fragments of even higher reactivity. Both, nonenzymatic reduction and oxidation in Maillard reaction systems can be explained by these intermediates. Oxidation leads to a 1,2,3-tricarbonyl compound. Thus, within the Maillard reaction it is exactly these structures with reductone character which direct the further course of reaction.

In Maillard literature, four main mechanisms of fragmentation have been proposed [5.8]:

1. Hydrolytic α-dicarbonyl cleavage
2. Retro-aldol cleavage

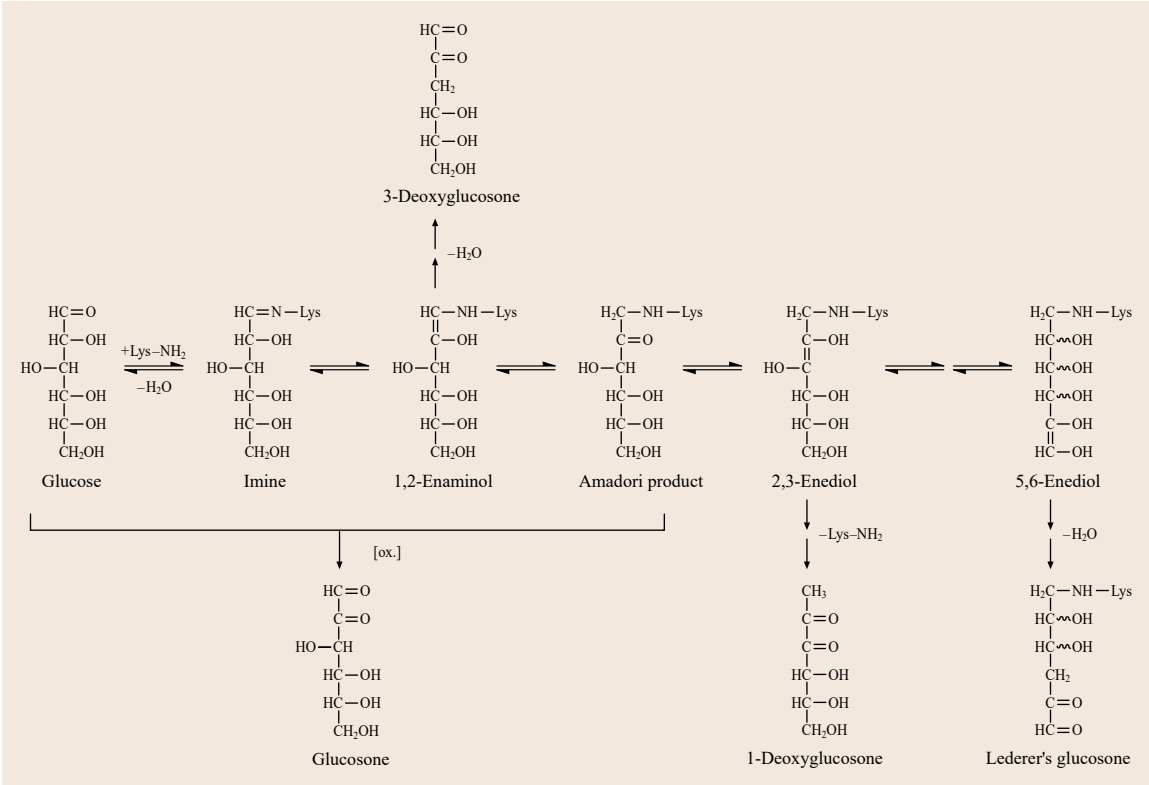

Fig. 5.2 Initial stage of Maillard reaction

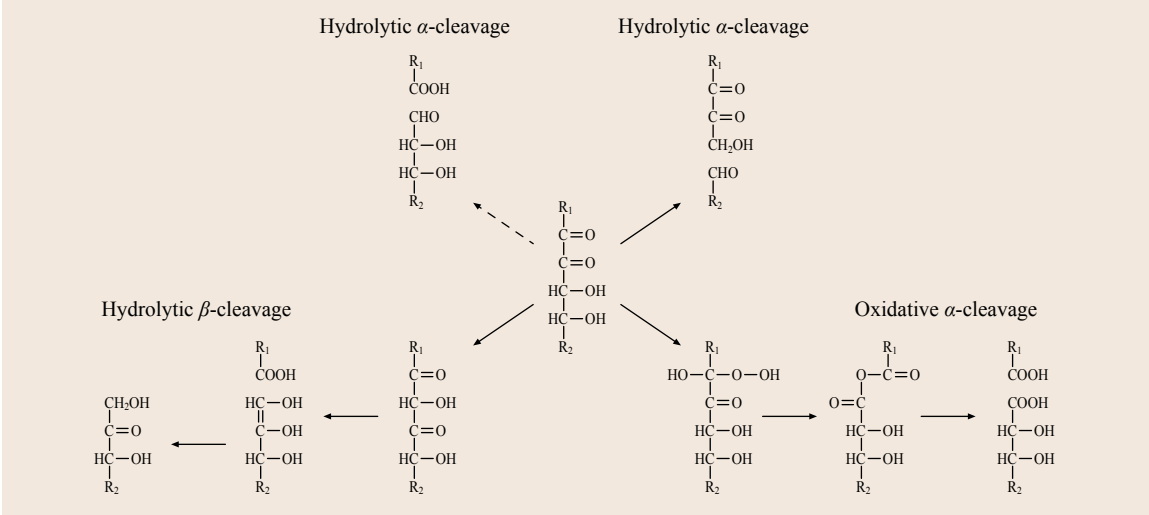

Fig. 5.3 Mechanisms of sugar fragmentation

3. Hydrolytic β-dicarbonyl cleavage
4. Oxidative α-dicarbonyl cleavage (Fig. 5.3).

Recently, the latter two have been refined by an additional amine-induced alternative route each.

The hydrolytic α-dicarbonyl cleavage describes the nonoxidative scission of an α-dicarbonyl motive to give a carbonyl and a carboxylic compound. It has been used very frequently especially in the older Maillard literature to explain the parallel formation of the two

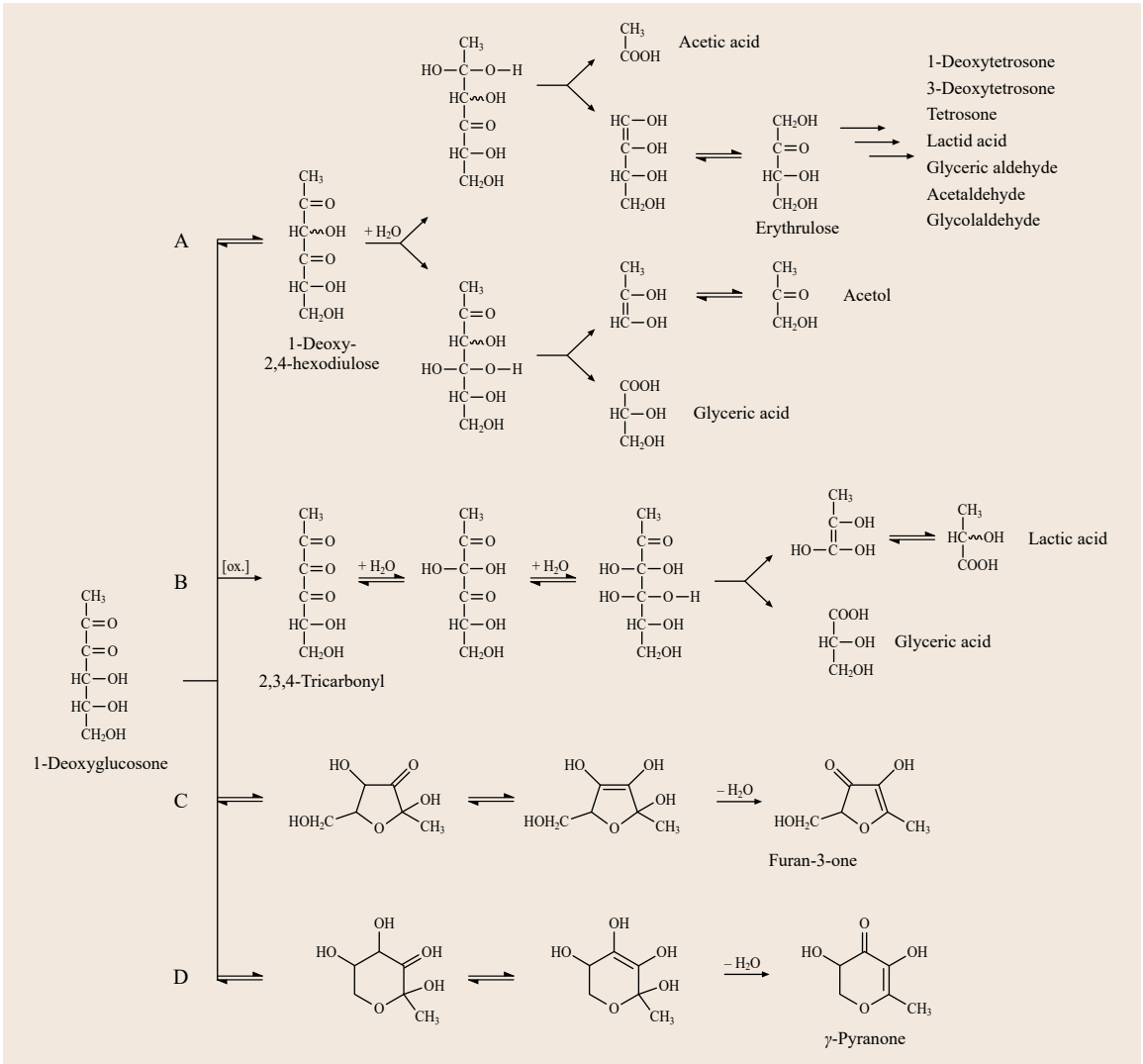

Fig. 5.4 1-Deoxyglucosone is the central intermediate of hexose Maillard chemistry: (A + B) β-dicarbonyl cleavage, (C + D) cyclization

molecule classes. However, recent investigations and also a comprehensive literature review have come to the conclusion that there is no experimental ground for this hypothetical mechanism and that its proposal is merely based on the coincidential, but non quantitative coexistence of structures with fitting complementary carbon backbone. Thus, this mechanism has to be excluded as void.

Retro-aldol cleavage is another mechanism that has been challenged recently. Any starting sugar shows the prerequisite β-hydroxycarbonyl moiety leading to two carbonyl fragments. Specifically from α-dicarbonyls, the mechanism leads to one α-dicarbonyl and one carbonyl molecule. This type of cleavage is commonly used to explain the generation of short-chained α-dicarbonyl compounds which are odor active as such, or are important intermediates involved in the formation of potent odor active compounds, that is, especially butane-2,3-dione (diacetyl), methylglyoxal, and 1-hydroxy-2-propanone (acetol). Aldol condensations are the reverse reaction and are frequently used in organic synthesis strategies under nonaqueous conditions. They have been also shown to proceed in aqueous systems at moderate temperatures, for example, leading from diacetyl and formaldehyde to 1,4-dideoxy-2,3-pentodiulose (1,4-dideoxypentosone) [5.9]. However, retro-aldol cleavages have been almost outruled for Maillard systems operated at moderate temper-

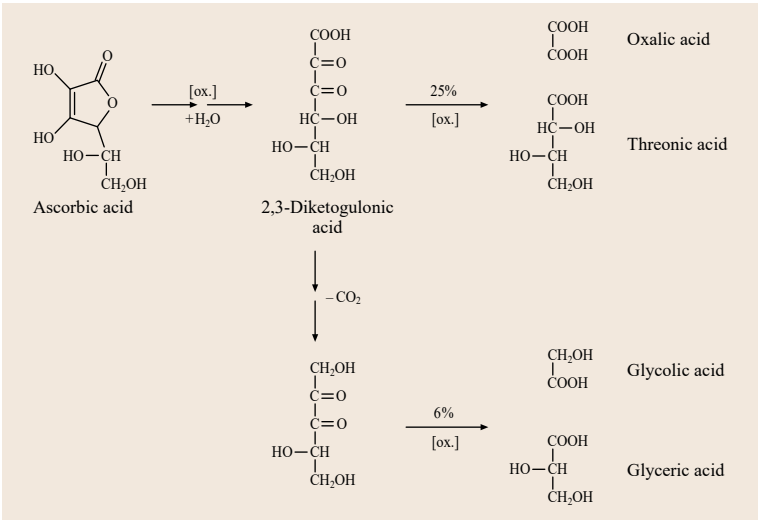

Fig. 5.5 Oxidative α-dicarbonyl cleavage is a major fragmentation mechanism during degradation of ascorbic acid

atures up to 50 °C. There was no scission of independently synthesized 1,4-dideoxy-2,3-hexodiulose (1,4-dideoxyglucosone) to give diacetyl and glycolaldehyde [5.4]. Instead, the educt was shown to be rather stable. In addition, incubations (< 50 °C) of authentic 1-deoxyglucosone and 3-deoxyglucosone both gave only negligible amounts of methylglyoxal [5.10, 11]. However, it is exactly these dicarbonyls which are frequently quoted as the main methylglyoxal precursors. Obviously, retro-aldol reactions are not favored at low temperatures and might require higher energy input found, for example, at boiling or roasting conditions.

β-Dicarbonyl cleavage reactions have evolved to one of the major fragmentation reactions already working at moderate temperatures. Although already mentioned in the earlier Maillard literature this mechanism has been studied first in detail by *Davidek* et al. in model systems using simple α- and β-dicarbonyl short-chained alkanes [5.12]. Later, these results were transferred by this and other groups to Maillard intermediates with a reductone motive. This allows an α-moiety to isomerize to a β-configuration, which is prerequisite for the β-scission. Figure 5.4 highlights reactions of 1-deoxyglucosone, which has to be evaluated as the major central intermediate of hexose chemistry with an exceptional high reactivity (e.g., half-life $\leq$ 30 min at 37 °C) [5.13]. Most important follow-up products can be rerouted to this structure. Detailed mechanistic studies were facilitated by a successful independent synthesis of 1-deoxyglucosone and allowed to explain about 70–80% of its total degradation under Maillard conditions. Path A starts from the isomeric 1-deoxy-2,4-hexodiulose. Hydration at the C_2-carbonyl function leads to acetic acid and an 1,2-enediol giving erythrulose. The yields of acetic acid were about 60% at 50 °C (up to 85% at 100 °C) independent of the presence of oxygen. This means that in hexose reactions this mechanism explains most if not all the acetic acid being formed. If the hydration proceeds at the C_4-carbonyl function glyceric acid and acetol result. While carboxylic acids have to be evaluated as stable Maillard endproducts the cleavage counterparts are α-hydroxycarbonyl intermediates which are prone to various follow-up reactions to give further carboxylic acids and dicarbonyls, but also important carbonyl structures as active odorants as such or as intermediates leading to odorants, for example, acetaldehyde, glycolaldehyde and glyceric aldehyde. Path B of Fig. 5.4 shows that β-dicarbonyl cleavage can also be facilitated from a double hydrated oxidation product of 1-deoxyglucosone (C_3 and C_4) to give lactic acid and as the counterpart glyceric acid in substantial yields. The putative cleavage starting from a C_2,C_3-dihydrate to give acetic acid and erythronic acid was experimentally disproven to be of importance. This also excludes a major alternative oxidative α-dicarbonyl cleavage reaction of 1-deoxyglucosone.

A forth cleavage reaction has been established only lately for Maillard systems, the oxidative α-dicarbonyl fragmentation. It was first evidenced from incubations of 2,3-pentandione to give acetic acid and propanoic acid in equimolar concentrations. The mechanism requires activated molecular oxygen stemming, for example, from UV irradiation or hydroperoxides. Incorporation at one of the carbonyl functions gives alkoxyradicals and after single-electron transfer reactions hydroperoxides. These rearrange via a Bayer–Villiger-type reaction to mixed asymmetric anhydride species, which are readily hydrolysed to two carboxylic acids (Fig. 5.3). Obviously, singlet oxygen is prerequisite

for the initial attack at the carbonyl function; however, if present, the reaction will also proceed at moderate conditions. This also must be the reason why during degradation of 1-deoxyglucosone this type of reaction is only of negligible importance. On the other hand, ascorbic acid is known to produce significant amounts of activated oxygen species. Consequently, *Smuda* and *Glomb* were able to verify 31% initiated by oxidative α-dicarbonyl cleavage out of a total of 75% of degradation related to fragmentation (Fig. 5.5) [5.14]. The remainder was attributed to decarboxylation and hydrolytic β-dicarbonyl cleavage. However, given the polarity of the resulting acids and the lack of carbonyl activity of the carboxylic acid function, it must be assumed that most products of the oxidative α-dicarbonyl fragmentation will not contribute to the odor profile.

5.3 Strecker Degradation

The Strecker degradation is by far the aspect of the Maillard reaction which accounts for most of the structures within the group of odor generalists and intermediaries listed in Table 5.1. 13 out of the 27 compounds can be explained directly from this mechanism, 7 by direct and indirect follow-up reactions. Basically, the term Strecker degradation describes the interaction of α-dicarbonyl compounds with amino acids including a decarboxylation step. This again underpins the importance of α-dicarbonyls as the central intermediates of Maillard reactions. After nucleophilic attack of the amino substituent at one of the carbonyl moieties and formation of an imine, the electron drawing second carbonyl function triggers the decarboxylation leading to an α,β-unsaturated imine, which hydrolyses to give the Strecker aldehyde and an eneaminol (Fig. 5.6, pathway A). This explains not only the formation of methional, 2-/3-methylbutanal, acetaldehyde, 2-methylpropanal, and phenyl acetaldehyde, but also formaldehyde from methionine, leucine/isoleucine, alanine, valine, phenylalanine, and glycine, respectively. Besides being aroma active with very low odor thresholds as such, these unpolar low molecular carbonyl structures are highly reactive and can perform, for example, aldol-type reactions to give further important aroma compounds. This also explains the spontaneous formation of 1,2-diethoxyethane from acetaldehyde in, for example, beer or wine. From the mechanistic point of view, the Strecker reaction represents a redox reaction. The amino acid is oxidized under decarboxylation, while the α-dicarbonyl gets reduced. This can be immediately realized from the comparison of the starting α-dicarbonyl to the α-hydroxycarbonyl structure, which is formed after tautomerism and hydrolysis of the resulting α-hydroxyimine to release ammonia already under moderate conditions (pathway B). If the tautomerism proceeds to an α-aminocarbonyl structure, condensation leads to dihydropyrazines, which spontaneously gets oxidized to give pyrazines (pathway C). These heteroaromatic structures contribute especially to the aroma profiles of baked and roasted foods. The basic reactions (A–C) have been continuously extended and have evolved today to a whole mechanistic cascade within the complex complete Maillard scheme. This is especially due to the fact that (I) many Strecker intermediates are very reactive, and that (II) the electronic isomerization reactions are reversible. In food systems of low water activity the intermediate hemiaminal prior to the release of the Strecker aldehyde cannot be formed (A) [5.15]. Instead, the α,β-unsaturated imine proceeds via ring formation to a 5-membered 4-oxazoline, which exists in an equilibrium to an 3-oxazoline (pathway D). Authentic synthesized oxazolines readily released the Strecker aldehyde upon addition of water (up to 56% at 37 °C). Other than by water content, the rate was strongly affected by pH and temperature. The existence of oxazoline derivatives was indeed verified in foods, for example, chocolate. Taken together, this alternative pathway explains the observation why especially from heat processed foods such as bread, snacks, and chocolates major amounts of odor active substances are formed during mastication and thereby add significantly to the retronasal perception.

In contrast to the α,β-unsaturated imine, the intermediate hemiaminal of pathway A represents an electron-rich eneaminol structure, which can be easily oxidized to give a heteroanalog α-dicarbonyl. Again, the electron-pulling carbonyl function initiates the intramolecular redox reaction to give the Strecker acid and the eneaminol after hydrolysis (pathway E) [5.16]. Thus, the ratio of formation of Strecker aldehyde to Strecker acid strongly depends on the reaction conditions. In-depth model reactions have indeed shown that in the presence of oxygen the ratio changed from about 1 : 1 under deaeration to 1 : 3–4 under aeration, that is, the formation of the Strecker acids represents a major mechanism for the processing of foods. The authors also excluded the notion that the formation of the acids might be an artifact of direct aldehyde oxidation by the use of stable isotopically labeled derivatives. Pathway E, therefore, explains the formation of the impor-

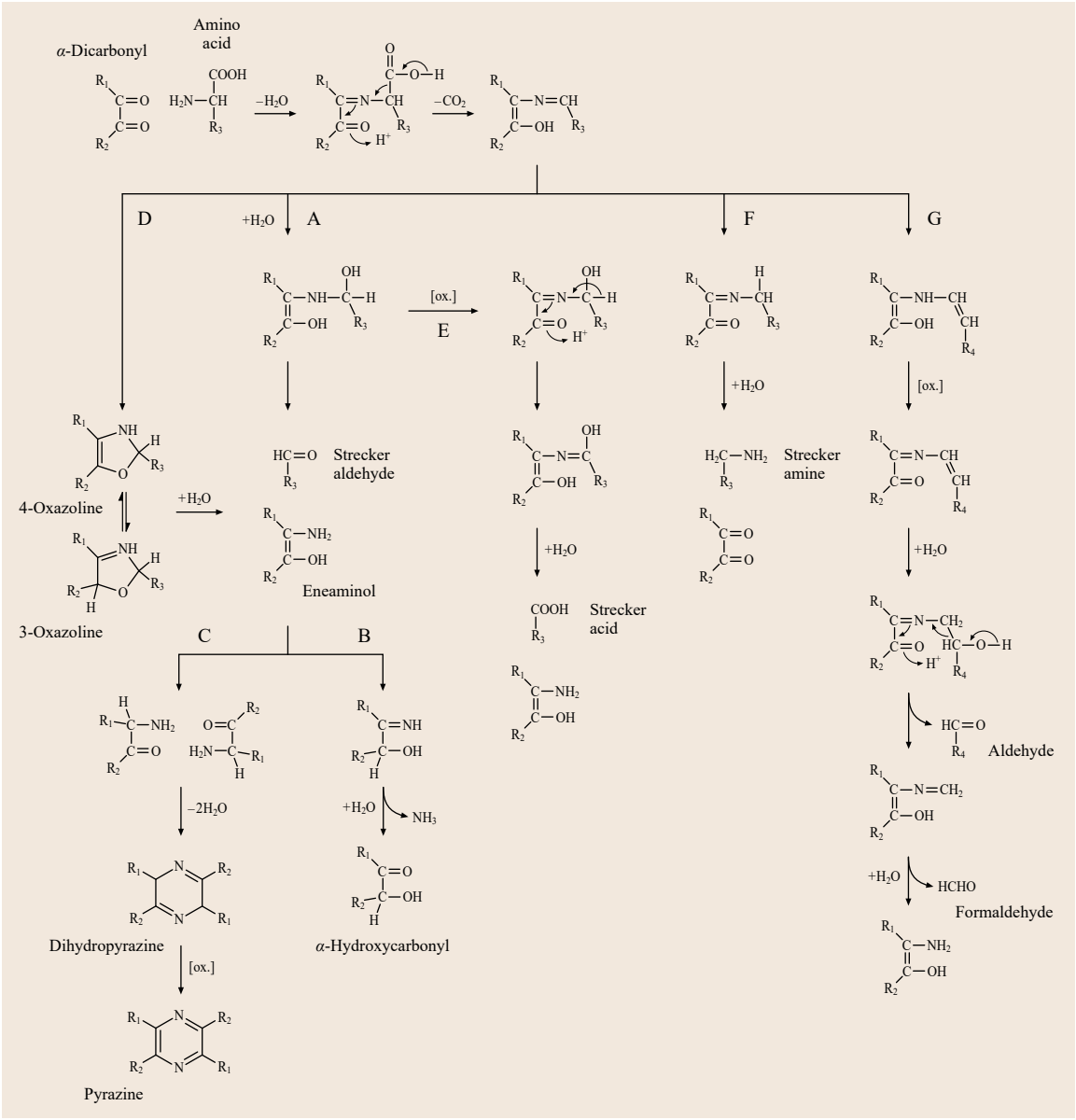

Fig. 5.6 The Strecker reaction cascade

tant odorants acetic acid, 2-/3-methylbutanoic acid, 2-methylpropanoic acid, phenylacetic acid from alanine, leucine/isoleucine, valine and phenylalanine, respectively. The acid formation also explains indirectly the detection of related methyl and ethyl esters in alcoholic beverages due to spontaneous esterification or transesterification from pectines induced, for example, under roasting conditions (ethyl-2,3-methylbutanoate, ethyl-2-methylpropanoate, methyl-2/3-methylbutanoate).

The reversibility of the above mentioned isomerization based on electronic rearrangement reactions is best envisioned by the detection of Strecker amines in amino acid/α-dicarbonyl mixtures (pathway F) [5.17]. This is obviously an alternative pathway to the pyridoxal phosphate-driven enzymatic generation of biogenic amines. However, the ratio of Strecker aldehydes to the respective amines ranged from about 1 : 100 (phenylalanine) to 1 : 1000 (other unpolar amino acids) in aqueous model systems. This and also the relatively high-odor thresholds might explain why Strecker amines are not found in above Table 5.1. The ratio might be related to the presence of electron pulling versus pushing groups,

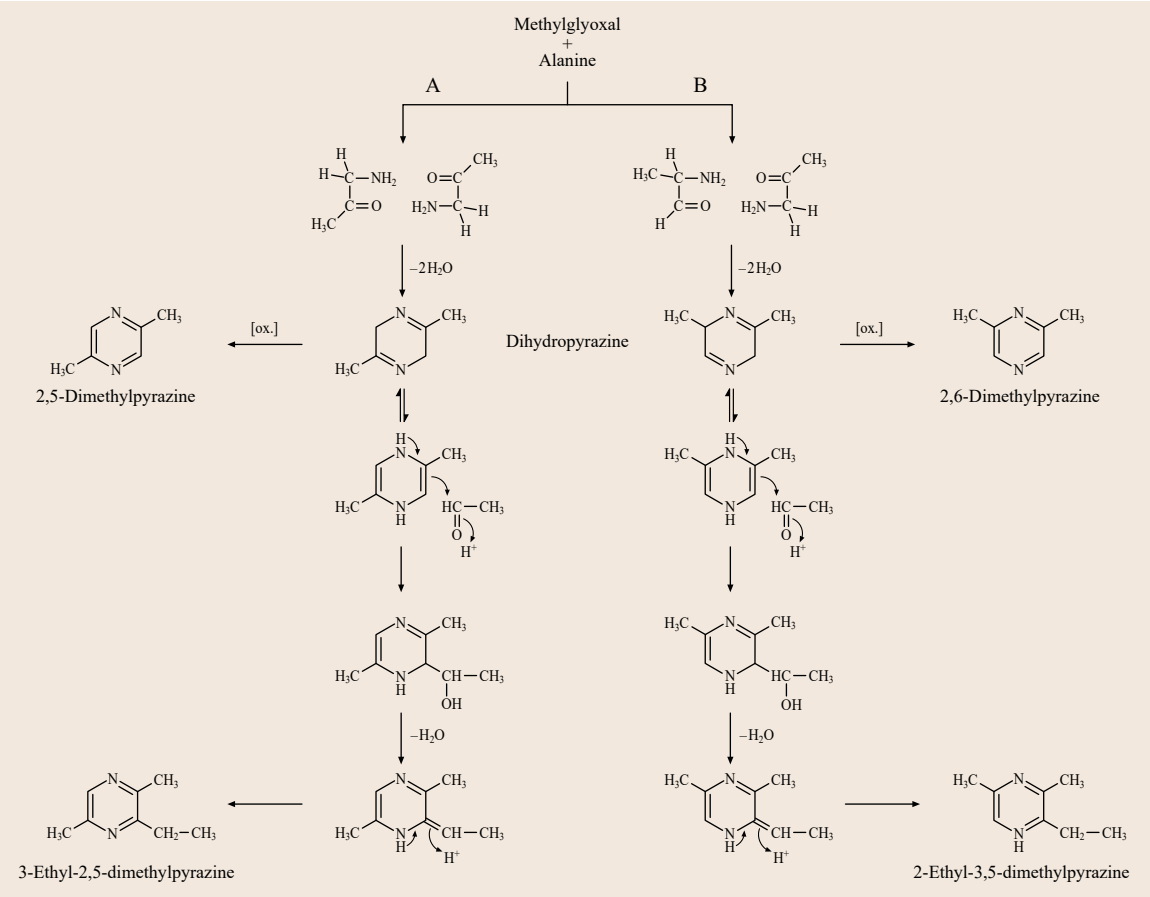

Fig. 5.7 Oxidative and nonoxidative formation of pyrazines

and also to a possible extension of unsaturation. This notion and also the reversibility were enlightened by the detection of substantial amounts of benzaldehyde (up to 7%) in reactions of 2-phenylethylamine in presence of methylglyoxal besides very small amounts of the respective Strecker aldehyde. Here (pathway G) after oxidation and addition of water, an intermediate half acetal was proposed to cleave off first benzaldehyde in a retro-aldol-type reaction and then formaldehyde to result again in the central eneaminol structure. Obviously, the cleavage of the half-acetal is strongly facilitated by the electron drawing dicarbonyl moiety.

It has to be mentioned that the Strecker pathways shown in Fig. 5.6 have been further extended today. One facet is that Strecker aldehydes have been detected in model systems of Amadori products [5.18]. This is expected, as Amadori products are transient intermediates in any Maillard system starting from carbohydrates leading to α-dicarbonyl compounds. However, the authors suggested a direct oxidative degradation route to explain different ratios of Strecker aldehydes to acids depending on the particular carbohydrate. On the other hand, Strecker-type reactions were identified in pyrolysis systems mimicking roasting processes [5.19] and in the interaction with fat autoxidation intermediates [5.20]. In contrast to earlier discussed reactions with a central eneaminol intermediate, here in both cases ylide intermediates were suggested as the important pivotal structures.

The oxidation of the dihydropyrazines to the aromatic derivatives in Fig. 5.6 occurs spontaneously in presence of atmospheric oxygen. In Fig. 5.7, this reaction is depicted in more detail for reactions with methylglyoxal and leads to 2,5-dimethylpyrazines in pathway A. Alternatively to 1-aminopropan-2-one, in pathway B condensation can also include 2-aminopropanal. However, this molecule is less abundant because the aldehyde function of methylglyoxal is more reactive than the keto moiety. After oxidation 2,6-dimethylpyrazine is formed. Thus in this oxidative pathway, the ring substituents must stem from the α-dicarbonyl compounds involved in the formation of the

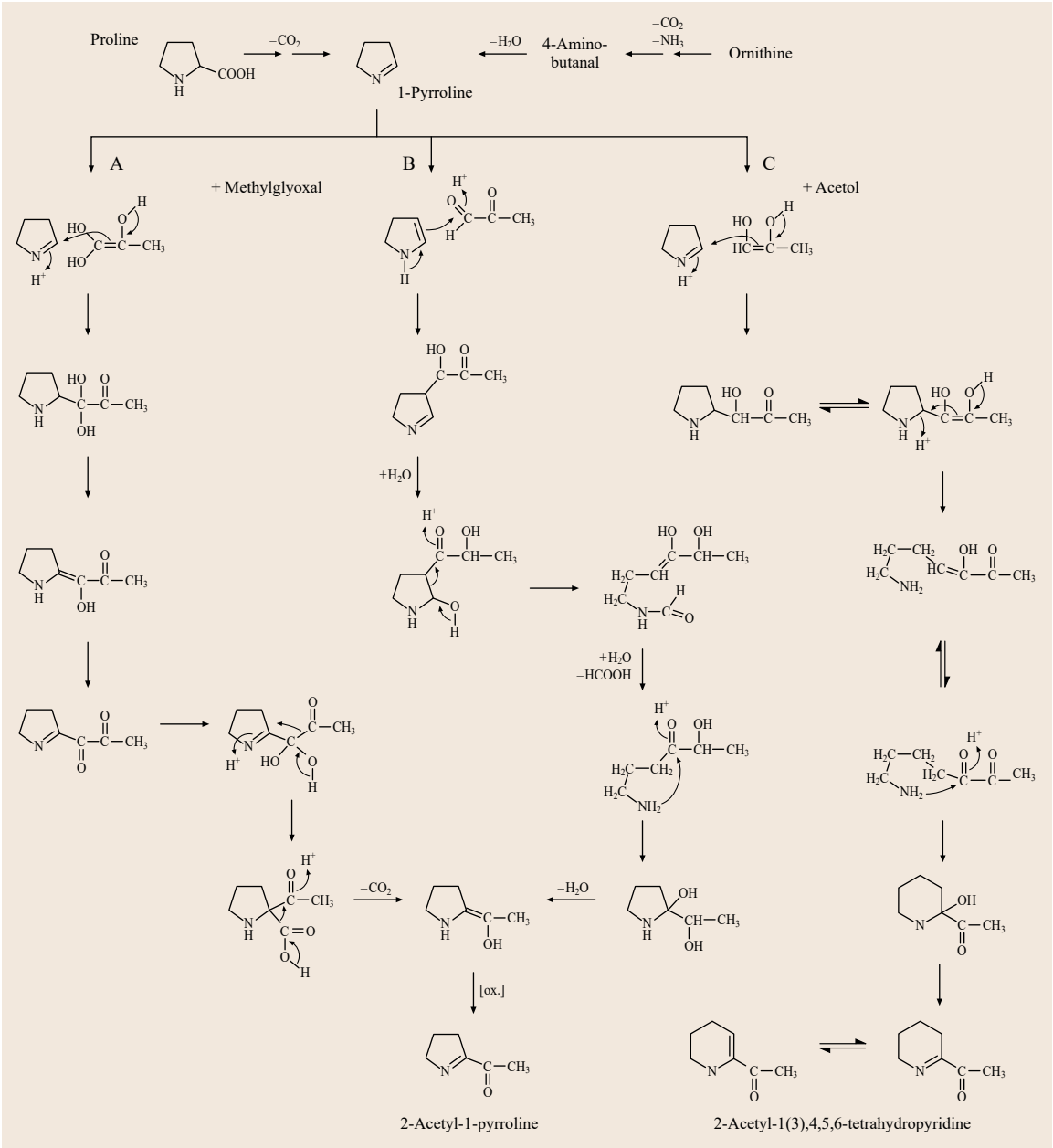

Fig. 5.8 Main routes of formation of 2-acetyl-1-pyrroline and 2-acetyl-1(3),4,5,6-tetrahydropyridine by Strecker degradation of proline with methylglyoxal

intermediate eneaminol [5.21]. As pyrazines are important agents of aroma profiles of baked, roasted, or fried foods with earthy odor qualities their mechanism of formation has been studied in detail and revealed additional nonoxidative pathways [5.22]. Here, the substitution pattern is argued by condensation of carbonyl structures to the electron rich dihydropyrazine ring system. As an example, the reaction with acetaldehyde is shown, which is the Strecker aldehyde of alanine, but may also originate from fermentative processes. Dehydration and tautomerization gives 3-ethyl-2,5-dimethyl- and 2-ethyl-3,5-dimethylpyrazine, respectively, without the need of an oxidative step. If the condensation reaction occurs with an α-dicarbonyl like methylglyoxal, the formation of acylated pyrazine derivatives can be explained.

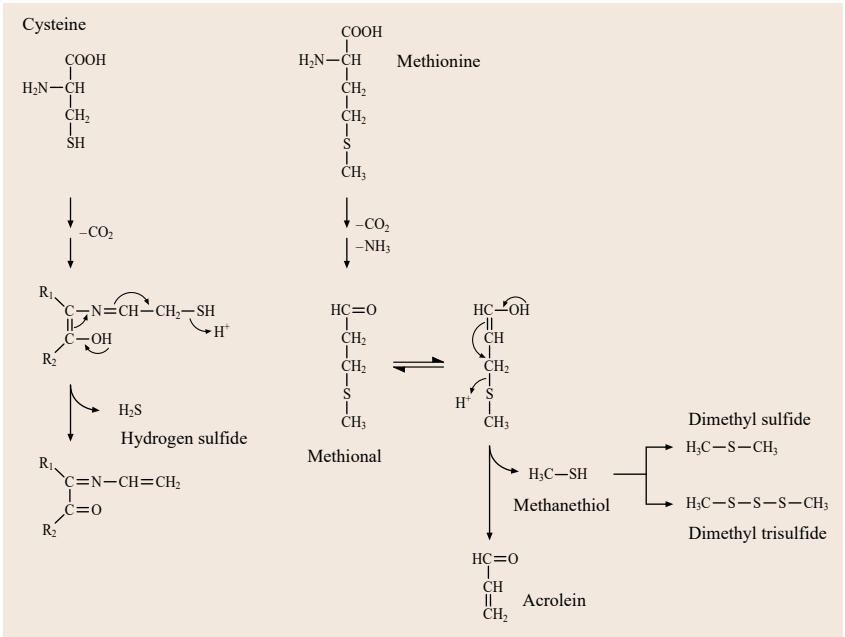

Fig. 5.9 Strecker degradation of cysteine and methionine leads to important odor active small nucleophiles

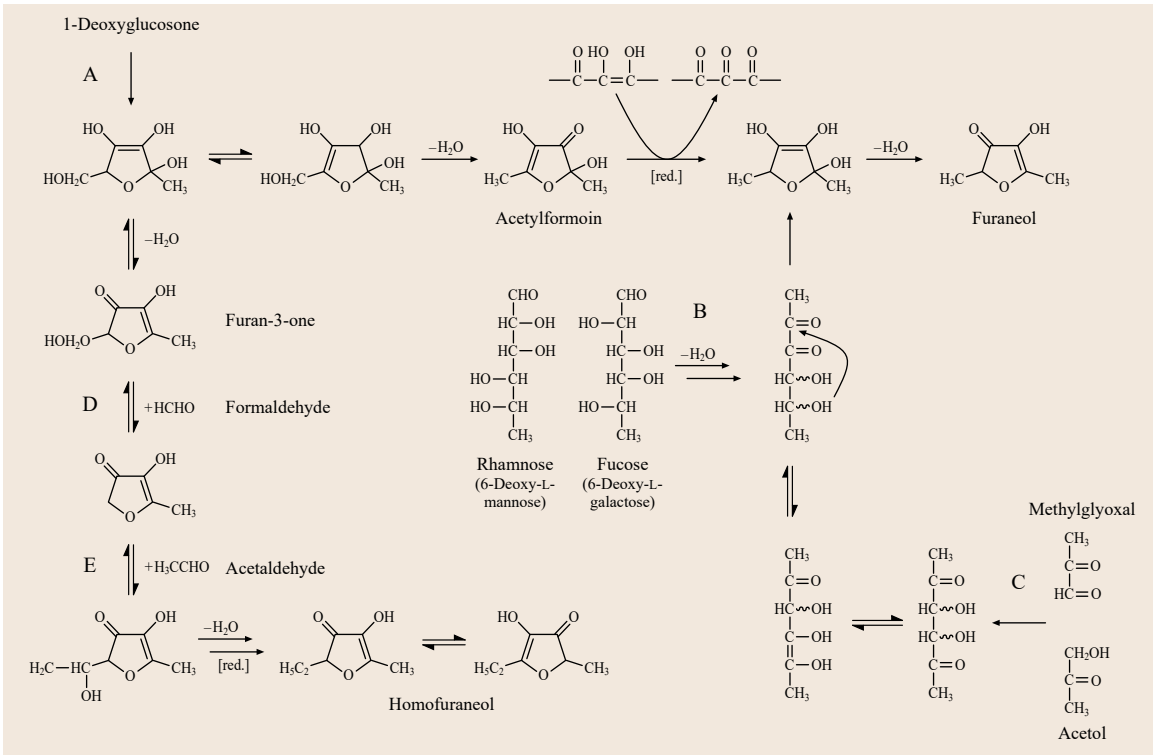

Fig. 5.10 Formation pathways of 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (furaneol)

2-Acetyl-1-pyrroline is the main representative of a class of N-containing heterocycles with roasty, popcorn-like odor notes and very low odor thresholds, especially for 2-acetyl-1-pyrroline which ranks 9 among the odorant generalists in Table 5.1 with 26% abundance. Central intermediate in the formation is 1-pyrroline,

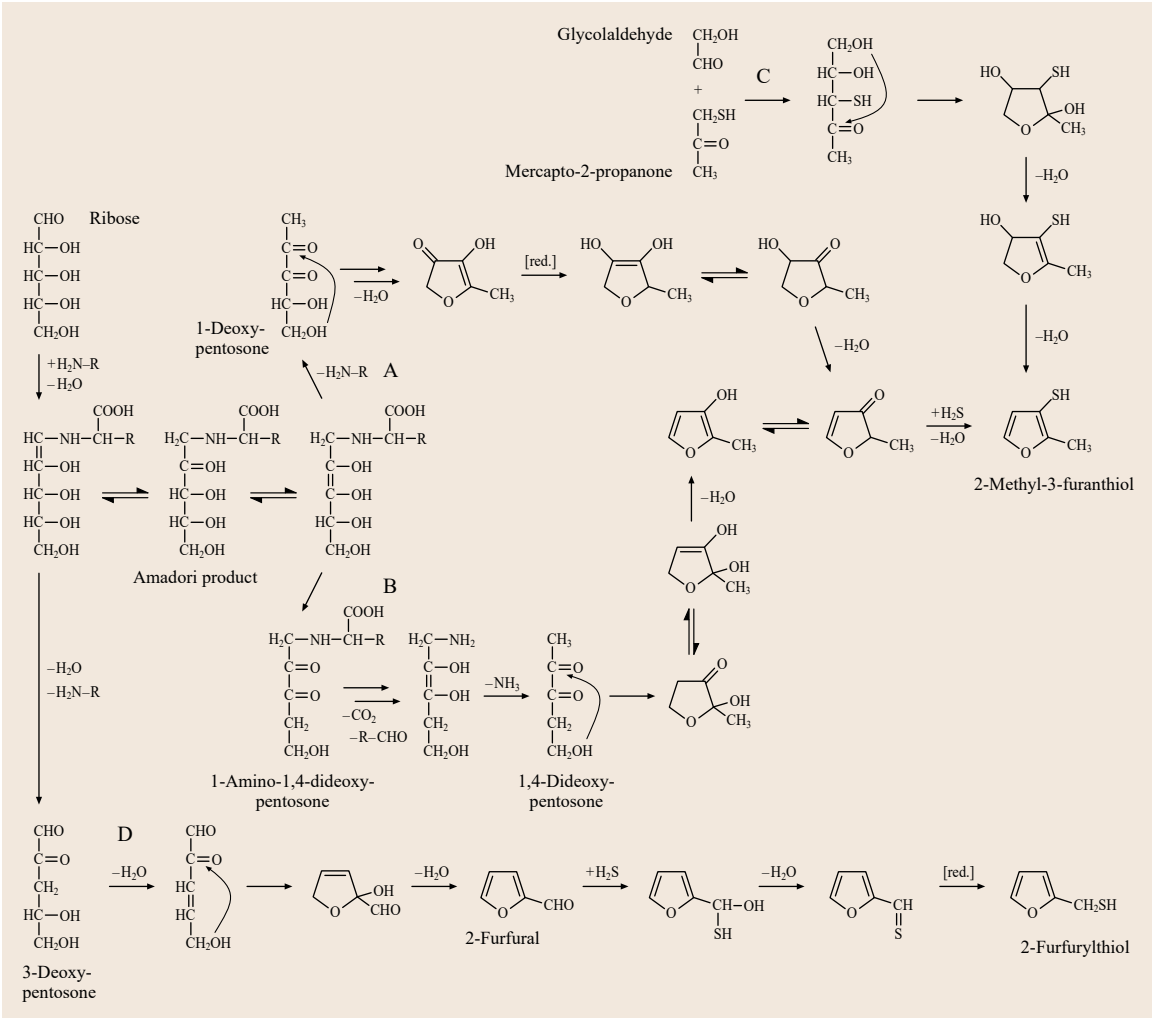

Fig. 5.11 Main formation mechanisms of 2-methyl-3-furanthiol and 2-furfurylthiol

which is the Strecker decarboxylation product of the amino acid proline [5.23]. Alternatively, but of less importance, 1-pyrroline can be formed by cyclization of the ornithine Strecker aldehyde 4-aminobutanal [5.24]. The reaction mechanism has been studied in-depth by the use of isotopically labeled educts elucidating two routes based on carbohydrate fragmentation C_3-products, that is, methylglyoxal and acetol (Fig. 5.8). The main course starts from the hydrate of methylglyoxal (pathway A) and thus allows a nucleophilic attack of the derivatized aldehyde function at 2-C of the pyrroline ring. Dehydration then leads to an N-analog reductone configuration, which can be easily oxidized to give a 1,2,3-tricarbonyl configuration. As known from other such configurations the central carbonyl function gets hydrated and allows the carbon backbone to rearrange to bring the carboxylic group in β-position to the carbonyl substituent of the acetyl group. Decarboxylation results in an electron rich eneaminol moiety that is oxidized to 2-acetyl-1-pyrroline. The loss of a C_1-fragment from the starting C_3-carbonyl has been independently verified by several authors using the CAMOLA approach to result predominately in the double labeled target compound [5.25]. However to a minor extent, a triple labeling was found too. This means that alternatively a second mechanism exists, which incorporates the intact C_3-carbon backbone of the starting carbonyl to the final pyrroline structure. The ratio of both pathways is 3/1 at pH 5.5 and changes to about 1/1 at pH 8.2, but is also influenced by other factors like the moisture content of the respective system.

Thus in pathway B, to explain the complete integration of the C_3 carbonyl, the reaction with native methylglyoxal is suggested. This allows the C,H-acidic

3-C position of pyrroline to nucleophilic attack the aldehyde function. Tautomerism and hydration lead to a hemiaminal function in β-position to the keto group of the former α-dicarbonyl backbone to enable a retro-aldol-type fragmentation. This means that in contrast to pathway A the C_1-fragment can now be cleaved off as formic acid. Cyclization, dehydration and oxidation give 2-acetyl-1-pyrroline. If the reaction starts at the oxidation level of acetol, the direct condensation of the hydroxymethylene function with the 2-C position of the ring can be realized (pathway C). In this case, the ring opening is facilitated by β-elimination of the amine function, which leads to ring expansion to a piperidine derivative, which after dehydration gives the tautomers 2-acetyl-1(3),4,5,6-tetrahydropyridine. These compounds can be found ranked under the individualists with 2.6% abundance in the earlier mentioned review on the total odorant space of foods.

The α-hydroxy carbonyl compound acetol is the result of the reduction of methylglyoxal during the Strecker cascade. As both carbonyls are common carbohydrate fragmentation products it was expected that both 2-acetyl-1-pyrroline and 2-acetyl-1(3),4,5,6-tetrahydropyridine were also found in reaction mixtures of higher sugars [5.26], but especially during the degradation of 1-deoxyglucosone and its follow-up product acetylformoin [5.27]. As explained in Figs. 5.4 and 5.10, acetol and methylglyoxal are immediate fragmentation products of these important sugar degradation intermediates. It has to be added that the described mechanisms have further been underlined by the detection of the propionyl pyrroline and propionyl piperidine analogs in reaction mixtures of proline with diacetyl and 1-hydroxy-2-butanone, respectively [5.28].

It is important to understand that the Strecker degradation leads to the production of very reactive small nucleophiles already under moderate conditions. This can be seen from the above described release of ammonia from the resulting α-hydroxyimine structure. However, even more important as educts for follow-up reactions leading to important odor active structures is the release of small sulfur compounds (Fig. 5.9). Cysteine is decarboxylated to the transient α,β-unsaturated imine. This brings the thiol substituent in β-position and leads to elimination of hydrogen sulfide. Besides being aroma active itself this potent nucleophile can condense with other Maillard-derived structures to give important odorants with extremely low thresholds like 2-methyl-3-furanthiol or 2-furfurylthiol (Fig. 5.11). Methionine gives the Strecker aldehyde methional, which easily eliminates methanethiol [5.29]. Oxidation and disproportionation then explains the formation of dimethyl sulfide and dimethyl trisulfide.

5.4 Other Mechanisms

Figure 5.4 depicts the main routes of degradation for 1-deoxyglucosone. Besides fragmentation, cyclization (C + D) leads to furanoic and pyranoic ring structures. 4-Hydroxy-2-(hydroxymethyl)-5-methylfuran-3(*2H*)-one (furan-3-one) is the main compound and reaches up to yields of 50% under moderate conditions. However, it is a nonstable intermediate and is quickly degraded at later stages. 3,5-Dihydroxy-6-methyl-2,3-dihydro-*4H*-pyran-4-one (γ-pyranone) shows the same reaction pattern, but at much lower levels (up to 8%). The formation of γ-pyranone within the 1-deoxy-route is closely related to the formation of 3-hydroxy-2-methyl-*4H*-pyran-4-one (maltol) from disaccharides, ranked within the individualists with 0.9% abundance in above odor signature study.

If alternatively to the formation of furan-3-one water is eliminated at the hydroxymethyl substituent of the furanoic half-acetal 2,4-dihydroxy-2,5-dimethylfuran-3(*2H*)-one (acetylformoin) results (Fig. 5.10, route A). This compound can easily react with nucleophiles to give, for example, pyrrol derivatives, but is also very redox active [5.30]. Disproportionation or reaction with other reducing structures or Strecker-type reactions followed by elimination of water then gives 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (furaneol) [5.31], which is one of the key generalist odorants with 41% abundance. The reduction step is prerequisite and explains why 6-deoxysugars like rhamnose or fucose are much more effective in generating furaneol [5.32]. Here, the 1-deoxy-route (B) directly leads to the target compound by repetitive water elimination without the need for a reduction step. The same accounts for the detection of furaneol in methylglyoxal or methylglyoxal-acetol incubations [5.33]. Aldol condensation gives the C_6-carbon backbone of the 1,6-dideoxy-hexodiulose intermediate (C). Interestingly, furan-3-one was reported to fragment to 4-hydroxy-5-methyl-3(*2H*)-furanone (norfuraneol), which is the pendant from the pentose 1-deoxy-route, and formaldehyde. This represents a retro-aldol reaction. Furaneol was also detected in pentose glycine reaction mixtures. Here, formaldehyde stems mainly from the Strecker degradation of glycine. Obviously, this is an equilibrium reaction, that is, formaldehyde condenses with norfuranol to result in furaneol via

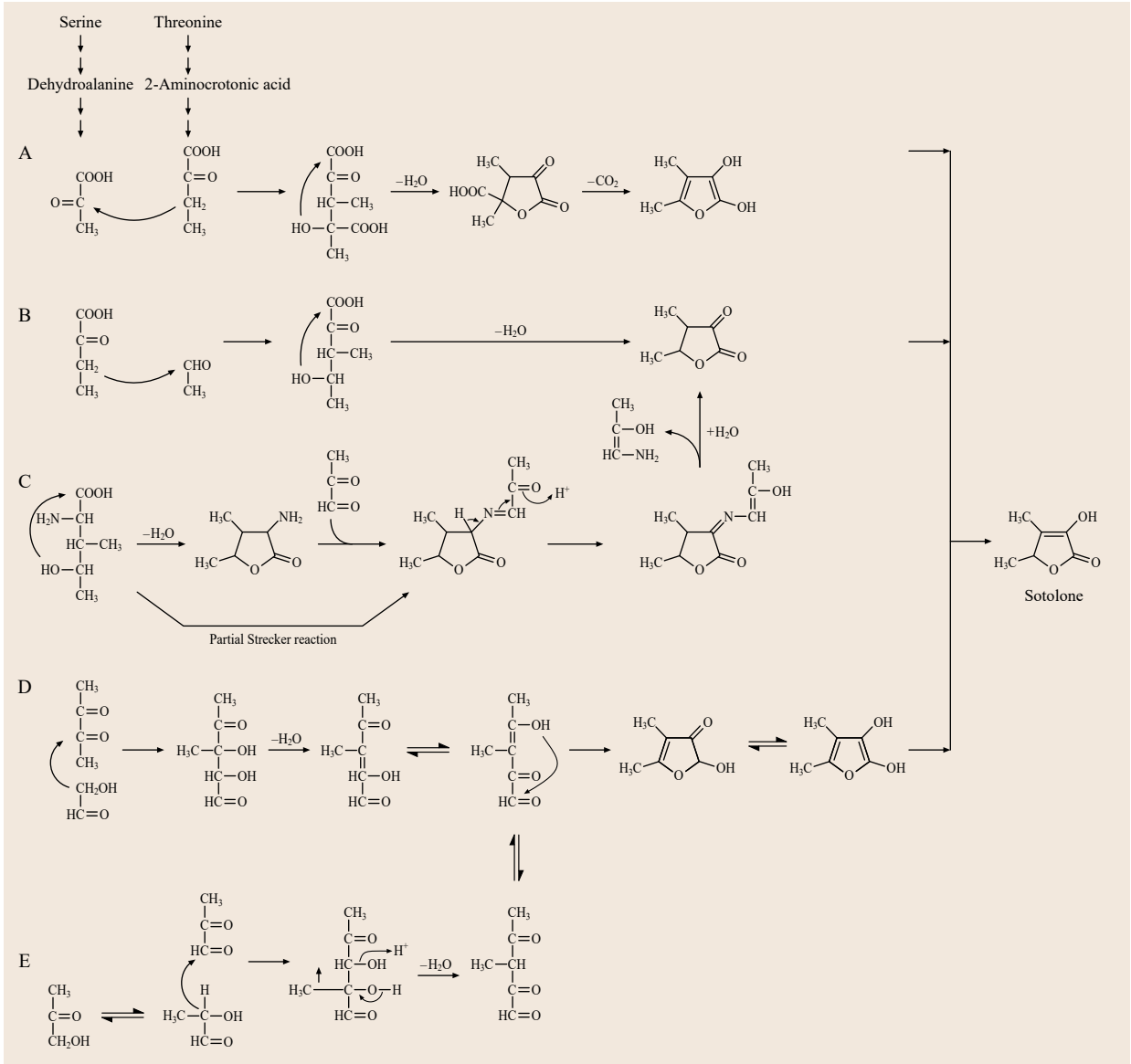

Fig. 5.12 Formation pathways leading to 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolone)

furan-3-one and acetylformoin (D) [5.34]. This aldol condensation can also occur with acetaldehyde and thus explains the formation of 4-hydroxy-2(5)-ethyl-5(2)-methyl-3(*2H*)-furanone (homofuraneol) in pentose alanine Maillard systems (E). This compound exists in two tautomeric forms in a ratio of 2 : 1.

3-Hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolone) is another furane derivative, which is of major importance to the odor composition of many foods. Figure 5.12 summarizes nonenzymatic formation pathways that have been reported in the literature. Most mechanisms include an aldol condensation step to build up the branched six-membered carbon skeleton. Relevant to strong heating processes combined with acidic pH values found, for example, under roasting conditions or protein hydrolysis is the formation of pyruvic acid from serine via dehydroalanine, enolization, and loss of ammonia (A). Threonine gives 2-oxobutanoic acid via 2-aminocrotonic acid. Both carbonyl molecules condensate to give sotolone after cyclization, decarboxylation, and rearrangement reactions. This represents an aldol-driven C_3+C_4 route, where the carboxylic acid function at 5-C is then eliminated due to its β-position to the other ring carbonyl

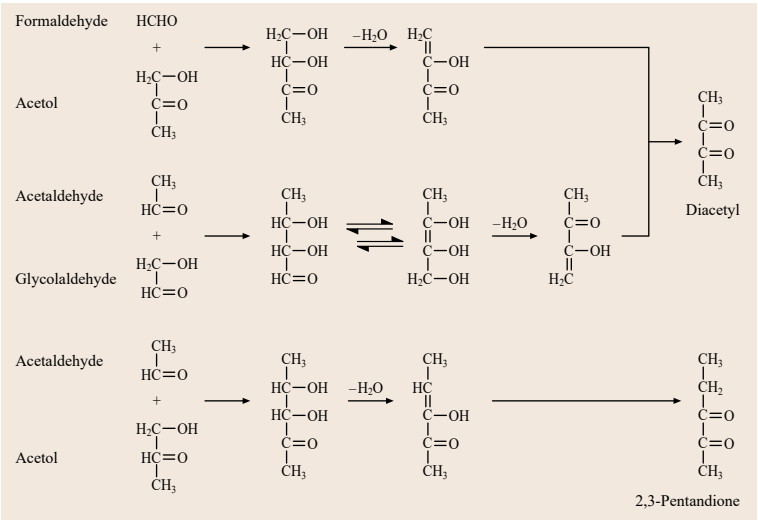

Fig. 5.13 Aldol condensation of short-chained carbonyl compounds explains the formation of diacetyl and 2,3-pentandione

functions. Alternatively, in pathway (B) for aged sake, a C_4+C_2 condensation product from 2-oxobutanoic acid and acetaldehyde was proposed to cyclize directly to the dimethyl substituted furanone [5.35]. Acetaldehyde may stem from acid degradation of threonine like the carboxylic acid or from alanine as the Strecker aldehyde. The positive correlation of acetaldehyde to the formation of sotolone was also reported for flor-sherry wines [5.36]. However, the most efficient pathway was reported for condiments as lovage and especially fenugreek (C) [5.37]. Here, the most abundant amino acid is 4-hydroxy-isoleucine, which already boasts the prerequisite functionalities. In-depth investigations of Blank et al. verified the intermediate 3-amino-lactone to be the most potent educt, to give sotolone in 40% yield at pH 6 in the presence of methylglyoxal. This unusual but very efficient deamination by a Strecker-type reaction can be argumented by the explicit C–H acidic position to give a conjugated 3-membered double bond system which is then hydrolyzed to give sotolone and the Strecker reaction typical eneaminol. On the other hand, incubations starting directly from 4-hydroxy-isoleucine were much less efficient, and a direct partial Strecker reaction was proposed eventually leading to the same ring intermediates in competition to the formation of high amounts of the Strecker aldehyde 3-hydroxy-2-methylbutanal. Alternatively, pathways D + E were proposed to explain the verified formation of sotolone in Maillard systems independent from the degradation of amino acids [5.38]. In (D), diacetyl (C_4) condenses with glycolaldehyde (C_2), dehydration then leads to a furanoic half-acetal to give sotolone after keto-enol-tautomerism. In case (E) of the condensation of acetol (C_3) and methylglyoxal (C_3) the methyl substitution pattern of the C_4-carbon backbone has first to rearrange to proceed via the same ring intermediates. In theory, this can be explained by a pinacol-pinacolone-type reaction. However to the best of our knowledge, such reactions have not yet been experimentally proven for food-relevant processing conditions.

It becomes obvious that aldol condensation reactions of short-chained Maillard intermediates play an important role in the generation of important flavor compounds. Alternatively to the above proposed retro-aldol fragmentation of 1,4-dideoxy-derivatives the formation of diacetyl was explained by the condensation of short-chained carbonyl structures, that is, formaldehyde or acetaldehyde with acetol and glycolaldehyde (Fig. 5.13) [5.28, 39]. This represents a C_1+C_3 and a C_2+C_2 route based on common sugar fragmentation products or Strecker aldehydes, respectively, to give the C_4 target structure after elimination of water and tautomerism. Aldol condensation of acetaldehyde (C_2) and acetol (C_3) also plausibly asserts the formation of 2,3-pentandione.

The Strecker degradation leads to the formation of very reactive small nucleophiles, that is, ammonia which can be released from the eneaminol intermediates, but especially hydrogen sulfide and methanethiol from the breakdown of cysteine and methionine, respectively. These nucleophiles can then easily react with carbonyl moieties to give odor intensive compounds of very low threshold, which are key agents of, for example, meaty aroma profiles. 2-Methyl-3-furanthiol was verified in incubations of 4-hydroxy-5-methyl-3(*2H*)-furanone, which is the main product of ribose via the 1-deoxypentosone route (Fig. 5.11, pathway A) [5.40]. However, this mechanism requires a reduction step to give a furan derivative of the ap-

propriate total redox level, which after elimination of water and substitution with hydrogen sulfide results in the aromatic target molecule. Although reduction steps in Maillard systems can be explained by the presence of reductone structures, this reasoning is a problem in this specific case, as the intermediate 4-hydroxy-5-methyl-3(*2H*)-furanone itself represents a cyclic reductone ether of explicit reducing capacity. Consequently, when *Cerny* and *Davidek* conducted an in-depth investigation based on the CAMOLA approach using ^{13}C-labeled sugars and authentic synthesized intermediates, they only observed very low amounts of 7% 2-methyl-3-furanthiol via this route in reaction mixtures of ribose, 4-hydroxy-5-methyl-3(*2H*)-furanone and cysteine at 95 °C and pH 5 [5.41]. Alternatively for the remaining 93%, they proposed that starting from the Amadori product the 2,3-enediol intermediate eliminates water at the position 4 to result in 1-amino-1,4-dideoxy-pentosone (pathway B). Although this α-dicarbonyl boasts an amino-reductone moiety, the prerequisite reduction step can in this case be explained by the Strecker reaction. Decarboxylation is here a strong driving force for the reduction to result in 1,4-dideoxypentosone after elimination of ammonia from the intermediate Strecker eneaminol [5.42]. Cyclization, water elimination, and incorporation of hydrogen sulfide then lead to 2-methyl-3-furanthiol. In their specific incubations starting from ribose the authors verified fragmentation and recombination reactions to be of negligible importance for the formation of the target furanthiol. However as stated earlier, sugar fragmentation is a general major aspect of Maillard sugar degradation. It is thus not unexpected that models with glycolaldehyde and mercapto-2-propanone lead to substantial amounts of 2-methyl-3-furanthiol (pathway C) [5.43]. Aldol condensation followed by cyclization and elimination of water directly leads to the target, while no reduction step is needed in this case. Mercapto-2-propanone represents an easily arguable acetol derivative. It has to be mentioned that in this study highest yields were obtained under extreme conditions of 180 °C in water-free systems.

A thiol furan structure of even higher importance to the odor space of foods is 2-furfurylthiol. For the pathway leading to this molecule above two studies were consistent with older literature [5.44], that is, the main precursor is 2-furfural, while fragmentation mechanisms were excluded. This means that starting from the *N*-glycoside of ribose the 1,2-eneaminol gives 3-deoxypentosone after elimination of water at the C-3 position and hydrolysis of the amine. Cyclization leads to 2-furfural, which condenses with hydrogen sulfide to result in 2-furfurylthiol after a reduction step (pathway D). To complete the discussion on both thiols, 2-methyl-3-furanthiol and 2-furfurylthiol, it has to be mentioned that minor pathways were reported to be valid in other reaction mixtures. This is owed to the fact, that (I) with, for example, thiamine or glutathione there are additional sources of sulfur than cysteine in foods [5.45], and (II) that hexoses and oligosaccharides can also generate substantial amounts of sugar intermediates with a five-membered carbon backbone following the fragmentation routes described earlier.

5.5 Conclusions

Major steps forward have been made in the elucidation of the complex Maillard reaction pathways resulting in nonenzymatic flavor formation. One major methodological driver was the use of stable isotopic labeled educts and intermediates to trace back their specific reaction pathways in the course of Maillard reaction. This has led to the characterization of 1-deoxyglucosone as the key reactive structure in hexose degradation systems based on insights into fundamental fragmentation mechanisms. Among others, short-chained molecules such as methylglyoxal, acetol, and acetaldehyde are thereby frequently found intermediates in the formation of major odorants. In this respect, hydrolytic β-dicarbonyl cleavage has evolved as the major scission reaction. In contrast, aldol and especially retro-aldol reactions were not substantiated to such an extent as would have been anticipated from the previous literature. Furthermore, it is important to note that the mechanistic degradation of higher sugars like disaccharides or oligosaccharides, which are much more relevant to many foods, is still not understood in full detail. On the other hand, the Strecker degradation as one aspect of the Maillard degradation of amino acids has been expanded from a simple decarboxylation and deamination reaction to a widely branched mechanism with all aspects of the general Maillard reaction scheme, including fragmentation, condensation, elimination, and redox reactions. Especially redox and rearrangement reactions are often important pieces in the puzzle of pathways explaining the formation of major odorants, but detailed insights into the underlaying mechanisms are still lacking and are awaiting to be uncovered.

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Part B Food and

Part B Food and Flavors

Food consumption is arguably the most dominant exposure to odors that human beings encounter on a daily basis. This section of the handbook further extends the general considerations of the previous section, but additionally specifically examines the molecular structures that play a major role as potent aroma contributors in foods. Humans experience food from a multisensory perspective, yet their initial encounter with a food requires an immediate appraisal on edibility, which is based on sight and smell and leads to a yes/no decision: does the food look and smell fresh and healthy and appear not to be harmful, or are there odor notes that may indicate deterioration processes, spoilage, or contamination? In addition to a hedonic rating (do I like it?), the consumer further needs to decide if a specific food item fits their current physiological status or if they need specific nutritional requirements. When a person is hungry, smells associated with energy sources such as sugar and fat provide information on where to gain energy. Meat, fish, and other protein-rich sources, on the other hand, tell us via potent odorants originating from amino acid breakdown that this food can serve as building material for our body, later constituting important parts of our bodily cells.

The course of human evolution from hunter-gatherer to domestication and farming, and ultimately industrialization, has seen a concurrent evolution of the food we eat. Early humans consumed foods just as they found them in nature, but with the discovery of tools, fire, and organisms to modify, ameliorate, cook, and conserve food for later usage the aroma and flavor impressions of foods encountered became altered, at times to a major extent, dictating a need for people to learn to accept and appreciate these modified foods. Most interesting in this context is the increasingly extended usage of microbial conversions that led to the generation of novel products such as bread, fermented meat and milk products, and acidic or alcoholic fermentation products, to name but a few. These processing steps not only helped to achieve the intended improvements such as better preservation, prolonged storage, improved digestibility, or increased nutritional effects, but also resulted in the formation of modified or new aroma profiles. Even accidental discoveries, for example, that some spoiled food was actually not really off, led to a dramatic increase in the pool of aroma and flavor impressions encountered. This evolution of food is still ongoing, with fermentation and food processing continuously undergoing development and leading to flavors and preference of novel products that our ancestors never encountered. Still, our omnivorous nature has meant that we have always been exposed to sensory variance; thus our ability to further broaden our sensory openness is an inherent trait. Exploration in this direction will surely continue as sensory curiosity is a major driver of human nature.

6. Coffee

Chahan Yeretzian

Coffee is a relatively young beverage that has only been known about since the 17th century. Initially consumed by the aristocracy, coffee has developed since the early 20th century into one of the world's most popular beverages and is now part of our daily routine and lifestyle. It also represents a major source of income for many coffee-producing countries and is a significant business sector in consumer countries. The triumph of this beverage may have been driven by various factors, but there is no doubt that its unique flavor is the prime reason for its amazing success. Here we will review current knowledge on the aroma of coffee from a chemical and analytical perspective and outline future trends. It is believed that most coffee aroma compounds have already been identified and quantified. Yet little is understood about how these aroma compounds are generated from green coffee precursors during roasting. A true definition of the aroma of freshly roasted and/or brewed coffee is very elusive and some aroma compounds start to degrade the moment they form. Furthermore, research on interindividual differences in the sensation and perception of coffee aromas is still in its infancy. After reviewing our current knowledge on coffee aroma compounds, we will outline recent developments in time-resolved analysis in three fields:

1. Aroma formation during roasting
2. Aroma extraction during espresso preparation and
3. In-mouth release during consumption.

Finally, we will address predictive models for sensory profiles derived from instrumental measurements – possibly the holy grail of aroma sciences.

6.1 Coffee Aroma – From the Seed to the Cup

The aroma of a freshly prepared cup of coffee is the final expression and perceptible result of a long chain of transformations that link the seed to the cup [6.1]. Figure 6.1 outlines various factors that impact the aroma of coffee. The final cup is the result of the interplay between genetic predispositions, environmental and climatic factors, harvest and post-harvest practices, sorting, grading, storage and transport, processing steps such as roasting, grinding and extraction and finally consumption practices. This long journey can be condensed into three key factors: *Predisposition* → *Transformation* → *Consumption*. All three play a specific and important role in the aroma of coffee.

Predisposition: The green coffee variety (genetics) with its specific set of chemical precursors forms the basis for the aromas that will later develop during the subsequent transformation steps.

Transformation: Even the best coffee will be ruined if not properly processed. The art and science of creating an exceptional sensory experience is to master every one of these transformations. The agronomy,

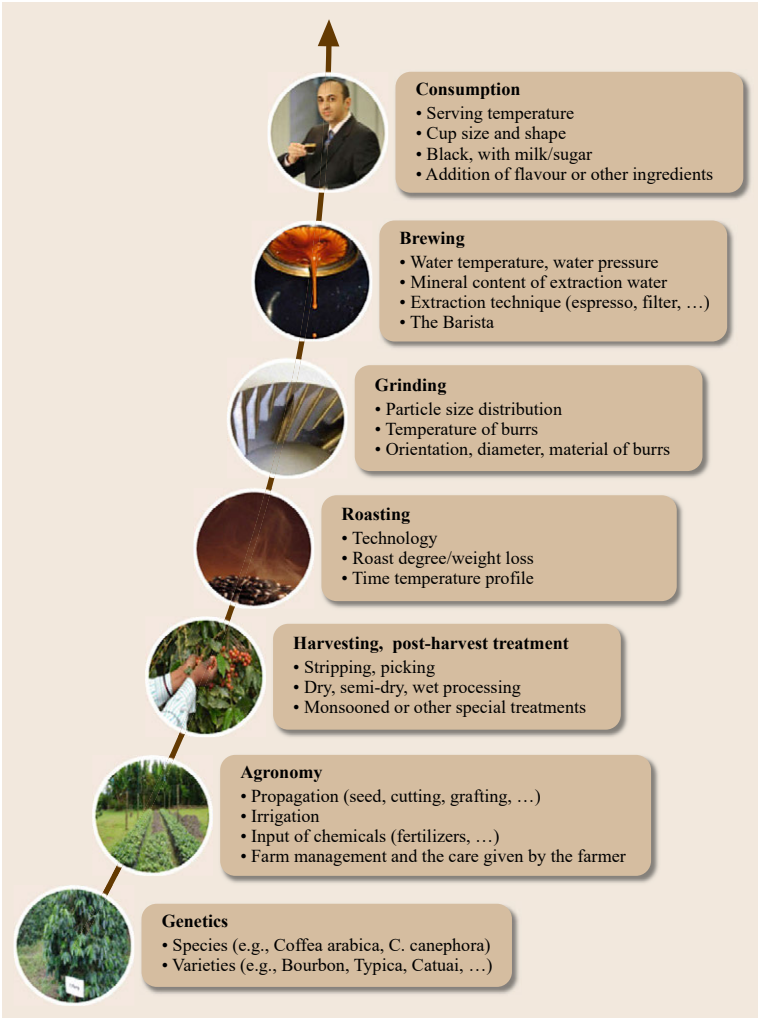

Fig. 6.1 The aroma of coffee is the final expression and perceptible result of a long chain of transformations, which link the seed to the cup. The aroma is influenced by genetic, agricultural, chemical and technical factors. However, the human factor in terms of the skill and care taken by all those involved in these transformations is just as important

climate, harvest practices and post-harvest treatment already start to modulate the genetic predisposition of the coffee beans. However, the smell and taste of green beans provide no cues as to what they might become once roasted. The most significant step in the transformation process is roasting. It unlocks the potential of the green bean and creates the coffee aroma.

Consumption: The way we prepare and consume our coffee greatly affects the flavor we perceive. However, beyond the perceptible quality in the cup, by choosing to buy a certain coffee, consumers show their support for all the work that has happened along the value chain and reward agronomic, ecological and business practices that they wish to reinforce.

6.2 The Sensory Experience of Coffee

There may be different reasons for the rapid and continuing rise of coffee's popularity. However, the prime driver for the increase is its unique set of sensory qualities. To better appreciate the multifaceted sensory experience elicited by a cup of coffee, one can schematically decompose it into four different components (Fig. 6.2).

1. *Chemical and physical properties*: This first and best understood factor of the sensory experience

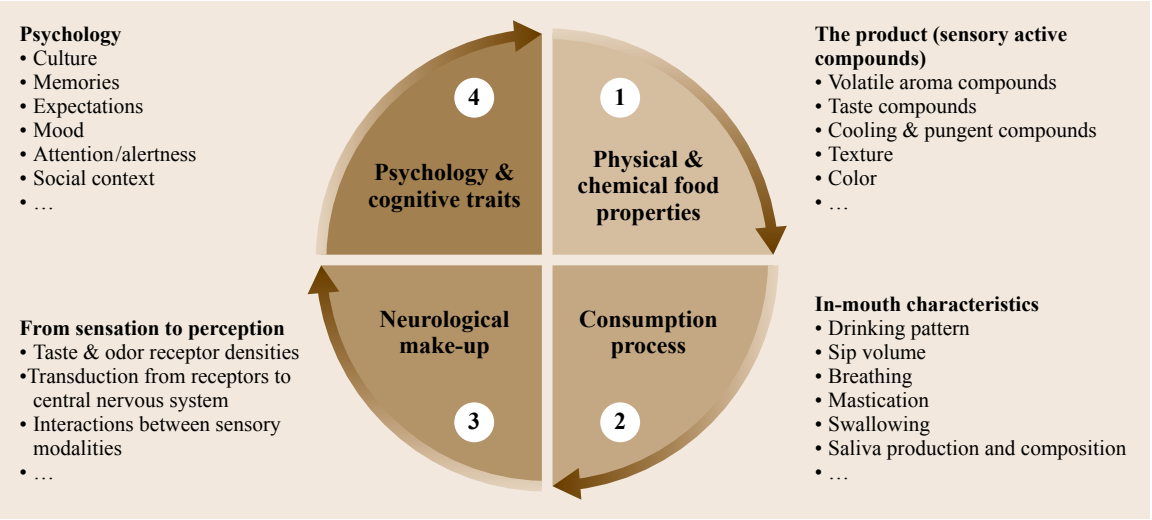

Fig. 6.2 The sensory experience of coffee is truly a holistic one. But, to understand it better, it can be schematically decomposed into four components

elicited from coffee is exclusively related to the product itself, without reference to how it is consumed and the actual consumer. These properties can be precisely measured by objective instrumental or analytical techniques and mainly focus on the concentration of aroma and taste compounds, temperature and texture (viscosity, % total solids, color). A lot of previous research into coffee aroma has focused on extracting, identifying and quantifying aroma active compounds in coffee.

2. *The consumption process*: A specific cup of coffee might have a precisely measurable composition of aroma compounds, yet these compounds may be released in-mouth and transported to the sensory receptors differently from person to person, leading to interindividual differences. Individual characteristics in consumption patterns (volume of sip, breathing rhythm, swallowing pattern, movement of the coffee in the mouth, etc.) and in-mouth physiognomy and physiology (shape of nasal cavity, amount and composition of saliva) will modulate the sensory experience and lead to interindividual differences in the sensory experience, even if consuming exactly the same coffee [6.2–10]. Indeed a given coffee will not *taste* the same for different people. This second factor therefore concerns the process of drinking and all aspects related to the physiological and physical environment in the mouth that leads to the liberation of the aroma and its transport from the oral to the nasal cavity, where the olfactory receptors are located. By recognizing the factors involved in a person's perception of coffee aroma, it has been possible to develop novel analytical approaches that open the way to individualized aroma science [6.11–13].
3. *The neurological make-up of individuals*: Besides individual differences in consumption patterns and in-mouth characteristics, the sensory experience is also modulated by individual differences in the initial sensation at the level of individual receptors as well as final and conscious perception. Sensation refers to the process of sensing our environment through touch, taste, sight, sound, and smell [6.14–16]. This information is sent to our brains in raw form where perception comes into play. Perception is the way we interpret these sensations and therefore make sense of everything around us. It is affected by a complex transduction process from activation of the multiple sensory receptors to the final response pattern at the level of the central nervous systems, where interactions from other senses may be integrated [6.17–19]. The individual sensory experience is therefore not only affected by the composition of sensory active compounds in the coffee and modulated by the individual's in-mouth characteristics and consumption habits. Individuals also differ in their neurological and physiological make-up. Like the in-mouth environment, this make-up will lead to an individually modulated sensory experience. Hence, the same coffee may elicit distinctively different sensory experiences for different people. Numerous reports have demonstrated the impact of anatomical and physiological differences on aroma perception [6.20–23]. A particularly well-studied case is bitter perception (sensitivity to 6-*n*-propylthiouracyl (PROP) or

phenylthiocarbamide (PTC). It has been established that individuals may vary in the extent to which they perceive bitter tasting compounds [6.24] and that this inheritable trait is related to the density of fungiform papillae on the tongue [6.25, 26]. There are reports that PROP sensitivity affects macronutrient selection [6.27]. Furthermore, the inability of some people to perceive specific odors, termed anosmia, is also well known [6.28, 29].

4. *Psychology and cognition*: Finally, individual history, past experiences, expectations, product familiarity [6.30, 31], psychosocial and cognitive factors such as culture, mood, and conditioning and social context can all affect the way a person experiences a cup of coffee [6.30, 32–39].

In Sect. 6.3, we will review the current status of *what makes coffee smell so good* [6.40, 41].

6.3 Coffee Aroma Compounds

Describing the sensory perceptions we experience whilst sipping a cup of coffee is not easy. It is the result of a complex multisensory experience involving all of our senses including olfaction, taste, touch, trigeminal sensation, vision, and possibly hearing.

Aroma is an odor; it is often referred to as a smell and is sensed by receptors in the nose. An important distinction is made between the orthonasal and the retronasal aroma. When we have coffee in front of us, we can smell it. We call this the orthonasal, or above-the-food aroma. VOCs, released from the coffee, enter our nasal cavity during inhalations and reach the olfactory epithelium. The olfactory centers reside very high along the roof of the nasal cavity just below and between the eyes. They cover an area of 5 cm^2 and contain some 10–20 million receptor cells. In order for these centers to be stimulated, the odor molecules have to be inhaled into the nose and carried up to the roof of the nose. The second type of aroma of significance to consumers is the aroma released in the mouth when drinking coffee. While coffee is in the mouth and also after swallowing, volatile compounds are released from the food and are transported by various airflows (mouth movement, respiration) from the oral cavity to the pharynx, passing the soft palate (*velum palatinum*). When exhaling, volatiles are swept up by the airflow coming from the oral cavity and the lungs and are released through the nose. During their transport from the oral cavity through the pharynx to the nasal cavity, VOCs pass along the olfactory epithelium and may trigger an olfactory perception. This aroma is termed the retronasal or in-mouth aroma, and is related to the aroma as perceived during eating or drinking.

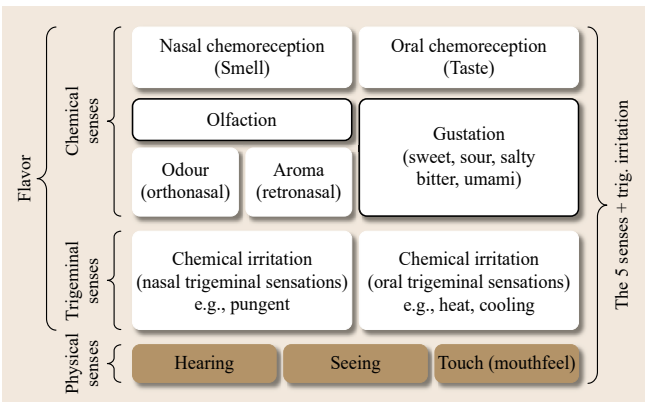

Fig. 6.3 Our senses represent our windows to the world and to our cup of coffee

Taste, or gustation, is the sensation of saltiness, sweetness, sourness, bitterness and umami (savoriness). Flavor is the combination of taste and smell. Another important additional sensation to describe the flavor of coffee is the body. It can be light, like a dry light wine, or it can be heavy, like a red wine. The perception of irritants is mediated not by taste and smell receptors, but by other chemosensitive receptors. The perceptual characteristics of chemical irritation, or chemesthesis, are mediated by nonspecific, multimodal somatosensory fibers and are a property of the skin [6.16]. These chemical senses are complemented by the physical senses of sight or vision, hearing and touch (somatosensation). Figure 6.3 summarizes the multidimensional space of the sensory experience.

Among the various sensory modalities, aroma (smell) is of paramount importance to the quality of coffee. Hence, in the remains of this chapter we will focus on volatile coffee aroma compounds. In order for coffee to be sensed by our nose (to have a smell) aromatic volatile organic compounds (VOCs) have to be released from the brew and reach the olfactory epithelium, a region in the upper part of the nasal cavity that contains the nerve endings that allow us to smell.

Scientific efforts to elucidate the origin of the rich and distinctive aroma of coffee, and ultimately to understand *what makes that coffee smell so good* [6.40, 41] can be traced back to 1880 when *Bernheimer*, a German scientist, identified the first few volatile compounds in coffee [6.72]. But the first significant progress can most

Table 6.1 Flavor active VOCs in Arabica coffee (after [6.42]). The first column lists 72 VOCs, classified into 15 different groups of chemical compounds (after [6.19, 30, 43–69])

Key odorant	CAS ID#	Concentration (ppb)[a]	Aroma descriptor	Sensory threshold (ppb)[b]
Aldehyde				
2-Methylbutanal	96-17-3	20 700	Rancid, almond-like, toasty	1.3
2-Methylpropanal	78-84-2	–	Toasty, caprylic, cheesy, dark chocolate, ethereal, fruity, malty, pungent	–
3-Methylbutanal	590-86-3	18 600	Fruity, almond-like, toasty, ethereal, chocolaty, peachy, fatty	0.35
(*E*)-2-Nonenal	18829-56-6	19	Fatty, green, cucumber, citrus	0.08
Acetaldehyde	75-07-0	139 000	Pungent, ethereal, fresh, lifting, penetrating, fruity, musty	0.7
4-Methoxybenzaldehyde	123-11-5	–	Sweet, powdery, vanilla, anise, woody, coumarin, creamy	27
Phenylacetaldehyde	122-78-1	–	Sweet, fruity, honey, floral, fermented	–
Propanal	123-38-6	17 400	Ethereal, pungent, earthy, alcoholic	10
Acid				
2-Methylbutyric acid	116-53-0	25 000	Acidic, fruity, dirty, cheese	10
3-Methylbutyric acid	503-74-2	18 060–32 180	Cheesy, dairy, acidic, sour, pungent, fruity, stinky	700
Ester				
Ethyl-2-methylbutyrate	7452-79-1	3.9	Fruity, berry	0.5
Ethyl-3-methylbutyrate	117442-70-3	14	Fruity	0.6
Furan				
Furfural	98-01-1	5880–19 370	Sweet, brown, woody, bread, caramellic	280
2-((Methylthio)methyl)furan/ 2-furfuryl methyl sulfurous	1438-91-1	–	Smoke, roast, onion, garlic, sulfurous, pungent, vegetable, horseradish	–
2-Furanmethanol acetate/furfuryl acetate	623-17-6	24 520–40 040	Onion, garlic, sulfurous, pungent, vegetable, horseradish	–
2-Methyl furan	534-22-5	–	Burnt, ethereal (mild), gasoline, acetone, chocolate	–
5-Methyl-2-furancarboxyaldehyde/ 2-methyl furfural	620-02-0	–	Sweet, caramellic, bready, brown, coffee-like	6000
Furfuryl formate	13493-97-5	–	Ethereal	–
Furfuryl methyl ether	13679-46-4	–	Roasted coffee	–
Furfuryl disulfide	4437-20-1	–	Sulfurous, coffee, roasted chicken, meaty, onion, cabbage	–
Sulfur-containing compounds				
Dimethyl trisulfide	3658-80-8	28	Sulfurous, cooked onion, savory, meaty, cabbage-like	0.001
Bis(2-methyl-3-furyl)disulfide	28588-75-2	–	Meaty, roasted scallion, onion, sulfurous	0.00076
Methional	3268-49-3	213–240	Boiled potato-like, musty, tomato, earthy, vegetable, creamy	0.2
Thiols				
3-Mercapto-3-methyl butyl formate	50746-10-6	130	Green blackcurrant, herbal, fruity, roasted, sweaty	0.0035
2-Furfurylthiol	98-02-2	1080–5080	Roasted (coffee-like), sulfurous	0.01
2-Methyl-3-furanthiol	28588-74-1	60–68	Sulfurous, meaty, fishy, metallic, boiled	0.007
3-Mercapto-3-methylbutylacetate	50746-09-3	7.5	Roasty, fruity, sulfurous, sweet	–
3-Methyl-2-butene-1-thiol	5287-45-6	13	Sulfurous, smoky, leek, onion	0.0003
Methanethiol	74-93-1	4550	Rotten eggs, meat or fish, cabbage, garlic, cheesy	0.02
Thiophene				
3-Methylthiophene	616-44-4	–	fatty, wine	–
Thiazole				
2,4-Dimethyl-5-ethylthiazole	38205-61-7	–	Nutty, roasted, meaty, earthy	–

Table 6.1 (continued)

Key odorant	CAS ID#	Concentration (ppb)[a]	Aroma descriptor	Sensory threshold (ppb)[b]
Furanone				
Dihydro-2-methyl-3(2*H*)-furanone	3188-00-9	7580–30 000	Sweet, bread, buttery, nutty	0.005
2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone (homofuraneol)	27538-10-9	16 800	Sweet, caramel, candy	20
3-Hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone)	28664-35-9	1.1–11 470	Extremely sweet, strong caramel, maple, burnt sugar, coffee	20
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (furaneol)	3658-77-3	10 930–112 000	Sweet, candy, caramel, strawberry, sugar	10
5-Ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone (abhexon)	144831-60-7	104–160	Seasoning-like, caramel-like	7.5
5-Ethyl-4-hydroxy-2-methyl-3(2*H*)-furanone	27538-09-6	17 300	Sweet, caramel, bread, maple, brown sugar, burnt	1.15
Ketone				
1-Octen-3-one	4312-99-6	–	Herbal, mushroom, earthy, musty, dirty	0.0036
2,3-Hexadione	3848-24-6	–	Burnt, buttery, caramel, chocolate cream, creamy, fruity, oily, pear, sweet	–
2,3-Butanedione	431-03-8	48 400–50 800	Buttery, creamy, fatty, oily, sweet, vanilla	0.3
2,3-Pentanedione	600-14-6	3540–39 600	Buttery, caramel, creamy, penetrating, sweet	20
4-(4′-Hydroxyphenyl)-2-butanone	5471-51-2	1	Sweet fruity, berry, jam, raspberry, ripe, floral (raspberry ketone)	1–10
1-(2-Furanyl)-2-butanone	4208-63-3	–	Rummy	–
Norisoprenoid				
(*E*)-β-damascenone	23726-93-4	195–255	Honey-like, fruity, apple, rose, honey, tobacco, sweet	0.00075
Phenolic compounds				
Guaiacol	90-05-1	2000–17 970	Phenolic, burnt, smoke, spice, vanilla, woody	2.5
4-Ethyl guaiacol	2785-89-9	800–24 800	Spicy, smoky, bacon, phenolic, clove	25
4-Vinyl guaiacol	7786-61-0	8000–64 800	Spicy, dry woody, fresh amber, cedar, roasted peanut	0.75
Vanillin	121-33-5	2290–4800	Sweet, vanilla, creamy	25
Pyrazine				
2,3-Dimethylpyrazine	5910-89-4	2580–6100	Nutty, coffee, peanut butter, walnut, caramel, leather	800
2,5-Dimethylpyrazine	123-32-0	4550–11 730	Cocoa, roasted nuts, roast beef, woody, grass, medical	80
2,3-Diethyl-5-methylpyrazine (hazulnut pyrazine)	18138-04-0	73–95	Roasted nuts, musty, meaty, vegetable, roasted hazelnut	0.09
2-Ethyl-3,5-dimethylpyrazine	27043-05-6	55-330	Roasted nuts	0.04
2-Ethyl-3,6-dimethyl-pyrazine (3,6-cocoa pyrazine)	13360-65-1	2570–5980	Potato, cocoa, roasted, nutty	8.6
2-Methoxy-3,5-dimethylpyrazine (3,5-cocoa pyrazine)	13925-07-0	1.1	Earthy, burnt, almonds, roasted nuts, coffee	0.006
2-Methoxy-3,2-methylpropylpyrazine	24683-00-9	–	Green, pea green, bell pepper	–
2-Methoxy-3-isopropylpyrazine	25773-40-4	2.4	Earthy, pea, bean	0.002
3-Ethenyl-2-ethyl-5-methylpyrazine	181589-32-2	–	Earthy	–
6,7-Dihydro-5-methyl-5*H*-cyclopentapyrazine	23747-48-0	–	Roasted nuts, earthy, baked potato, peanut, roasted	–
Ethylpyrazine	13925-00-3	–	Peanut butter, musty, nutty, woody, roasted cocoa	4000
Pyridine				
Pyridine	110-86-1	21 280—65 520	Fishy	77
Pyrrole	109-97-7	–	Sweet, nutty, ethereal	–
1-Methyl pyrrole	96-54-8	–	Smoky, woody, herbal, negative notes; defective beans	–

Table 6.1 (continued)

Key odorant	CAS ID#	Concentration (ppb)[a]	Aroma descriptor	Sensory threshold (ppb)[b]
Terpene				
Linalool	78-70-6	–	Flowery, citrus, orange, terpene, waxy, rose	0.17
Limonene	138-86-3	–	Citrus, herbal, terpene, camphor	4
Geraniol	106-24-1	–	Sweet, floral, fruity, rose, waxy, citrus	1.1

[a] The second column shows the range of reported concentrations in ppb (μg/g) in roasted Arabica coffee. For some VOCs no information on concentrations could be found in the literature.
[b] In cases where different sensory detection thresholds were found, the one included in the table corresponds to the lowest one reported. All sensory thresholds were determined in water except:
1. Matrix unknown
2. Threshold measured by first diluting compounds in ethanol in a defined concentration and then dissolving them in water (for linalool → as R-linalool) 3. Determined in ethanolic solution 9.5%
4. Determined in cellulose
5. Determined in beer
6. Determined in air.

likely be attributed to *Reichenstein* and *Staudinger*, who, in 1926, identified and patented several important aroma active compounds in coffee [6.73, 74]. Mainly fueled by progress in analytical techniques, in particular gas chromatography (GC), the number of publications on coffee aroma and the number of identified coffee VOCs has rapidly increased since then. Today, around 1000 VOCs have been reported in coffee, which includes compounds from both green and roasted coffee [6.75].

For many years, scientists concentrated on identifying the VOCs in coffee. But already by the 1970s it had become clear that only a small fraction of these volatiles – perhaps 5% – are odoriferous and hence relevant to the aroma. As a result, the focus has shifted towards these few sensory relevant aroma-active compounds in the headspace (HS) of coffee (the airspace above the coffee).

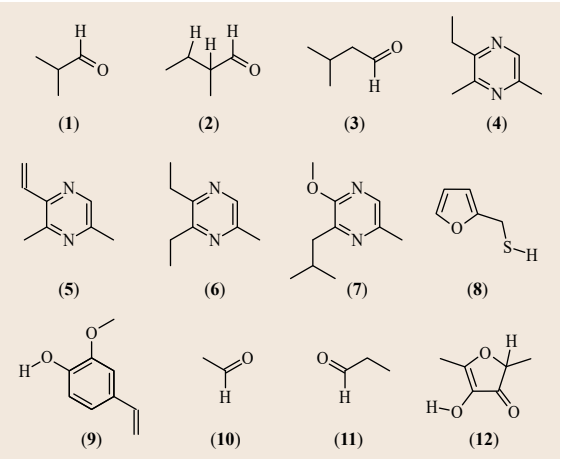

Fig. 6.4 These 12 compounds are considered of particular importance to the aroma of coffee. The numbers in brackets, below the formula, correspond to the numbers behind the names in Table 6.2 (first column)

Several instrumental methods have been developed to achieve the following objectives: to identify and quantify the odor-relevant volatiles, to assess their odor impact and note, and to recombine coffee aromas from the identified and quantified main aroma compounds. Given the extensive differences in coffee genetics, geographical origins, cultivation practices and processing techniques, it is not surprising that publications on coffee aroma composition differ in terms of the relative importance placed on the main aroma compounds. Consequently, while there has been extensive investigation into the main aroma volatiles in coffee, individual studies often report slightly different sets of volatiles that are representative of the particular aroma of the coffee being studied. Furthermore, the different methodological strategies and analytical approaches used for measuring VOC compositions are an additional source of variability in the ranking of the main aroma compounds. Hence, in order to *eliminate* variability due to differences between varieties for example, we have focused in Table 6.1 on just the Arabica variety.

In-depth studies by Grosch and his coworkers on the identification and quantification of flavor active VOCs, the determination of their odor thresholds and extraction yields, already concluded in the mid-1990s that less than 30 VOCs are important to the aroma of roasted coffee [6.44, 46, 48, 76–79]. Omission experiments further suggest that the actual number of indispensable coffee aroma compounds could be as small as nine [6.49, 51, 52, 80]. Based on this more focused and detailed work, a condensed list of coffee aroma VOCs has been com-

Table 6.2 Quantitative composition of roasted coffee, ground coffee and coffee extract/beverage, for the 28 most potent coffee aroma compounds, determined for a medium roasted Columbian Arabica coffee. (a) Mass fraction of the aroma compound in roast and ground coffee powder (after [6.70, 71]). (b) Concentration of aroma compounds in the extract/beverage (54 g of R and G extracted with 1 l of hot water) (after [6.70]). (c) Mass fraction of aroma compounds in the extract/beverage (calculated from [6.70]). (d) Extraction yield (after [6.70]). (e) Compounds whose absence was significant for most panelists when omitted from the aroma model (after [6.49]). The numbers in brackets, behind the compound names, correspond to the numbers of the structures shown in Fig. 6.4 (last column)

Compound name	Powder (µg/kg) (a)	Extract (µg/l) (b)	Extract (µg/kg) (c)	Extraction yield (%) (d)	Aroma model (e)
Sweet, caramel notes					
2-Methylpropanal (1)	24 000	760	14 000	59	x
2-Methylbutanal (2)	26 000	870	16 000	62	x
3-Methylbutanal (3)	17 000	570	11 000	62	x
2,3-Butandione	49 000	2100	39 000	79	
2,3-Pentandione	35 000	1600	30 000	85	
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	140 000	7200	130 000	95	
2(5)-Ethyl-4-hydroxy-5(2)-methyl-3(2*H*)-furanone	16 000	800	15 000	93	
Vanillin	4100	210	3900	95	
Earthy notes					
2-Ethyl-3,5-dimethylpyrazine (4)	400	17	310	79	x
2-Ethenyl-3,5-dimethylpyrazine (5)	53	1	19	35	x
2,3-Diethyl-5-methylpyrazine (6)	100	3.6	67	67	x
2-Ethenyl-3-ethyl-5-methylpyrazine	15	0.2	4	25	
3-Isobutyl-2-methoxypyrazine (7)	120	1.5	28	23	x
Roasted, sulfurous notes					
2-Furfurylthiol (8)	1700	17	320	19	x
2-Methyl-3-furanthiol	60	1.1	20	34	
Methional	250	10	185	74	
3-Mercapto-3-methylbutylformat	130	5.7	105	81	
3-Methyl-2-buten-1-thiol	13	0.6	11	85	
Methanthiol	4400	170	3100	72	
Dimethyltrisulfid	28	–	–	–	
Phenolic					
Guaiacol	2400	120	2200	73	
4-Ethylguaiacol	1800	48	900	49	
4-Vinylguaiacol (9)	45 000	740	14 000	30	x
Fruity, flowery					
Acetaldehyde (10)	120 000	4700	87 000	73	x
Propanal (11)	17 400	–	–	–	x
(*E*)-β-Damascenone	260	1.6	30	11	
Sharp					
3-Hydroxy-4,5-dimethyl-2(5*H*)-furanone/sotolone	1900	80	1500	78	
5-Ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone/furaneol (12)	104	–	–	–	x

piled in Table 6.2, where compounds are grouped into sensory families. Besides concentrations in roast and ground coffee, concentrations in the liquid coffee extract and the extraction yields have also been included. The last column in Table 6.2 marks the 12 compounds that were considered by *Grosch* and his coworkers to be particularly important – shown in Fig. 6.4 [6.49, 51, 80]. Omitting these compounds from a coffee aroma model (individually or as groups) leads to a significant difference in the coffee aroma profile and so they are considered to be of particular importance in terms of coffee aroma.

6.4 Analytical Techniques for Coffee Aroma Analysis

6.4.1 Gas Chromatography

Without doubt the classical *work horse* in coffee aroma analysis has been gas chromatography (GC). Over the past 30 years it has led to the elucidation, characterization and quantification of the compounds that are relevant to coffee aroma. Two potential weaknesses of such an approach are that it is slow, making it unsuitable if fast processes need to be monitored in real time. Furthermore, it neglects possible interactions between aroma compounds, such as masking and enhancement. Yet, in spite of these and other drawbacks, GC analysis is still considered an excellent approach for understanding and reconstituting the flavor of coffee. The ultimate proof that we *understand* the aroma of coffee is when we are able to reconstitute this aroma [6.51, 80].

6.4.2 Olfactometry

Many aroma active compounds in coffee only appear in the HS at very low concentrations, some of which can hardly be detected by analytical detectors. The only detector capable of sensing these highly potent coffee aroma compounds is our nose. Hence, aroma chemists use their nose (or a panel of *sniffers*) to detect compounds that are eluted at the end of the GC column. Trained sniffers also provide a sensory description of the compound. In combination with the retention time and mass spectral profile, the aroma note often allows the compound to be unambiguously identified. Techniques that combine separation by capillary column chromatography and the human nose as a detector are called GC-olfactometry – GC/O [6.81] and are explained in the following section.

Two major GC/O screening techniques have been developed: one by *Grosch* and his coworkers (called aroma extract dilution analysis, AEDA) [6.52, 82] and the other by *Acree* and his coworkers (called comprehensive high-throughput arrays for relative methylation, CHARM analysis) [6.83–91]. These techniques are shown schematically in Fig. 6.5. Both evaluate a dilution series of an original aroma extract using GC/O. The occurrence of an aroma in each dilution is noted. As the dilution increases, the compounds with lower odor potency successively cease to be sensed, and only the most potent are detected at higher dilutions. The number of occurrences of each odorant across dilutions are then added together. The greater the number of dilutions in which an odorant is sensed, the higher is the odor potency in both AEDA and CHARM analysis. This leads to plots of dilution- or CHARM-values. Both AEDA and CHARM analysis originally proposed that the larger the dilution- or CHARM-value, the more important the contribution of the respective aroma compound to the overall aroma. While this interpretation has slightly evolved since its introduction, both techniques are still widely used to estimate the relative importance of various volatile aroma compounds to the aroma of coffee [6.1, 84, 86, 87, 89, 92]. An alternative technique, called gas chromatography – surface of nasal impact frequency (GC-SNIF), was introduced by *Chaintreau* and his coworkers [6.93]. In this method, the intensities of the aromagram peaks were based on the detection frequencies of the odorants perceived at the sniffing port by a panel of assessors. This approach allowed standard deviations to be calculated and hence led to more quantitative data analysis than previous GC/O methods. For a review of GC/O, with a specific discussion of coffee, please refer to [6.94].

An interesting application of GC/O was the identification of the chemicals responsible for the moldy/earthy off-notes found in lots of green Mexican coffees [6.1, 92]. GC profiles obtained from a reference sample – a sample free of off-notes – and a moldy sample, showed minor differences, and no indication of

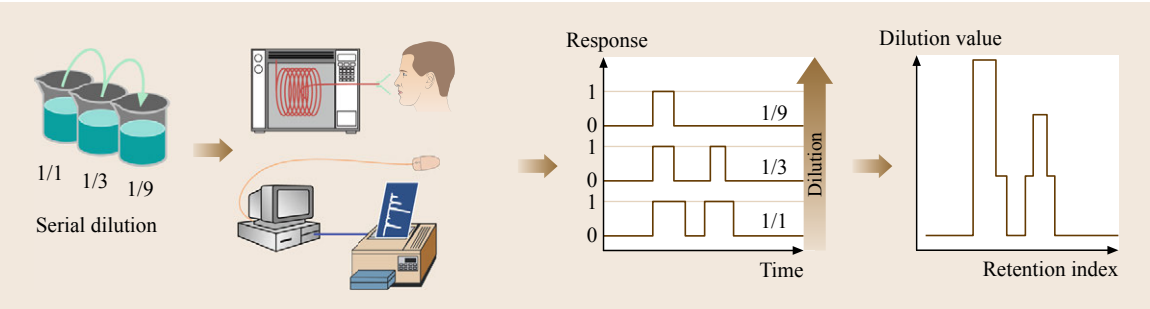

Fig. 6.5 Schematic description of gas chromatography olfactometry, GC/O. Aroma extract dilution analysis and CHARM analysis are the two most common realizations of GC/O

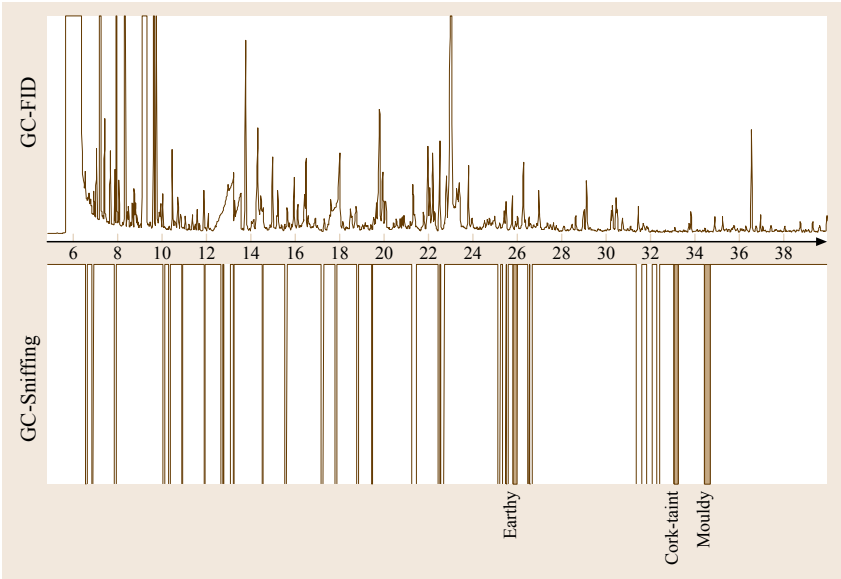

Fig. 6.6 Comparison of a GC chromatogram on the top with the GC/O profile analyzed by one sniffer on the bottom for a sample with a moldy, earthy defect. All sniffed signals without sensory descriptors represent signals that are typical to coffee and are not related to the off-note

the off-note was found. The same samples were then subjected to a GC/O sniffing analysis. This allowed several differences between the extracts that are related to the off-flavor to be identified (Fig. 6.6). In particular, earthy, green, chemical and moldy chromatographic zones were located that could be identified as 2-methyl isoborneol, 2,4,6-trichloroanisole, geosmin and various pyrazines.

The example in Fig. 6.6 demonstrates one of the strengths of GC/O: the identification of VOCs responsible for olfactory off-notes in a defective sample. It offsets the lack of sensitivity to low concentration flavor active compounds encountered with other detection systems. In this study, it was clear that instrumental detection failed to recognize the defects documented in the sensory profile [6.1, 92].

6.5 Trends and New Developments in Coffee Aroma Analysis

Through a sustained and concerted research effort over more than 30 years our understanding of the aroma of coffee has steadily grown. Today we believe that the list of aroma active compounds is essentially complete. Nevertheless, research into coffee aroma and aroma in general is still in its infancy. Major efforts are currently being made to develop a range of emerging technologies and analytical strategies. We would like to highlight three major trends that we believe will shape the future of (coffee) aroma research. These are: development of time-resolved analytical technologies (Sect. 6.5.1), progress on individualized aroma science (Sect. 6.5.2) and mathematical and statistical models to predict sensory profiles from instrumental measurements (Sect. 6.5.3).

6.5.1 Time-Resolved Analytical Techniques

Novel analytical technologies and strategies are currently being introduced, which go well beyond established chromatographic techniques and introduce time-resolved methods based on direct injection mass spectrometry using optical and laser ionization [6.95–101] and chemical ionization [6.11, 102–116]. Here we will discuss two applications of time-resolved approaches. In particular, we will present examples for coffee roasting and extraction. Both are dynamic processes requiring a time resolution of approximately one second. Both processing steps are also crucial to the transformation of coffee from the seed to the cup.

Aroma Formation During Roasting

One of the crucial steps for creating a good cup of coffee is the roasting process, where various physical and chemical changes lead to the formation of the desired coffee aroma molecules. One possibility for studying the formation of VOCs during roasting is to take samples at different times during the roasting process and analyze them offline with gas chromatography [6.70, 117–121]. However, these techniques are not only time-

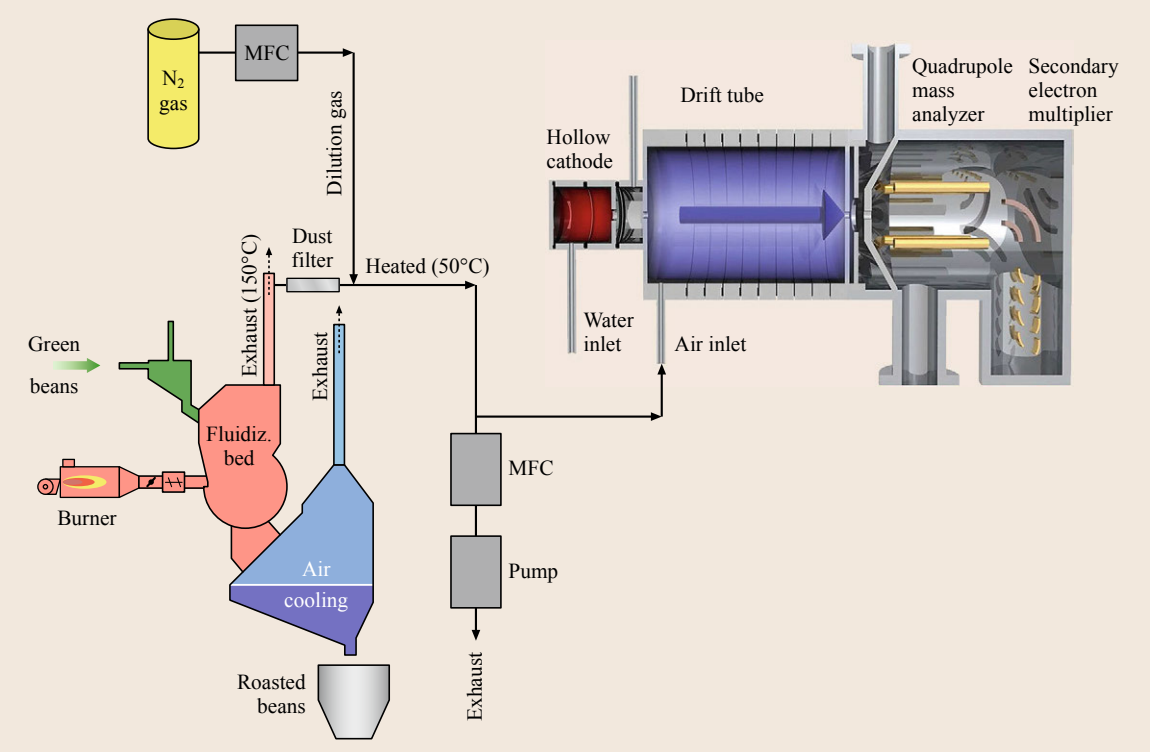

Fig. 6.7 Experimental setup for online measurements of the roaster off-gas during roasting on a 200 g/batch fluidized bed sample roaster from Neuhaus Neotec. The gas from the roaster off-gas is sampled through a dust filter and diluted with nitrogen gas to reduce the temperature and humidity of the sampled gas. All sampling lines are heated to 50 °C to prevent condensation. While most of the sampled gas is sent through the mass flow controller (MFC) and the pump to the exhaust, a small fraction is sampled via the air inlet into the drift tube of the PTR-MS. In the drift tube, VOCs are ionized by proton transfer from H_3O^+ and mass analyzed by a quadruple mass filter

consuming, but often require complex sample preparation processes before analysis, with the additional risk that irregularities in these processes might affect the outcome of the analysis. In contrast, direct online measurements of the roaster off-gas provide a direct insight into the dynamics of VOC formation in real time, are very sensitive and do not require sample preparation (hence avoiding potential distortion of information). An already well-proven technology for online analysis of coffee roasting, introduced by *Yeretzian* and his coworkers, is proton-transfer-reaction mass spectrometry (PTR-MS) [6.102, 122–125]. Furthermore, in collaboration with *Zimmermann* et al., alternative techniques based on resonant laser ionization coupled to time-of-flight mass analysis [6.95, 99–101], or ion trap mass spectrometry [6.126] have also been applied to explore the coffee roasting process. More recently, with single-photon ionization time-of-flight mass spectrometry (SPI-ToF-MS), single bean roasting has been performed on Arabica as well as Robusta beans [6.101]. Combining soft ionization via proton-transfer reaction with high mass resolution of a time-of-flight instrument (PTR-ToF-MS) [6.127] provides the advantage of a fast analytical technique to record information about VOCs formed during roasting in just one single mass spectrum. Online monitoring of coffee roasting with PTR-ToF-MS allows the formation dynamics of numerous VOCs to be followed in real time [6.114, 116, 128, 129].

Two examples of online analysis by PTR-MS of roaster off-gas during coffee roasting are provided below. The first example represents a Columbian Arabica coffee, roasted using different roaster gas temperatures to achieve the same dark roast degree (CTN 67 on the Neuhaus scale). The temperature of the roaster gas was set to 228, 238, 248 and 258 °C (isothermal roasting) for four roasting trials and more than 50 VOCs were measured simultaneously during each trial. Figure 6.7 shows the experimental setup, while Fig. 6.8 shows the time–intensity profiles for two selected compounds – furfural and 5-methylfurfural – during the four different isothermal roasting trials. While the cof-

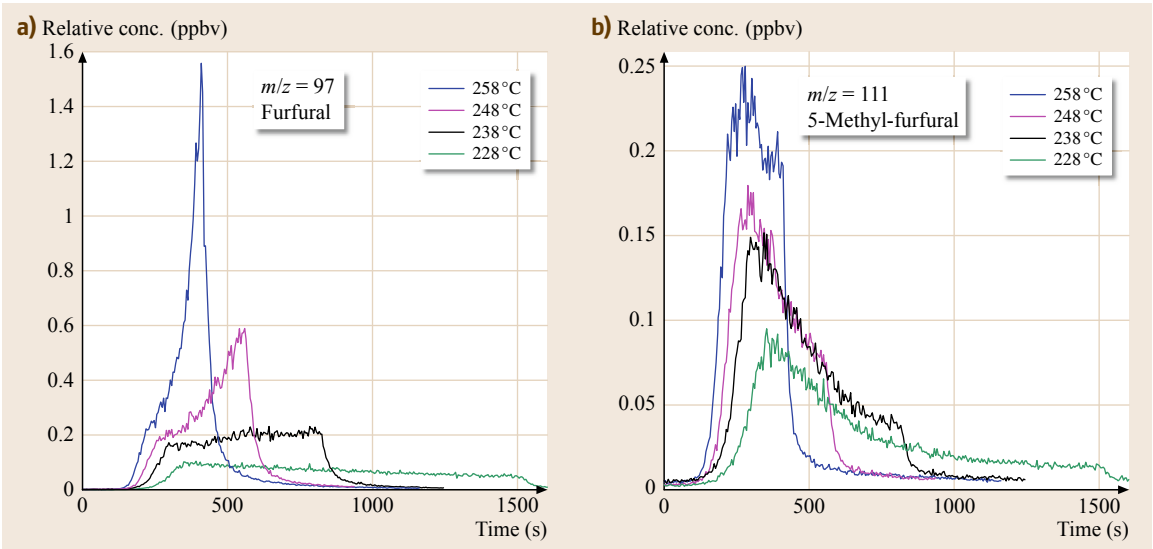

Fig. 6.8a,b The PTR-MS ion traces that are shown were monitored online and in real time in the off-gas of a fluidized bed sample roaster (200 g). Shown are two compounds (tentative assignment: furfural **(a)** and 5-Methyl-furfural **(b)**) during four different roasting trials for different hot air temperatures

fee reaches the same dark roast degree of CTN 67 in all four trails, the roasting times differed. At 228 °C (Fig. 6.8, green line), the roasting time corresponded to 25 minutes (1500 seconds). In contrast, roasting the coffee at 238, 248 and 258 °C (black, pink and blue lines), the time to reach CTN 67 was reduced to ≈ 14, ≈ 10 and ≈ 7.5 min respectively. Besides the obvious reduction in roasting time with increased temperature, the time–intensity profiles for the two selected compounds differed in two major aspects: (i) A strong increase in the intensity of the VOCs in the off-gas with increasing temperature, which corresponds to a concomitant increase in the compound's concentration in the roasted coffee [6.116]. Clearly short-time high-temperature (STHT) roasting generates coffee that exhibits significantly higher aroma intensity, in comparison to long-time low-temperature (LTLT) roasting to the same roast degree. The impact of the time–temperature profiles (for identical roast degree) has been confirmed in gas chromatographic [6.116] and sensory (to be published) studies. (ii) Modifying the time–temperature roasting profile may alter the dynamics of the compound formation and hence modulate the formation of intermediates. By exploring the formation of a range of phenolic compounds online, it has been possible to demonstrate the sequential formation of compounds, from precursors via various intermediate stages to the final VOC [6.101].

The second example examines the roasting of three distinct single-origin Arabica coffees. Figure 6.9 shows the time–temperature profiles of two example VOCs: formic acid as an example of a volatile organic acid formed during roasting and methanol as an example of a VOC with alcohol functionality. The coffees were always roasted to the same medium roast degree (103 Pt on the Probat scale), using either a medium or a low burner intensity/temperature. The left frame in Fig. 6.9 shows the PTR-MS ion traces of formic acid. Roasting at a medium burner intensity, the first to reach the target roast degree was the Central American coffee from Guatemala, followed by the coffee from South America (Colombia). The coffee that took the longest time was the Yirga Cheffe from Ethiopia. In a second series of experiments, the same coffees were roasted at a low burner intensity, which in general led to longer roasting times. However, besides extending the time to reach the target roast degree of 103 Pt, we also observed that the order (time) in which the coffee reached the target roast degree changed. The Yirga Cheffe, which clearly took the longest at the medium burner intensity, was the fastest at the lower burner intensity. This change in the order that the target roast degree was achieved for different burner intensities has been observed for many more VOCs other than those shown in Fig. 6.9. In a recent publication, the implication of such observations on split versus mixed roasting was discussed [6.116].

Extraction Kinetics of Coffee Aroma Compounds

Using proton-transfer-reaction time-of-flight mass-spectrometry (PTR-ToF-MS), we investigated the extraction dynamic of 95 ion traces in real time (time

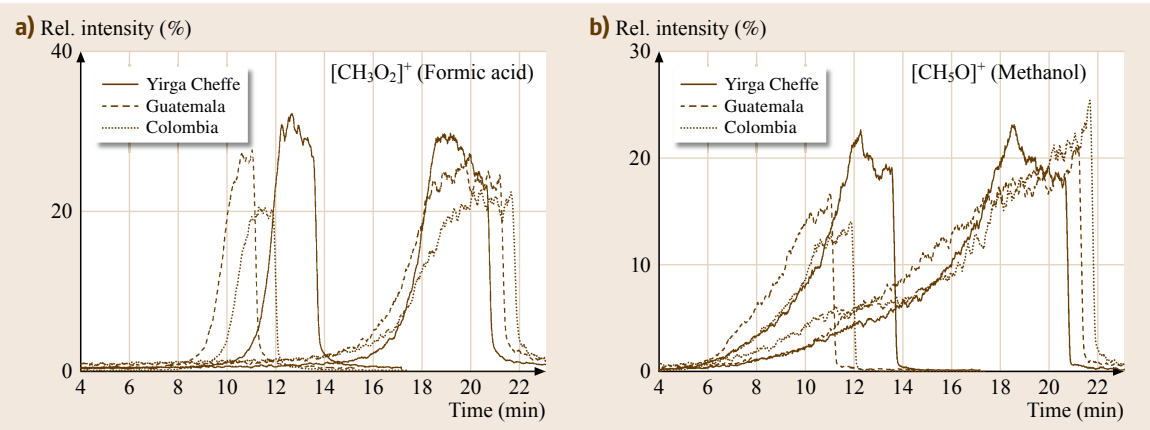

Fig. 6.9a,b Three different pure coffee varieties, all of the species Coffea arabica, were roasted to the same medium roast degree of 103 Pt on the Probat scale. Once, the three coffees were roasted by applying a lower roasting temperature (which corresponds to a lower burner intensity), which led to roasting times longer than 20 min. Alternatively, the same three coffees were roasted at the higher roasting temperature (which corresponds to a medium burner intensity), leading to roasting times between 11 and 14 min. Two example compounds are shown here: **(a)** formic acid and **(b)** methanol

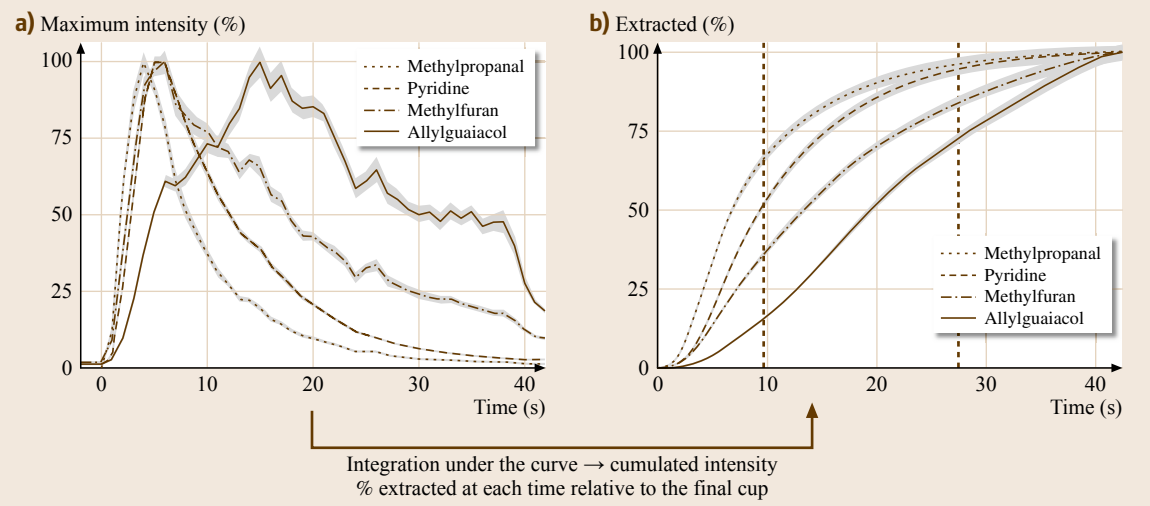

Fig. 6.10a,b Time–intensity profiles for one specific capsule (lungo, 42 second extraction time, 110 ml extraction volume) showing differences in extraction kinetics. **(a)** Data normalized to the maximum intensity of each of the four VOCs. **(b)** Integration of the area under the curve at each time point as a percentage of the total area at the end of the extraction. The shaded ribbons show the 95% confidence interval

resolution: one second) during espresso coffee preparation [6.130]. Fifty-two of these ions were tentatively identified. This was achieved by online sampling of the VOCs close to the coffee extract flow, at the exit of the extraction hose of the espresso machine (single serve capsules). The results show considerable differences in the extraction kinetics for different compounds, which led to a fast evolution of the volatile profiles in the extract flow and consequently to an evolution of the final aroma balance in the cup.

The time–intensity profiles in Fig. 6.10 show different extraction dynamics for the analyzed VOCs (Fig. 6.10a). Four example compounds are shown: methyl propanal, pyridine, methyl furan and a guaiacol. The time to reach the maximum intensity ranged from 2 to 20 seconds. Once the maximum had been reached, the intensity fell at different rates, depending on the compound. This decrease in intensity provides information on how the compounds are extracted. A fast decrease implies that the compound is extracted over

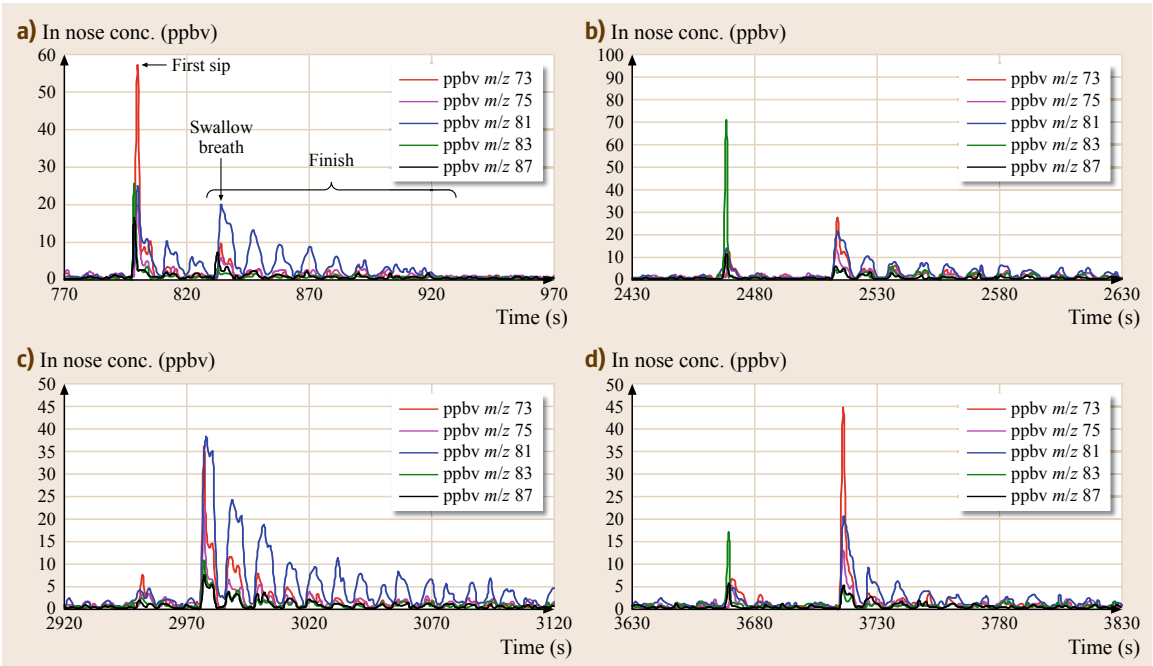

Fig. 6.11a–d Nose-space profile of four different assessors while drinking an espresso coffee. All four assessors drank the same coffee and the breath-air exhaled through the nostrils was measured online by PTR-MS. Three characteristic moments of the consumption process are outlined. *First sip* corresponds to the nose-space profiles of the exhalation just after taking coffee into the mouth. *Swallow breath* corresponds to the nose-space exhalation following just the swallowing of the coffee. *Finish* corresponds to the sequence of nose-space profiles after swallowing when the assessor no longer has any coffee left in their mouth

a relatively short time period while a slow decrease implies that the compound is extracted over a longer period. This approach will allow the extraction dynamics of VOCs to be explored under various extraction conditions, such as different pressures and temperatures and for different mineral contents.

6.5.2 Moving Towards an Individualized Aroma Science

By taking a closer look at the actual flavor experience that occurs whilst coffee is being drunk, it becomes clear that it is a highly dynamic experience, constantly changing and evolving in the mouth. In order to develop a better understanding of the aroma perceived by a consumer, it is important to develop techniques that capture the temporal evolution of the aroma during the actual process of consumption [6.11, 131–135].

Coffee aroma evolves in the mouth during drinking and leaves a typical after-odor or finish in the mouth for several minutes after swallowing – we have termed this dynamic evolution of the in-mouth aroma *the melody of coffee* [6.11, 12]. The nose-space technique allows these dynamic processes to be visualized, and provides a vivid insight into aroma release and its temporal evolution in the mouth.

Figure 6.11 shows the nose-space profiles from four different assessors (four male assessors aged 33–43 and labeled A to D when drinking the same coffee [6.11]. The time axis runs through all four experiments without interruption and shows the relative time of analysis for the four assessors. Assessor A drank the coffee first, from second 770 to 970; assessor B drank the coffee between seconds 2430 to 2630, and so forth. It is obvious that the four nose-space profiles are different. The main differences pertain to: (i) the absolute intensities of the three characteristic moments during the in-mouth consumption experience – the first sip, the swallow-breath and the after-odor, and (ii) the relative intensities of the individual VOCs (balance) for each of these characteristic moments. For assessors A and B, the first sip dominates, while assessor C perceives hardly any aroma during the first sip. For assessor C, the coffee aroma is essentially composed of the swallow-breath aroma and the persisting after-odor. Comparing assessors A and C with B and D, we notice that their first sip and swallow-breath aromas are different in terms of aroma balance. Five m/z ion traces are shown and qualified by their

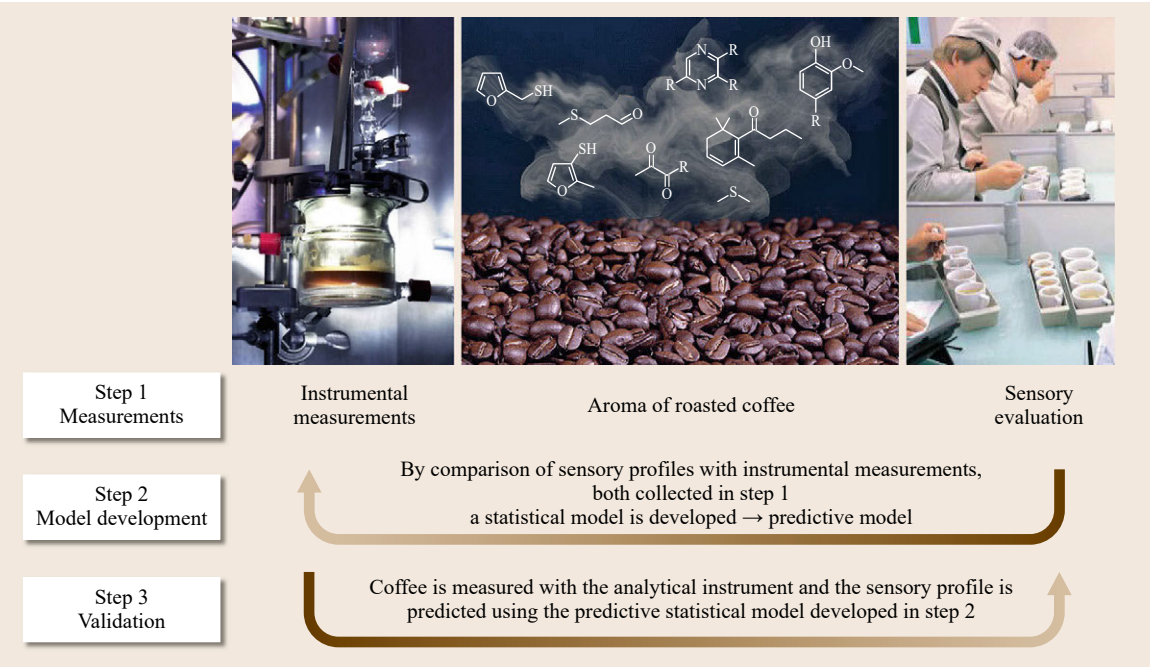

Fig. 6.12 Predicting sensory profiles from instrumental measurements: A three-step approach

m/z values. They have tentatively been assigned as: m/z 73: 2-butanone; m/z 75: methylacetate; m/z 81: furfurylalcohol; m/z 83: 2-methylfuran; m/z 87: 2-, 3-methylbutanal (57%); diacetyl (43%) [6.11]. The time resolution used for recording the nose-space spectra was 0.5 seconds.

This example demonstrates that the coffee aroma that reaches the olfactory receptors can vary over a large range between assessors. The fraction and composition of VOCs that are actually released from a food in the mouth and transported to the olfactory receptors depends not just on the composition of the food but is strongly modulated by anatomic and physiologic characteristics of the person, and may be further modulated by the person's consumption and breathing pattern. This is a demonstration of what we may already all know – a given coffee or food does not taste the same to everybody.

6.5.3 Predicting Sensory Profile from Instrumental Measurements

Improved strategies and methods for the correlation of sensory and instrumental analysis are being developed with the ultimate objective of predicting the sensory profile from instrumental measurements. While this represents a truly challenging endeavor, it is often considered the holy grail of flavor science [6.109–111].

The strategy presented in [6.109] can schematically be described as a three-step process, as outlined in Fig. 6.12. In the first step a range of coffees were analyzed by instrumental techniques (e.g., PTR-MS and/or GC-MS) and are profiled by a trained sensory panel. The second step concerns the development of a mathematical or statistical model that predicts the sensory profiles based on measured instrumental data. In the third step, in order to validate the predictive model, a se-

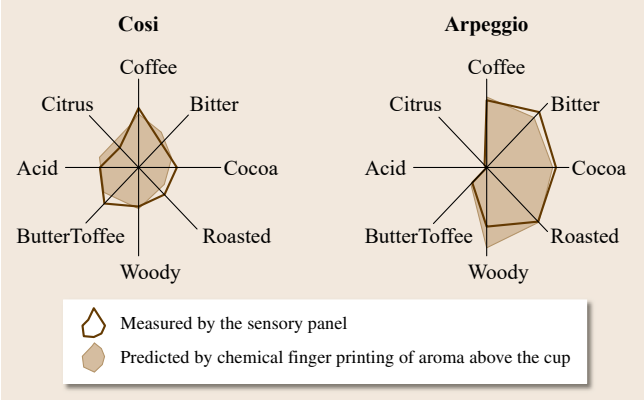

Fig. 6.13 Comparison of predicted sensory profiles, based on instrumental measurements (PTR-MS) and sensory profiles for two selected coffees. The coffees analyzed here were two single serve coffee capsules from Nespresso, a Cosi and an Arpeggio

ries of coffees were measured by instrumental methods and the sensory profiles predicted based on the model developed in step 2. Subsequently, the same coffees were profiled by the sensory panel and the profiles compared to the predicted ones. If the match was considered satisfactory, the model was successfully validated and can be applied to predict the sensory profiles of coffee, based on measured instrumental data.

In Fig. 6.13, the application of a model that was developed specifically for Nespresso single serve coffees was applied to two capsules [6.109, 110]. Predicted sensory profiles, generated using a formerly established predictive model based on PTR-MS measurements, are superimposed on the sensory profiles of the same capsules created by a sensory panel. Clearly, a very good match was achieved.

6.6 What Next?

For much of the past, research into coffee aroma focused on the identification, quantification and qualification of main coffee aroma compounds, and it is believed that essentially all relevant compounds have been identified. Consequently, the focus is shifting towards new fields. We see three major trends (among others) that we believe will dominate research into coffee aroma in the years to come.

Technological and analytical progress in instrumentation, and online techniques with high time resolution and very high sensitivity will certainly be one of the most prominent and relevant instrumental developments.

Understanding individual coffee flavor perception and preferences is a second major field of research that will attract significant attention. Novel tools and strategies will be developed to measure the volatile aroma compounds delivered breath-by-breath to the nose at an individual level. Understanding the basis of the differences in aroma delivery during coffee drinking and sensation/perception will contribute to the development of individualized aroma science.

Predicting the sensory profile of coffee from instrumental measurements is possibly the most significant challenge in flavor science and will certainly attract major attention and effort for many more years to come.

Today, flavor science is moving into a discipline that is truly multidisciplinary and that requires a new breed of scientists [6.12]. What was once the playground of food and flavor scientists and analytical chemists is today a complex scientific platform where experts from biology, psychophysics, psychology, organic chemistry, analytics, material sciences, physics, mathematics and health meet with food and flavor scientists to work in concert.

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7. Beer

Michael Dresel

This chapter presents some background information on the aroma-active volatiles in beer. It explains the evolution of the flavor-active compounds that are derived from the raw materials, namely water, malt, hops, yeast, and adjuncts. Especially the utilization of hops as well as hop itself as a key ingredient for the production of beer will be discussed. Furthermore, the flavor changes that occur during the most important manufacturing steps are summarized. Additionally, selected volatiles causing desired flavors as well as volatiles responsible for undesired off-flavors will be highlighted. Finally, this chapter will provide a detailed view on the impact of beer lagering and aging on the volatile profile of aroma-active compounds in beer.

With a worldwide production of approximately 2.0×10^9 l/yr, beer is one of the most consumed alcoholic beverages in the world. The most important beer-producing countries are Germany, the United States, and China [7.1].

Although beer is produced most of the times with only four ingredients (water, malt, hops, and yeast), it is an extremely complex mixture of aroma- and taste-active, volatile and nonvolatile components. The major constituents of traditional beer are water (91%), ethanol ($\approx$ 5.5%), carbohydrates (3%), proteins (0.5%), and carbon dioxide (0.5%). However, many minor compounds have been identified that are influencing the overall aroma perception of beer. These components can either originate from the raw materials, or can be formed de novo during the beer production or can be the result of the storage conditions of beer. Understanding the contributions of the raw materials and the processing conditions to the flavor of beer is essential for the brewers to ensure consistent product quality of existing products and to successfully introduce new products in the market. Although concentration of various flavor-active compounds in beer varies from g/l to ng/l, the importance of a compound for the flavor of beer is not its concentration but the impact that a specific component has on aroma and/or taste. In this context, consistent terminology to describe and compare the sensory properties of beer is essential [7.2].

Despite the fact that many compounds have already been identified and their origin has been elucidated, many volatiles still remain unknown; moreover, it is generally accepted that the flavor of beer depends on the balance between all theses sensorially active compounds. Also, the flavor profile changes during storage and aging of beer. These changes strongly depend on the storage conditions, rendering beer an extremely complex beverage. Reaction initiated by light, oxygen, and temperature can result in the de novo formation of volatiles or can lead to increasing or decreasing concentrations of aroma compounds. Although the phe-

nomenon of beer aging has been studied since decades, it was found to be extremely complex and is far from being understood.

This chapter deals with the origin and the evolution of the dominant volatile (off-) flavors in beer. Special attention will be drawn on hops as a raw material, as the hopping technology was found to influence the overall flavor perception of beer significantly. Furthermore, flavor changes that occur during storage and aging of beer will be highlighted.

7.1 Raw Materials

In order to obtain an insight into the aroma of beer, one must have a closer look at the respective raw materials used in brewing as the aromas are either present naturally in these materials or formed during the production. This section will especially deal with the brewing water, malt, hops, and yeast.

7.1.1 Brewing Water

Water accounts for around 90% of the beers volume. Its mineral content is of crucial importance for the brewing process: It influences the formation/degradation of bitter compounds and it contributes to the overall flavor of beer [7.3–7]. While the quality of brewing water depends on the respective local water supply, the mineral composition is nowadays very often adjusted in order to meet specific desired requirements. In this context, common treatments comprise pH and mineral adjustment, removal of iron and insoluble particles, as well as microbiological controls.

7.1.2 Malt and Wort

Next to water, malt is the most important ingredient from a quantitative perspective. It is rich in starch that will be transformed into fermentable sugars during wort production. The malt-derived aroma depends strongly on the type of malt used for wort production. Malt is rich in aldehydes but the majority of these compounds are already lost during the kilning process [7.8]. During this process, many other volatiles are formed, for example, phenolic acids [7.9, 10] and lipid-derived degradation products [7.11]. Especially, lipid oxidation products might remain in the final product and are believed to be involved in the evolution of typical staling aroma during beer aging [7.11]. Over the years, different methods of producing malt have been developed leading to a huge range of individual malt types meeting the demands of beer producers. Due to the formation of Maillard reaction products, a darker malt color results in a lower fermentable sugar content. However, most volatiles will be evaporated during wort production. Yet, studies proved that the flavor intensities of descriptors such as bread-, caramel-, burnt-like increased with an intensified malt color and that the flavor profile will be more divers [7.12, 13]. Compounds that were clearly connected to an increasing malt color and therefore wort color are furfural, 5-methyl-furfural, pyrazine, methylpyrazine, and 3-methylbutanal and its aldol condensation product 2-isopropyl-5-methyl-2-hexenal [7.12] (Fig. 7.1).

Another important compound that is formed during kilning and wort production is dimethyl sulfide (DMS; Fig. 7.2). This compound can be beneficial to the overall flavor of beer at concentrations above 30 µg/l but below 100 µg/l. Concentrations above 100 µg/l result in a flavor that is often associated with cooked vegetables or cooked sweet corn. Much research has therefore focused on the parameters that influence the formation and release of DMS during beer production [7.14–16]. Especially, two formation pathways are important that are significantly influenced by the brewing conditions [7.17]:

1. *S*-methyl-methionine (SMM; Fig. 7.2) can release DMS, under the temperature conditions necessary

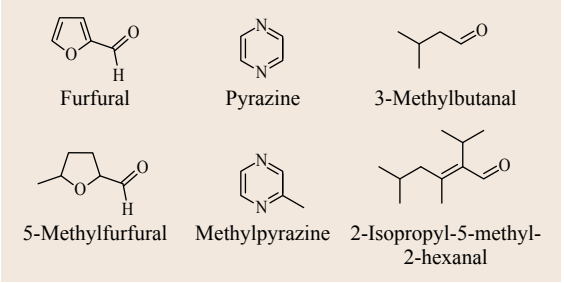

Fig. 7.1 Chemical structures of volatiles clearly influenced by wort color

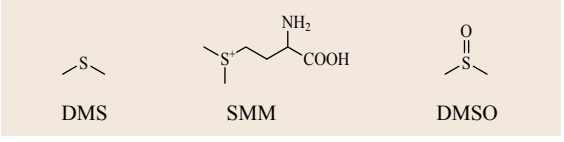

Fig. 7.2 Chemical structures of dimethyl sulfide (DMS) and two important precursors, *S*-methyl methionine (SMM) and dimethyl sulfoxide (DMSO)

during wort production. This precursor is present in grains which are relevant for the brewing industry such as barley, wheat, and oat.

2. DMS can be liberated from dimethyl sulfoxide (DMSO; Fig. 7.2) by both eukaryotic and prokaryotic microorganisms [7.18]. This precursor is especially produced during kilning at temperatures above 60 °C.

Both SMM and DMSO are very soluble in water and are therefore easily extracted during mashing. Especially DMSO is heat-stable and nonvolatile under these conditions and will be reduced during fermentation to give rise to DMS.

Besides these two, more than 100 compounds, among them sulfur compounds, alkenals, aldehydes, ketones, esters, alcohols, and furans, can be detected in wort [7.13]. However, their concentrations are comparably low and their contribution to the overall flavor of wort and beer remains unclear until today.

7.1.3 Hops

Although normally used in rather small quantities, hops play a major role for the beer aroma. Especially its essential oil is responsible for the unique aroma of hops and beer. The essential oil content typically ranges from 0.2–2.0 ml/100g for aroma hop varieties and 0.7–3.0 ml/100 g for bitter hop varieties.

Currently, over 450 volatiles have been identified in hop's essential oil [7.19], but estimations suggest that the hop oil actually comprises over 1000 compounds [7.20]. The most important compound classes are hydrocarbons, oxygenated compounds, and organosulfur compounds [7.21]. With a share of up to 75%, the hydrocarbon fraction is the most predominant group of the essential oil. Within this group, the monoterpene β-myrcene and the sesquiterpenes α-humulene and β-caryophyllene are the most important compounds (Fig. 7.3). β-Myrcene is only relevant for so-called dry hopped beers (Sect. 7.2.3), as it is almost completely lost during the wort boiling, due to its high volatility, and remaining levels will be further reduced during beer production. Despite the fact that the shares of these three compounds vary a lot between the different hop varieties, it is believed that a low β-myrcene and a high α-humulene content is connected to a pleasant aroma perception of beer [7.8].

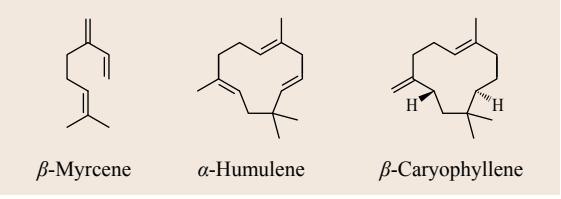

Fig. 7.3 Chemical structures of β-myrcene, α-humulene, and β-caryophyllene

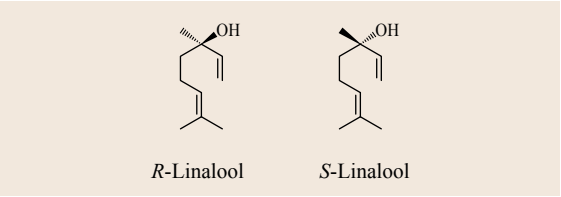

Fig. 7.4 Chemical structures of *R*-linalool and its stereoisomer *S*-linalool

The second largest group comprising up to 50% of the hop oil are the oxygenated compounds. Among them, many esters of straight and branched chain fatty acids and alcohols can be found. One important oxidation product of β-myrcene that is already present in hops is *R*-linalool (Fig. 7.4) and represents around 95% of the total linalool content. This compound has been suggested to be a marker compound to measure the hop flavor in beers, whereas its stereoisomer *S*-linalool (Fig. 7.4) is less flavor-active [7.22]. Like β-myrcene, other terpene and sesquiterpene hydrocarbons are sensitive toward oxidation (Sect. 7.2.1) and enzymatic biotransformation (Sect. 7.2.2).

Interestingly, it was recently shown that hop pellets that were slightly aged and used for dry hopping (Sect. 7.2.3) led to more pronounced fruity, floral, and herbal aroma impressions than a beer that was produced the same way with regular vacuum-packed hops [7.23].

Branched esters like 2-methylpropyl isobutyrate and 2-methylbutyl isobutyrate contribute to the fruity aroma of hops. Furthermore, free short-chain fatty acids such as 2-methylbutyric acid are responsible for a cheesy aroma perception when hops age.

Nowadays, with the discovery of 4-mercapto-4-methylpentan-2-one (4-MMP) (Fig. 7.5) [7.24, 25], organosulfur compounds moved into focus of interest [7.26, 27]. Especially 4-MMP polarizes brewers as this compound is characteristic for the blackcurrant-like aroma of especially American hops and beer. Unfortunately, this compound is perceived as catty, when

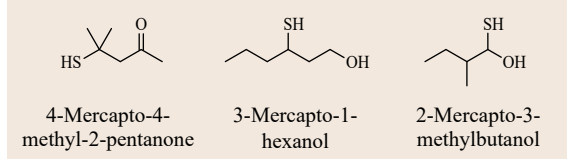

Fig. 7.5 Chemical structures of 4-mercapto-4-methyl-2-pentanone, 3-mercapto-1-hexanol, and 2-mercapto-3-methylbutanol

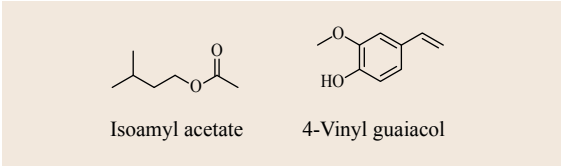

Fig. 7.6 Chemical structures of isoamyl acetate and 4-vinyl guaiacol

present in higher concentrations. The main problem in this context is the huge inter-individual variation of the odor threshold value. As a result, one will perceive the aroma of 4-MMP as pleasant in a beer, whereas another person will reject this beer. Currently over 40 polyfunctional thiols, for example, 3-mercapto-1-hexanol, 2-mercapto-3-methylbutanol (Fig. 7.5), are known. However, they seem to play a role only for a small number of hop varieties.

Yet, it is impossible to estimate the relevance of these compounds as they are only present in relatively low concentrations, but possess extremely low flavor threshold concentrations of a few ng/l [7.28]. Also, additive and synergistic effects might be of relevance for this substance group if different compounds are present in beer.

Besides the mentioned volatiles, hop aroma compounds are present in a glycosidically bound form in hops [7.29, 30]. The amount differs from variety to variety and also depends on the hop product. For example, a supercritical carbon dioxide extract of hops contain very little glycosidically bound compounds, whereas in ethanolic extracts, they will be present.

7.1.4 Yeast

Although yeast is vital for the production of beer, it does not contribute on its own to a specific beer aroma. Instead, due to its versatile metabolic pathways, yeast might generate specific volatile flavors that are characteristic for a specific beer style. A traditional German wheat beer will offer banana-like as well as clove-like and phenol-like flavors originating from isoamyl acetate [7.31] and 4-vinyl guaiacol [7.32], respectively (Fig. 7.6). These compounds are produced in elevated concentrations by specific yeast strains.

Furthermore, the nature of the yeast is of crucial importance, as it is known that ale strains (top fermenting yeast) naturally tend to produce more sensory-active esters and higher alcohols as lager yeast strains (bottom fermenting yeasts). In contrast, typical lager strains produce less of these sensory-active compounds [7.33]. However, the fermentation temperature plays a major role as studies gave proof that a higher temperature also results in an increased production of esters and higher alcohols [7.33]. These findings apply for both top- and bottom-fermenting yeast species. Traditionally, bottom-fermented beers are produced at temperatures around 10 °C, whereas top-fermented beers are fermented between 15 and 25 °C. This procedure favors clearly the production of esters and higher alcohols in ales. Yet, some beers are pushing the borders resulting in beers with special flavor characteristics, for example, German Kölsch (top fermenting at lower temperatures) or *German Alt* (bottom fermenting at higher temperatures).

7.1.5 Adjuncts and Other Additives

Whereas malts, which do not necessarily have to be derived from barley, are used to influence the overall aroma and taste perception, specialty malts can also contribute to the fermentable sugar content. In contrast to that, adjuncts are nonmalted materials and are mainly used to save costs or to enhance the brewhouse capacity. Furthermore they are used to influence color, foam stability, colloidal stability, flavor stability, and sweetness.

In order to create unique tasting experiences, other products might be used at different time points to introduce special flavors. The term *Gruit* refers to an old-fashioned herb mixture that can be used instead of or additionally to hops. These adjunct herbs commonly include sweet gale (*Myrica gale*), ground ivy (*Glechoma hederacea*), mugwort (*Artemisia vulgaris*), horehound (*Marrubium vulgare*), yarrow (*Achillea millefolium*), and heather (*Calluna vulgaris*). Sometimes other herbs as juniper berries, ginger, caraway seed, aniseed, nutmeg, or cinnamon are also used. Besides, other additives can be orange peel and coriander in Belgian Wit Beers or fruit beers, especially cherries, in case of Belgian-style Fruit Lambics.

7.2 Flavor Evolution of Hoppy Aroma

As the production scheme of the first wort, the unhopped wort, can hardly be altered in order to provide sufficient saccharification for the later fermentation step, the composition of flavor-active volatiles is barely affected by the technological steps. At this point, the flavor of the wort is influenced by the choice of specialty malts providing caramel-, biscuit-, toffee-, chocolate- and coffee-like as well as other roasted flavors. In con-

trast to that, not only the choice of the hop variety but also the precise hopping regime has a huge impact on the overall beer flavor.

7.2.1 Cooking

Traditionally, hops are added to the hot wort after the spent grain has been removed by filtration. During the following boiling step, the congeners of the humulones are extracted and isomerized to form the *cis*- and *trans*-iso-humulones (Fig. 7.7). These transformation products are addressed as the bitter principle in beer [7.34, 35]. In order to achieve sufficient isomerization of the humulones, boiling times over 60 min are required.

Accordingly, most hop-derived volatiles, especially terpenes like β-myrcene, β-pinene, and *cis*-ocimene, are almost completely lost during this production step [7.36]. Moreover, these compounds are very sensitive toward oxidation. Especially, the epoxidation of double bonds in compounds like α-humulene and β-caryophyllene leads to compounds that have been detected in beer and are believed to contribute to the flavor of beer [7.37]. However, it is not easy to estimate the transfer rate into beer, as those volatiles can also be formed during fermentation.

As mentioned before, *R*-linalool (Fig. 7.4) is believed to be a marker substance for hop flavor in beer [7.22]. However, it must be taken into account that the flavor-active *R*-isomer undergoes a racemization during boiling toward the less flavor-active *S*-isomer (Fig. 7.4), leading to a significantly lower amount of *R*-linalool and its concentration will decrease further during beer aging [7.38]. Nevertheless the absolute decrease of compounds like linalool is hard to estimate. *Kollmannsberger* et al. discovered that these compounds are present in hops in a glycosidically bound form and can be released later during fermentation due to glycosidase activity and liberation of the flavor-active aglycones. This could explain increasing concentrations of several hop-derived aroma compounds in beer over time [7.29, 30].

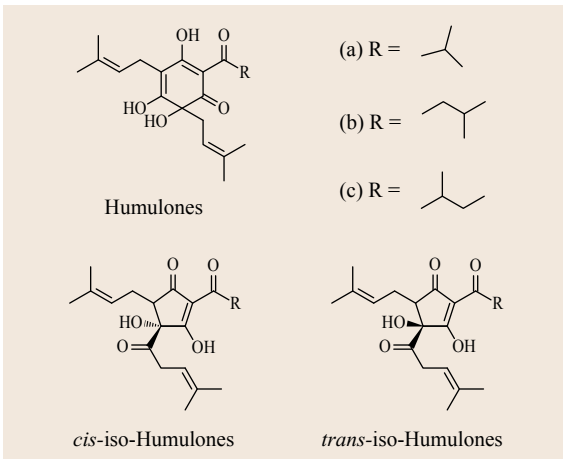

Fig. 7.7 Chemical structures of the co-(a), n-(b), and ad-(c) congeners of the humulones and their transformation products, the corresponding congeners of the *cis*- and *trans*-iso-humulones

7.2.2 Fermentation and Lagering

Another important process step, during which the hop-derived aroma profile significantly changes, is the fermentation. The most abundant terpene hydrocarbons β-myrcene, α-humulene, and β-caryophyllene are almost completely lost during fermentation. So far, no biotransformation products of these compounds have been detected in beer [7.39, 40]. Losses are believed to be caused by adsorption to the hydrophobic biomass and migration to the foam layer [7.39, 41]. The more hydrophilic oxidation products of those compounds that are already formed during wort boiling are more likely to remain in the final beer and have been proposed as potentially important flavor-active compounds [7.42]. Interestingly, humulol II might be an exception as it was produced in model fermentation studies with α-humulene, but not obtained during hydrolyzation experiments with humulene epoxides [7.37].

Several saturated and unsaturated aldehydes (e.g., neral, β-citronellal, and citral (= geranial) [7.43] (Fig. 7.8) and ketones (especially methyl ketones) occur in the hop essential oil. These carbonyl compounds will be transformed by the yeast due to various dehy-

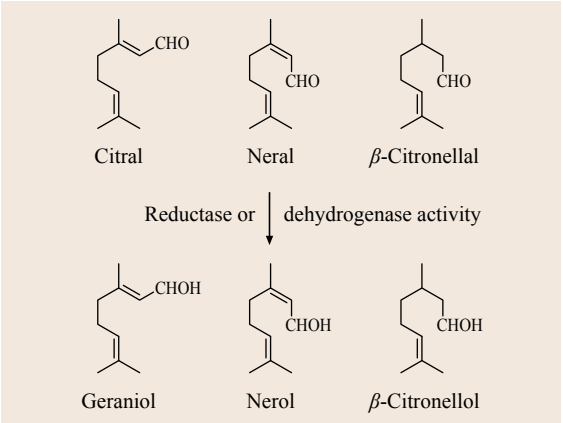

Fig. 7.8 Biochemical reduction of citral, neral, and β-citronellal to the corresponding alcohols geraniol, nerol, and β-citronellol

drogenase and reductase enzymes to the corresponding alcohols [7.44–46] .

Another important compound class are saturated and unsaturated esters. Especially methyl esters are found in hops and undergo both hydrolysis toward acids and transesterification toward ethyl esters during fermentation [7.46]. However, contradictory results are described for terpene-derived esters. While, *Peacock* and *Deinzer* found that geranyl acetate is hydrolyzed during fermentation [7.47], *King* and *Dickinson* describe that geranyl and citronellyl acetate are formed during fermentation with lager yeast but not during fermentation with ale yeast [7.42].

Interestingly, concentrations of geraniol and linalool decrease during fermentation, whereas the concentration of β-citronellol increases [7.48]. *King* and *Dickinson* [7.42, 49] reported earlier that during the fermentation, geraniol can be reduced to β-citronellol or can undergo a isomerization and give rise to linalool which reacts further to α-terpineol and terpin hydrate (Fig. 7.9). Furthermore, the cis–trans isomerization of nerol to geraniol is described as a nonstereospecific biotransformation during fermentation. Accordingly, it is generally accepted that terpenes do not transform spontaneously under the conditions occurring during beer production.

Norisoprenoids, such as β-ionone and β-damascenone (Fig. 7.10), are highly flavor-active degradation products of carotenoids. Yet, these two compounds are already present in hop oil. Nevertheless, β-ionone and β-damascenone have been found in beers at levels that might be relevant for the hoppy aroma [7.43]. Moreover, studies revealed a significant increase in β-damascenone during fermentation [7.50, 51] and it is believed that this phenomenon is due to the release of compounds from glycosidically bound aroma precursors [7.51]. *Biendl* et al. [7.30] and *Kollmannsberger* et al. [7.29] showed that the glycosidic fraction of beers produced with ethanolic hop extract or hop pellets, is susceptible to acidic and enzymatic hydrolysis. Moreover, a series of aroma-active compounds was obtained under these conditions: Aliphatic alcohols (e.g., 1-octen-3-ol, *cis*-3-hexen-1-ol), aromatic compounds (e.g., benzyl alcohol, phenyl ethyl alcohol, methyl salicylate, vanillin), monoterpene alcohols (linalool, *cis*- and *trans*-linalool oxide, α-terpineol, geraniol), and norisoprenoids (e.g., β-damascenone, 3-hydroxy-β-damascenone, 3-hydroxy-7,8-dihydro-β-ionol). Yet, another study indicates that the absolute concentrations of compounds released during the brewing process strongly depend on the hop variety [7.52]. However, knowledge on the evolution of hop glycosides during fermentation and the influence of the yeast type is fragmentary and more profound research is required to determine the impact of those compounds on the actual flavor perception of beer [7.53].

Finally, another group of oxygenated hop oil derived compounds are ethers (e.g., rose oxide), which are flavor-active compounds that have been detected in beer [7.54, 55] and might contribute to the hoppy aroma [7.43, 54]. Although major biotransformation pathways and biotransformation products have been revealed, it is not yet fully understood which volatile compounds are responsible for certain aroma impressions that are evolving during fermentation.

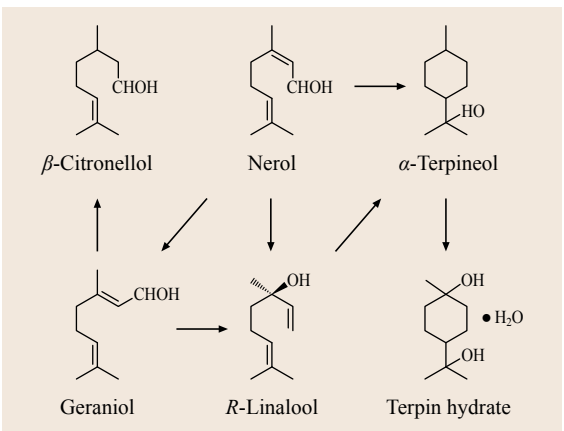

Fig. 7.9 Biochemical transformations of some monoterpenoids

7.2.3 Impact of Different Hopping Technologies and the Hop Variety

As mentioned before, the composition of the hop oil is quite divers and a lot of changes occur during beer production. Accordingly, different hopping technologies will have a huge impact on the flavor of the final beverage. Depending on the hop product, different flavor-active fractions might not be introduced into the beer. For example, a hop extract obtained by extraction with supercritical CO_2 contains rather unpolar compounds, mainly α- and β-acids, whereas the vast majority of flavonoids, polyphenols, and glycosides remain in the residue. In contrast to that, an ethanolic extract comprises to a certain extent polyphenols and

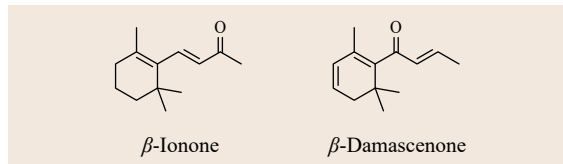

Fig. 7.10 Chemical structures of β-ionone and β-damascenone

glycosides [7.56]. However, such an extract does not reflect the original composition of hop cones or pellets and therefore lacks several flavor-active compounds. Accordingly, the flavor profiles of beers produced with those extracts will differ from a beer hopped with hop cones or hop pellets. However, it is not known if and how those compounds may influence the aroma of the final beer products.

Furthermore, the time point of adding hops has a great influence on the compounds transferred into beer. In order to reach an isomerization of the hops α-acids toward the better soluble iso-α-acids (Fig. 7.7), the bitter principle of beer, a sufficient boiling time is required. To achieve this transformation and depending on the recipe as well as the beer style, boiling times between 60and 120 min are necessary. It is easily conceivable that such a long process at high temperatures will affect hop-derived volatiles in many ways. Most vulnerable are compounds with low boiling points, such as short-chain ketones, aldehydes, and most importantly, monoterpene hydrocarbons (e.g., β-myrcene and β-pinene) and monoterpene alcohols (especially linalool and geraniol). A hop addition at the beginning of the boiling process will result in an almost complete loss of those compounds. Furthermore, during this so-called *early hopping*, sesquiterpene hydrocarbons will be oxidized. Especially, the oxidation products of α-humulene and β-caryophyllene have been studied thoroughly and many of those compounds have been suggested to be at least partly responsible for the spicy aroma of early hopped beers [7.44, 57, 58].

In order to counteract a complete loss of these compounds, an additional hop dosage can be applied toward the end of the boiling process. Such a hop addition is usually done during the last 5−15 min and does not essentially contribute to the formation of iso-α-acids. On the other hand, the more volatile compounds will at least partly remain in the hopped wort. However, all compounds that are introduced by *early* and/or *late hopping* might be metabolized during fermentation.

Besides, a hop addition toward the end of the fermentation or even in the lager tank is possible. This so-called *dry hopping* can be seen as an extraction of hop (oil)-derived compounds by an ethanolic solution. Accordingly, especially polar compounds (glycosides and monoterpene alcohols) will be extracted, influencing the overall flavor in a unique way.

In this context, not only the time point of adding hops is essential, but also the chosen hop variety. As the original hop oil content varies between 0.4 and 3.7 ml/100 g [7.59, 60], the amount of hops added during each hop addition is crucial regarding odor threshold concentrations. Furthermore, the hop oil composition strongly depends on the variety. For instance, farnesene, α-selinene, β-selinene, and aromadendrene are compounds whose occurrence is limited to certain varieties [7.61]. A strong varietal-dependency was also found for the release of glycosidically bound compounds during the beer production, such as linalool, β-citronellol, nerol, β-damascenone, geraniol, eugenol and terpinene-4-ol [7.52].

Moreover, sulfur-containing compounds have been found to be especially characteristic for hop varieties from America or New Zealand. For example, 4-mercapto-4-methylpentan-2-one (catty/black cassis-like aroma) and 3-mercaptohexan-1-ol (grapefruit-like aroma) enhance the hoppy aroma derived from the hop varieties *Tomahawk*, *Cascade*, or *Nelson Sauvin*, respectively [7.26, 62]. Another study suggests that β-citronellol might be characteristic for *dry-hopped* beers derived from particular flavor hop varieties in comparison to other hop varieties [7.63].

7.3 Special Flavors

Revealing the contribution of the raw materials on the final flavor is a necessity in order to understand how the flavor of beer develops and changes over time. This knowledge is the basis to understand how specific flavors are formed. This section deals with the development of wanted and unwanted flavor-active compounds during beer production, storage, and aging.

7.3.1 Desired Flavors

Ethyl alcohol is the most predominant alcohol in beer. However, ethanol is not considered to be an important odorant. While the production of ethanol is a common feature of practically all yeast strains used for beer production, the production of volatile, higher alcohols, so-called fusel alcohols, depends on the yeast strain. Yet, the vast majority of these compounds are only present in concentrations below their odor threshold concentration. Higher alcohols that might contribute to the overall aroma are 2-methylpropanol, 2-methylbutanol, 3-methylbutanol, and 2-phenylethanol. These compounds are often considered having a warming effect on the taste of beer [7.33].

More importantly, higher alcohols are direct precursors of esters found in beer. Considering the yeast metabolism, acetates and ethyl esters are the most

important esters produced during fermentation. Especially, ethyl acetate, 2-methylbutyl acetate, and 3-methylbutyl acetate (= iso-amyl acetate; banana-like aroma) are important esters as their concentrations are normally above their flavor threshold value. However, concentrations of the esters strongly depend on the yeast strain, the density of the wort (= original gravity in degree Plato) and the amount of oxygen available during fermentation [7.33]. As already mentioned in Sect. 7.1.4, ale strains tend to produce more fusel alcohols. Other esters commonly found in beer are, for example, ethyl hexanoate (sweet apple aroma), ethyl octanoate (sour apple aroma), and 2-phenyl ethyl acetate (roses and honey-like aroma impressions) [7.33]. All these esters are normally associated with fruity aroma impressions. During lagering and beer aging several new esters can originate from ethanol and organic acids, whereas the concentrations of other flavor-positive esters decrease. The formation of winy flavors for example has been related to ethyl 3-methylbutyrate and 2-methylbutyrate [7.64].

Another group of relevant volatiles are aldehydes, which are formed as reactive intermediates during various stages in the brewing process. The most important pathways are the degradation of malt-derived fatty acids and lipids as well as the oxidation of corresponding alcohols. However, only one aldehyde is of greater importance for the beer aroma. Acetaldehyde is found in concentrations between 2 and 10 mg/l and gives beer a typical green apple flavor, if the flavor threshold value is exceeded. This value strongly depends on the beer and varies between 5 and 50 mg/l [7.33]. Other aldehydes, so-called staling aldehydes, play an important role in the aging of beer [7.65].

7.3.2 Controversial Flavors

While the above-mentioned compounds are commonly described as positive attributes, the appreciation of some other compounds strongly depends on the beer style. A good example is 4-vinylguaiacol, typically found in especially German wheat beers. These beers are made with top-fermenting yeast strains as well as wheat or malted wheat and have a phenolic, clove-like flavor. During the manufacturing of German wheat beers, significant amounts 4-vinylguaiacol are mainly produced by an enzymatic decarboxylation of ferulic acid during fermentation. In contrast to that only low levels of 4-vinylguaiacol are liberated thermally induced from barley-derived ferulic acid during wort boiling (Fig. 7.11) [7.66]. Interestingly, the same flavor is considered to be an off-flavor in Pilsner-style beers. Moreover, levels above 10 µg/l are regarded as an indicator that the affected beer was probably contaminated with wild yeast strains [7.67].

The sensorial impact of vicinal diketones is also a controversial topic. While they are regarded as a defect in lager beers, they are desired in certain heavily hopped beers, such as British ales. The most important vicinal diketones are 2,3-butanedione (= diacetyl) and 2,3-pentanedione. Especially diacetyl causes a buttery, butterscotch-like aroma in beers. This compound is derived from α-acetolactate, which is excreted by the yeast cells and decomposes to diacetyl in the wort. Yet, healthy yeast has a great potential to reduce diacetyl to acetoin and subsequently to 2,3-butanediol. The same applies for 2,3-pentanedione, which is also metabolized to its diol. However, the reduction of vicinal diketones requires enough time and in almost every beer, diacetyl is detectable. The flavor threshold concentration varies for the different beer styles and was found to be as low as 0.03 mg/l for Pilsner-style beers. Although both, diacetyl and pentanedione are normally present in rather low concentrations in beer, they are supposed to contribute to the sensory sensation and flavor balance of the final product [7.68].

While most of the aroma compounds formed during fermentation are believed to have a positive effect on the aroma perception of beer, sulfur compounds, formed during the fermentation, can be a serious issue regarding off-flavors [7.43]. These sulfur-containing components are very often potent flavor-active substances with low flavor threshold values. Yet, a series of sulfur components present in beer might contribute positively to the overall beer flavor when present in small amounts.

The predominant sulfur-containing volatile is sulfur dioxide with concentrations up to 15 mg/l. It is naturally produced during fermentation and is able to counteract the formation of oxidation flavors due to its capacity to bind carbonyl compounds [7.69, 70] formed during beer aging. This ability is used to increase the shelf life of beer, by adding metabisulfite salts, which will release sulfur dioxide. However, the addition has to be in accordance with the legal requirements.

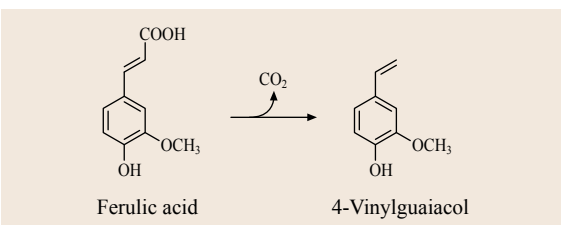

Fig. 7.11 Formation of 4-vinylguaiacol by thermal and enzymatic decarboxylation of ferulic acid

In contrast to that, hydrogen sulfide (H_2S) is a substance that should not be present in beer, because of its very unpleasant flavor. Fortunately, hydrogen sulfide normally is no serious issue, because of its high volatility; during fermentation its concentration will dramatically drop, due to the fact that the substance will evaporate from the fermentation tank together with carbon dioxide.

Another critical sulfur compound as already mentioned in Sect. 7.1.2 is DMS. Despite the fact that it will be evaporated during the wort boiling, it still remains a crucial compound. During the kilning step, DMS can be oxidized to DMSO. As DMSO is heat-stable and nonvolatile during wort boiling, it will be reduced during fermentation to give rise to DMS [7.17]. However, some people appreciate the sulfury note of DMS in beer, when it is present in a rather low concentration.

7.3.3 Aging Flavors

As mobility of our society increases, local products are no longer consumed regionally, but worldwide. Accordingly, products are transported over large distances before they reach the consumer. Flavor consistency and stability are therefore important criteria for the success of a product. Unfortunately, beer is not a static system and changes will occur in a time-dependent manner. Regarding the flavor of beer, those changes can either be the development of new flavor-active compounds or the degradation of initially present flavor-active compounds [7.71]. The decrease in positive flavors such as floral, fruity, and estery notes, and the evolution of staling flavors exceeding their odor threshold values, is further complicated by the interactions of single compounds [7.72, 73]. Furthermore, strong and dominant flavors as they occur for example in dark beers, can mask to a certain extend the perception of aging flavors. Accordingly, these products seem to have a better sensorial flavor stability. Yet, the concentrations of staling compounds are also increasing thereby going relatively unnoticed.

Aging of beer strongly depends on the beer style and the conditions under which the aging occurs, for example, temperature, oxygen content, or the presence of iron and other metal salts [7.74]. As beer is a mixture of many different compounds, many different reactions can occur depending on the aging conditions. An overview on the evolution of basic aging flavors in beer was given by *Dalgliesh* (Fig. 7.12) [7.75].

Yet, this figure is a generalization of the possible sensorial sensations occurring during beer storage. Accordingly, it is not applicable to every beer. For most beers, a constant decrease in the bitter intensity can be observed during aging. At the same time, a sweet, caramel, toffee-like aroma develops. Very often a rapid formation, followed by a fast decline in the so-called ribes flavor occurs, which reminds of blackcurrant leaves (*Ribes nigrum*) [7.76, 77]. Later, a flavor associated with wet cardboard can be perceived [7.74]. Experiments revealed that (*E*)-2-nonenal (Fig. 7.13) gives beer a cardboard flavor when added to beer [7.78]. Later it was shown, that (*E*)-2-nonenal is already present in wort as its Schiff bases and is transferred into the final beer, where it is steadily released until the compound exceeds its odor threshold concentration [7.79, 80].

Even if they usually remain below their odor threshold concentration, furfural and 5-hydroxymethylfurfural (Fig. 7.9) are thought to be marker compounds for beer staling and heat load during the brewing process [7.81]. Both compounds are typical Maillard reaction products that are formed during wort production; they can then be transferred into beer in a chemically bound state (as Schiff bases or SO_2-adducts). Especially, the Schiff base will release furfural and 5-hydroxymethylfurfural during beer aging contributing to a sweet, caramel-like flavor. Accordingly, the vast majority of furfural and 5-hydroxymethylfurfural is formed during beer aging. The formation rate has

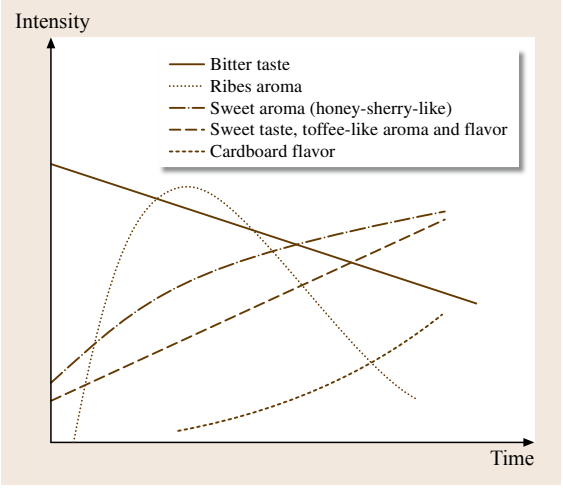

Fig. 7.12 Development of aging flavors of beer during time (according to *Dalgliesh* [7.75])

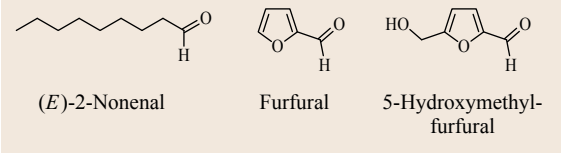

Fig. 7.13 Chemical structures of (*E*)-2-nonenal, furfural, and 5-hydroxymethylfurfural

been reported to be almost linear and to be temperature dependent [7.82].

Furthermore, several other staling aldehydes are formed during lagering [7.67, 74]. Thereby it is known, that the malt quality seems to be of essential importance regarding the formation of staling aldehydes. Yet, it is not clear which factors are responsible for significant differences in the brewing properties of malts [7.84].

In general, lipids are considered to be less important, as they are practically insoluble in water and lost to a large extent due to adsorption to solids. However lipid-derived oxidation products of aged hops are thought to be precursors for typical staling flavors [7.11]. In contrast to that, it is suggested that malt-derived polyphenols contribute to the flavor stability of beer [7.8].

When beer is exposed to sunlight, it quickly develops an unpleasant aroma, commonly referred to as the *sunstruck-flavor*. This off-flavor is predominantly caused by the skunky smelling 3-methyl-2-buten-1-thiol (MBT), formed in a light-induced reaction in the wavelength range of 350–550 nm involving isohumulone, riboflavin, and cysteine. The formation mechanism has been studied since decades (see [7.83] for a review) and even today, studies try to unravel the mystery of the respective conditions causing *sunstruck-flavor* [7.85]. The commonly accepted formation mechanism is shown in Fig. 7.14. A photosensitized cleavage of the C–C bond by light is suggested to be followed by a loss of carbon monoxide and a subsequent recombination with sulfur radicals.

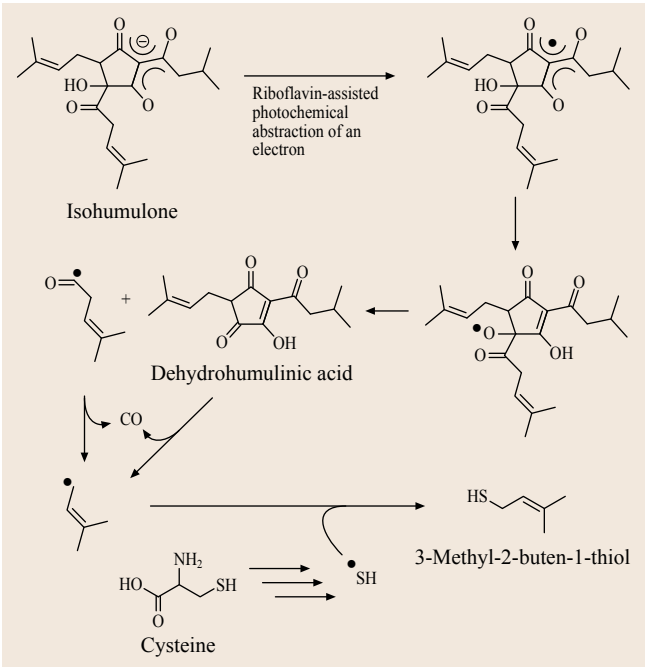

Fig. 7.14 Postulated mechanism of formation of 3-methyl-2-buten-1-thiol, responsible for the so-called *sunstruck flavor* (after [7.83])

As soon as beer is exposed to daylight, a rapid formation of MBT is initiated and due to its extremely low odor threshold (5–10 ng/l), even trace amounts of MBT usually have a noticeable effect on the aroma of beer.

In order to prevent this reaction, beer is either bottled in green or brown bottles, the latter being more effective. Naturally, full protection is only offered by metal cans. However, some beers are bottled in white glass. To prohibit the formation of MBT, either ultraviolet(UV)-filters must be embedded in the glass or the beer must be produced with so-called light-stable hop products. In these products, the unstable isohumulones are reduced to give rise to more light-stable dihydro-, tetrahydro-, and hexahydroisohumulones (Fig. 7.15) [7.34, 35]. However, it was shown, that tetrahydroisohumulones undergo the same reaction, as the α-hydroxy-keto function is still intact [7.83]. Furthermore, the authors reported that dihydroisohumulones are only partially light stable.

Other sulfur compounds can also be relevant for the aging flavor of beer, due to their very low odor threshold levels. Dimethyl trisulfide (fresh-onion-like) may increase above its flavor threshold of 0.1 µg/l [7.86, 87]. Other sulfur-containing volatiles that have been related to beer aging (catty, ribes-like aroma) include 3-methyl-3-mercaptobutyl formate [7.88] and 4-mercapto-4-methyl-pentane-2-one [7.89].

Although beer is a rather microbiologically stable beverage, a few microorganisms are able to grow in beer. Their growth can have a negative effect on

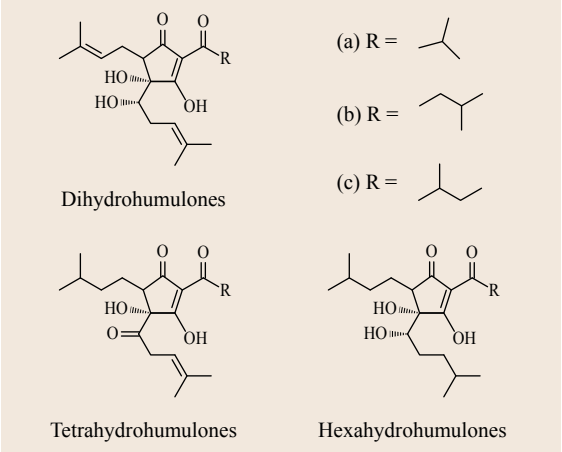

Fig. 7.15 Chemical structures of the co-(a), n-(b) and ad-(c) congeners of the dihydro-, tetrahydro- and hexahydroisohumulones

beer quality in general as well as on the flavor stability in particular. Possible infections include mycotoxin-producing fungi derived from the malting process, wild yeasts and bacteria, including certain lactic acid bacteria as well as anaerobic Gram-negative species [7.90].

7.4 Influence on the Sensory Sensation due to Other Constituents

As already mentioned, several hop-derived compounds can interact and cause synergistic effects. Moreover, even different compounds classes can influence each other. So far, studies have shown that carbon dioxide concentration influences the perception of the beer's bitterness, the warming perception of ethanol as well as the sweet taste [7.91]. Furthermore, studies indicated that the flavor complexity is mainly influenced by the ethanol concentration. These findings are supported by an investigation showing, that the ethanol concentration is crucial for the odor threshold concentration of several hop-derived volatiles, such as β-damascenone, β-carophyllene, and α-humulene [7.92]. But the concentrations of other nonvolatile compound classes, such as hop bitter acids or sweeteners, also influence the overall flavor complexity [7.91]. Another study provided evidence that the sourness plays a substantial role on how special flavors are perceived [7.93]. Interestingly, it was of great relevance which acid was used for the pH adjustment. Moreover, *Mojet* et al. found evidence suggesting that all tastants might also elicit a smell sensation [7.94], and therefore an influence on the overall aroma perception cannot be excluded. Such effects are strongly related to multisensorial perceptual effects as discussed in Chap. 47 of this book.

Finally, the temperature of beer obviously has a noticeable influence on the overall flavor perception, firstly due to the fact that the composition of volatiles in the head space above the aqueous layer will change, but also due to multisensorial interactions; for example, temperature can drastically influence the perception of tastants and this, in return, can again modify the perception of overall aroma of beer. Such considerations reveal that beer as a rather complex product is still far from being understood in view of its aroma and flavor properties; coming decades will surely unravel a multitude of knowledge in our attempt to understand why we savor beer for its unique flavor sensation.

7.5 Outlook

While many mysteries concerning the flavor of beer have been resolved, it is only partly possible to control and predict its flavor. Being an extremely complex beverage, many different compounds contribute to the overall flavor. Moreover, varying concentrations are making it almost impossible to fully characterize raw materials or even to link detectable, analytical changes to the sensorial perception elicited in the consumer. Although flavor thresholds and aroma impressions of single compounds have been studied thoroughly for certain compounds, interactions leading to flavor enhancement, suppression, or masking are not yet comprehensively understood. However, progress in analytical and sensorial evaluation methods will undoubtedly help to optimize and control the flavor evolution of the final beverage [7.95]. This information can also be used to adjust flavor profiles to the preferences of the consumers.

Flavor stability remains another problem that is not fully understood and, moreover, controlled. Yet, it is of major importance for a brewer to supply the consumer with consistent quality. Nevertheless, some mechanisms responsible for the aging of beer have been elucidated so that brewers will likely be able to increase the shelf life of beer in the near future. Possible sources of antioxidants that might be suited for this purpose are malts and hops as they are already naturally rich in antioxidants [7.61, 96].

Overall, increased efforts in multisensorial research will have most likely a strong impact on our common understanding of what specifically drives the complex perception of beer flavor.

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8. Wine

Philippe Darriet, Alexandre Pons

Wine aroma is related to human cognition through multimodal stimuli, particularly in the case of volatile compounds detected by orthonasal and retronasal perception. In fine wines, aroma may be associated with associations of complexity, finesse, and elegance, sometimes attaining the level of uniqueness that makes them a source of a great pleasure. This chapter reviews the diversity of volatile components constituting wine aroma, including compounds originating from grapes, the metabolism of wine microorganisms during alcoholic and malolactic fermentations, implicating *Saccharomyces cerevisiae* and *Oenococcus oeni*, respectively, and oak barrels during wine aging. It will also address those associated with off-odors. The impact of all these compounds on wine aroma and quality is considered, including recently described perceptual interaction phenomena (i. e., masking, synergistic effects, and perceptual blending) and the influence of nonvolatile compounds in the wine matrix on aroma perception.

The quality of wine aroma is a matter of great importance. The first sensory impressions of a wine are color, followed by aroma, via orthonasal, then retronasal sensations. Tasters are capable of detecting a broad palette of aromas, including spicy, woody, flowery, and fruity components originating from the grape variety, soil, and climatic conditions, as well as vinification and aging processes [8.1]. Sometimes, tasting a wine is a source of great pleasure and represents such a unique experience that it may be considered a veritable work of art [8.1].

Wine tasters may use either hedonic or analytical criteria, depending on their preferences and knowledge of wine tasting. In this context, they also appreciate the intensity and complexity of the aromatic nuances and assess overall wine quality [8.1]. Analysis of the language used by a group of professional wine tasters revealed that they focused less on describing wines than categorizing them in relation to types they have already memorized [8.2]. Tasters unconsciously seek wine aromatic components related to specificity and originality, in order to associate each wine with an ideotype (stored reference). This aspect is related to the concept of typicality [8.3–5]. However, not all nuances are perceived in the same manner by different tasters; each taster has a unique personal sensitivity to aromas [8.6]. The context of the wine tasting may also have an impact on flavor perception [8.1, 7]. The perception of wine aroma and typicality is a complex cognitive process involving all the senses including somatosensory perceptions.

So, on what basis are a wine's aromatic nuances perceived? The aromatic palette is initially associated with the many – from one to several hundred – volatile compounds in the headspace above a glass of wine. Highly reputed wines usually have a complex composition consisting of a larger number of compounds. These compounds are stimuli for the human olfactory system, commencing with the olfactory epithelium before being transformed into nerve impulses in the olfactory bulb and becoming conscious sensations [8.8, 9]. However, volatile compounds do not contribute equally to wine aroma. Some, present in trace amounts (in the order of $\mathrm{ng\,l^{-1}}$ or $\mathrm{pg\,l^{-1}}$), have a very low olfactory

detection or recognition threshold, while others, detected at higher concentrations (several mg l^{-1}), have much higher detection or recognition thresholds [8.10, 11]. This paradoxical situation is due to the specificity of detection by the human olfactory system. However, the perceived intensity of a compound usually increases at higher concentrations [8.12]. Also, the presence of other wine compounds (ethanol, polyphenols, acidity, etc.) may affect the composition of the volatiles present in the headspace above the wine [8.13–15] and, potentially, the sensory perception of its aromas.

Nevertheless, the aroma perceived by the brain is not an algebraic sum of all the volatile compounds, but is related to a complex combinatorial process in the unconscious stage of perception [8.16]. Thus, the ultimate perception and verbalization of aromas involve complex, unconscious combinations of volatile compounds, as well as cognitive processes and memories of past experiences [8.10].

This chapter presents an overview of wine aroma components, including compounds originating from grapes (varietal compounds), those resulting from the fermentation metabolism of wine microorganisms, or associated with aging in oak barrels and bottles, and chemicals responsible for off-odors. The relationship between volatile compounds and their aromatic attributes in wine at each stage in tasting is discussed on the basis of recent research. The complex interaction of some key volatile compounds involved in overall perception is explored, focusing on combinatorial perception, as well as the impact of nonvolatile compounds on wine aroma.

8.1 Composition of the Wine Matrix

8.1.1 Fermentation Aroma

Compounds produced by the fermentation metabolism of yeasts (*Saccharomyces cerevisiae*, *Saccharomyces uvarum*) and lactic bacteria (*Œnococcus oeni, Lactobacillus* sp.) contribute first to the vinous character of a wine and thus its sensory specificity in comparison to other fermented beverages, as well as, in some cases, its fruity character. These compounds are primarily alcohols, particularly higher or fusel alcohols, esters formed by the esterification of higher alcohols with the acetic acid produced during fermentation, such as acetate esters of higher alcohols, esters formed from fatty acids and ethanol, such as ethyl esters of fatty acids, and, finally, carbonyl and sulfur compounds.

The key higher alcohols are 3-methyl-butan-1-ol (isoamyl alcohol) and 2-methyl-butan-1-ol (bug-like and heavy solvent odor), 2-methylpropan-1-ol (heavy solvent odor), 2-propan-1-ol (heavy solvent odor), 2-phenylethanol (rose flower, *pot pourri*), and 3-methylthiopropan-1-ol (cooked cabbage). These compounds with high-odor detection thresholds (ranging from mg l^{-1} to tens of mg l^{-1}) can affect wine aroma, as concentrations may be as high as several tens or hundreds of mg l^{-1}. Total concentrations of these compounds vary between 100 and 550 mg l^{-1} [8.11, 17]. Overall, with the exception of 2-phenylethanol, these compounds are considered to have a detrimental effect on quality, due to their pungent aromas when concentrations in wines exceed 300 mg l^{-1} [8.11, 17].

Esters of fatty acids (mainly butanoic, hexanoic, octanoic, and decanoic acids) and acetic esters of higher alcohols contribute through fruity and flowery nuances to the fermentation aroma of wines [8.11, 18]. The most significant acetic esters of higher alcohols in wine aroma are isoamyl acetate, responsible for the well-known banana aroma in some *primeur* wines, 2-phenylethyl acetate, which sometimes exhibits pungent, fruity, rose-like odors, and, less significantly, hexyl acetate (pear) and isobutyl acetate (banana). Globally, these compounds may be present at concentrations above the detection threshold (close to mg l^{-1}), sometimes masking the perception of varietal aroma nuances. Ethyl acetate (solvent *vinegar like*), also produced during alcoholic fermentation, may contribute to the fruity aroma of young wines at concentrations below 100 mg l^{-1}, while concentrations over 150 mg l^{-1} are always related to problems due to acetic or lactic bacteria, involving the esterification of ethanol with acetic acid [8.11].

Until 10 years ago, ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate, all known for their flowery aromas, were considered the most significant ethyl esters of fatty acids. The detection thresholds of these esters are in the μg l^{-1} range in water, and mg l^{-1} in young wines, significantly exceeding their individual detection thresholds. The amount of esters in young wines depends mainly on parameters related to alcoholic fermentation: *S. cerevisiae* yeast strain, and fermentation temperature, as well as composition and turbidity of the grape must [8.19–21].

More recently, the contribution of branched esters was identified, usually with higher concentrations in red wines. These compounds include hydroxylated ethyl esters, including ethyl 3-hydroxybutanoate [8.22, 23], ethyl 4-hydroxy butanoate (ethyl leucate) [8.24],

ethyl 2-hydroxyhexanoate, and ethyl 6-hydroxyhexanoate [8.22, 23], keto esters, such as ethyl 4-oxopentanoate (ethyl levulinate) [8.22, 23], and methylated esters, such as ethyl 2-methylpropanoate (ethyl isobutyrate), ethyl 2-methylbutanoate, and ethyl 2-hydroxy-4-methylpentanoate. While the individual olfactory detection thresholds of these compounds are similar to those of conventional esters, their contribution to the fruity aromas of red wines through perceptual interaction phenomena was recently reported [8.23]. Ethyl 2-methylpropanoate and ethyl 2-methylbutanoate are implicated with ethyl propanoate in blackberry aromas, while ethyl 3-hydroxybutanoate, together with ethyl butanoate, ethyl hexanoate, and ethyl octanoate contribute to red berry aromas [8.23]. Moreover, ethyl 2-hydroxy-4-methylpentanoate, only recently identified, was demonstrated to be involved in blackberry aroma via perceptual interaction phenomena [8.25]. The organoleptic impact of the enantiomers of this compound has also been studied [8.26]. Branched ethyl esters present lower olfactory detection thresholds than nonbranched compounds [8.22].

Globally, the contribution of the fermentation esters to wine aroma is limited in time, as these compounds are quite quickly degraded through hydrolysis during aging [8.27, 28]. However, contradictory results were recently reported in red wines by *Antalick* et al. [8.29], who observed high ester levels in aged red wines. In fact, other ethyl esters of branched acids may also be formed via chemical esterification during aging, including ethyl 2-methylpentanoate, ethyl 4-methylpentanoate, ethyl cyclohexanoate [8.30, 31], ethyl isobutyrate, ethyl 2-methylbutyrate, ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl isovalerate, butyl acetate, and ethyl phenylacetate [8.32, 33]. Their contribution to wine aroma has not yet been precisely qualified.

Chemical esterification mechanisms may also affect various acids present in wine, such as cinnamic acid, chemically esterified to ethyl cinnamate (reminiscent of cinnamon, sweet-balsam, sweet-fruit, plums, and cherries), which is also formed during alcoholic fermentation [8.36, 37]. Ethyl phenylacetate has a strong, flowery flavor and is formed from phenylacetic acid, produced by the oxidation of phenylacetaldehyde [8.38, 39].

8.1.2 Other Fermentation Compounds

Other volatile sulfur- and carbonyl-based compounds, produced by wine microorganisms during alcoholic and malolactic fermentation, may significantly impact wine aroma (Sect. 8.1.5). Among the latter, diacetyl (2,3-butanedione) and acetoin, an intermediate in diacetyl formation, contributes to buttery, lactic aromas in both dry white and red wines following malolactic fermentation by *Œnococcus oeni* [8.11, 18]. The olfactory detection threshold of diacetyl is in the $\mu g\,l^{-1}$ range in water and $mg\,l^{-1}$ in model wine. A few $mg\,l^{-1}$ diacetyl ($2-3\,mg\,l^{-1}$ in dry whites and $5\,mg\,l^{-1}$ in reds) may contribute positively to wine aroma [8.11, 17, 40]. Another key volatile compound is acetaldehyde, but its contribution to wine aroma is generally limited, as it combines easily with the sulfur dioxide used as a preservative, except in oxidized wines. However, in specific situations, where wines are subjected to extreme oxidation during aging (e.g., Sherries from Xerez in southern Spain, Marsala, from Sicily, and *vin jaune* from the Jura region of France), acetaldehyde is present at concentrations of several hundreds of $mg\,l^{-1}$, contributing, with other volatile compounds (sotolon), to their typical oxidized apple and nut flavor [8.41].

8.1.3 Compounds Originating from Grapes

Methoxypyrazines

Methoxypyrazines are nitrogen heterocycle compounds belonging to the pyrazine group, largely represented in both animals and plants [8.42]. Among the various methoxypyrazines, some alkylated methoxypyrazines, such as 2-methoxy-3-isobutylpyrazine (IBMP), 2-methoxy-3-sec-butylpyrazine, and 2-methoxy-3-isopropylpyrazine (IPMP) are highly volatile with very low-odor thresholds, in the nanogram per liter range in water (Table 8.1). The odors of these methoxypyrazines are vegetable-like, reminiscent of pea pods, green peppers and, depending on the concentration and the compound, earthy nuances. These same substances have also been identified in bell peppers, pea pods, potatoes, and carrots as well as blackcurrants, raspberries, and blackberries [8.43–45].

These compounds, particularly 2-methoxy-3-isobutylpyrazine (IBMP), have been identified in various grape varieties such as Cabernet Sauvignon, Cabernet Franc, Sauvignon blanc [8.46–49], Merlot, Carmenère, and Verdejo [8.45, 50–52]. IBMP has also been found in Pinot Noir, Chardonnay, Riesling, Chenin Blanc, Traminer, Syrah, and Pinotage [8.53, 54], but in very low concentrations. Among the most odorous methoxypyrazines, IBMP is proportionally the most abundant compound in grapes and wine. Its concentration in Sauvignon blanc wines varies from 0.5 to $40\,ng\,l^{-1}$ and from 0.5 to $100\,ng\,l^{-1}$ in Cabernet Sauvignon wines. This compound can be detected at the highest concentrations (up to $160\,ng\,l^{-1}$) in Carmenère wines, which are frequently marked by herbaceous flavors [8.45, 50]. In Sauvignon blanc, as in Cabernet Sauvignon, the pea-pod, pepper-like aroma of IBMP

Table 8.1 2-Methoxy-3-alkylpyrazines identified in grapes and wines

Name	Structure	Descriptors	Detection threshold ($ng\,l^{-1}$)
			
2-Methoxy-3-isobutylpyrazine	R: $CH_2CH(CH_3)_2$	Greenpeper/pea pod	1[a]
2-Methoxy-3-isopropylpyrazine	R: $CH(CH_3)_2$	Greenpeper/pea pod/earthy	2[a]
2-Methoxy-3-sec-butylpyrazine	R: $CH(CH_3)CH_2CH_3$	Greenpeper/pea pod	1[a]
2-Methoxy-3-ethylpyrazine	R: CH_2CH_3	Greenpeper/pea pod	40[b]
2-Methoxy-3-methylpyrazine	R: CH_3	Greenpeper/pea pod	4000[c]

[a] Determined in water at ISVV, University of Bordeaux
[b] Concentrations (after [8.34])
[c] Concentrations (after [8.35])

contributes to their herbaceous expression. A negative effect has been noticed on Cabernet Sauvignon wine flavor at concentrations as low as $15\,ng\,l^{-1}$ [8.55]. IBMP can also affect the flavor of Tempranillo and Grenache grown in Spain [8.13]. On the other hand, IBMP can contribute, to some extent, to desirable varietal aromas in Sauvignon blanc wines [8.56, 57]. As noticed by *Escudero* et al. [8.58] and *Pineau* et al. [8.59], perceptual interaction phenomena between 2-methoxy-3-isobutylpyrazine and other compounds, β-damascenone, dimethylsulfide can modulate the perceived herbaceous flavor of this compound by tasters (Sect. 8.2).

During vinification, IBMP is easily extracted during pressing or prefermentation maceration [8.60, 61]. Neither alcoholic fermentation nor aging modify the original concentration of IBMP in the must by more than 30% [8.60, 61]. This means that conventional winemaking techniques do not contribute to a decrease of IBMP in must, from the original amounts in grapes to the finished wine. However, the settling of must during the fermentation of white or rosés wines can reduce IBMP levels by about 50% [8.60, 61]. As for red wines, thermo-vinification can lead to a decrease of IBMP by evaporation [8.60, 61]. During aging, and because this compound is nonoxidizable, oxygenation has no impact on its concentration [8.60, 61]. Moreover, IBMP content is very stable during aging and can affect wine aroma for many years [8.60, 61].

Furthermore, other methoxypyrazines, such as 2-methoxy-3-methylpyrazine [8.48] and 2-methoxy-3-ethylpyrazine [8.62, 63], have been identified in Sauvignon blanc grapes, but these compounds are much less odoriferous than IBMP (Table 8.1).

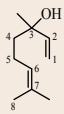
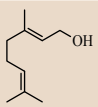
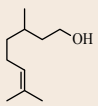
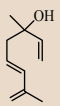
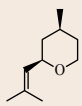
Also, numerous studies have been published concerning the impact of natural (climate, soil) and viticultural factors on IBMP concentrations in grapes and wine. *Allen* et al. [8.54] first noticed lower IBMP concentrations in wines obtained from grapes ripened at higher temperatures, whereas *Falcao* et al. [8.64] discovered varying IBMP levels in wines obtained from grapes grown at different altitudes. The sensitivity of IBMP to UV light and its resulting degradation, leading to the formation of 2-methoxy-3-methylpyrazine, a much less odoriferous compound [8.45, 65, 66] should be noted. Climate-related variations in IBMP content in grapes and wine thus represent a key factor [8.55, 67–69]. Moreover, other physiological parameters of grape vines, such as yields and the availability of water and nitrogen [8.45, 70] also impact IBMP development. IBMP content in ripe grapes may also vary significantly, depending on the clonal origin of the vines [8.50].

Terpenic Compounds

Terpenes are a very large widespread group of compounds. Working from an early hypothesis by *Cordonnier* [8.71], various enological research teams have conducted research to learn more about these compounds [8.72–75]. There are 40 main monoterpene compounds in grapes. The most important in terms of odor are certain monoterpene alcohols and oxides, such as linalool, geraniol, citronellol, (*E*)-hotrienol, (*E*) and (*Z*)-rose oxide and nerol (3,7-dimethyl-2(*Z*),6-octadien-1-ol), which develop floral aromas. The detection thresholds of these compounds are quite low, ranging from tens to hundreds of micrograms per liter (Table 8.2).

The monoterpene alcohols mentioned above play a major role in the aroma of grapes and wines from the Muscat family (Muscat de Frontignan, also called Muscat à Petits Grains or Muscat d'Alsace, Muscat of Alexandria, also called Muscat à Gros Grains, Muscat d'Ottonel, White Muscat from Piemonte, etc.) as well as crosses between Muscat of Alexandria and other varietals (including such varietals as Muscat de Hambourg (crossed with Frankenthal [8.78]), and the Argentinian variety Torrontes (a cross with the Mission also called Pais variety [8.78]). The concentrations of

Table 8.2 Characteristics of some monoterpenes identified in grapes and wine

Name	Structure	Descriptors	Detection threshold[a] ($\mu g\,l^{-1}$)	Concentrations in Muscat wines (Min–Max)[b] ($\mu g\,l^{-1}$)
Linalol 3,7-Dimethyl-1,6-octadien-3-ol		Coriander seed-rose	20/3[c]	170–815
Geraniol 3,7-Dimethyl-2(*E*),6-octadien-1-ol		Rose	90/9	223–1056
Citronellol 3,7-Dimethyl-6-octen-1-ol		Rose-lemongrass	20/15	nd-20
(*E*)-Hotrienol 3,7-Dimethyl-1,5(*E*)7-octatrien-3-ol		Linden-rose	300/70	5–300
(2*S*, 4*R*)-(−)-*cis*-Rose oxide		Floral-green	8[d]/0.2[c]	0.2–10[c]

[a] Determined in water at ISVV, University of Bordeaux
[b] Concentrations (after [8.72–75])
[c] Detection threshold in wine model solution and water, respectively
[d] Determined on racemic mixture
[e] Values (after [8.76, 77])

the main monoterpenes in these grapes and wines are much greater than the odor-detection threshold of these compounds. Monoterpenes also have a more or less pronounced impact in the flavor of Gewürztraminer, Albariño, Scheurebe, and Auxerrois grapes and wines, and to some extent those of Riesling, Muscadelle, and some clones of Chardonnay. These monoterpene alcohols are often found in many varieties (Sauvignon, Syrah, Cabernet Sauvignon, etc.) at levels generally below the olfactory perception threshold. In addition, as some monoterpenes have an asymmetric carbon, varying degrees of enantiomeric forms of monoterpenes can be found in grapes [8.79]. For example, linalool, hotrienol, cis-, and trans-rose oxide are predominantly present (88–97%) in a single enantiomeric form in the various varieties of Muscat that were analyzed (*S* form for linalool and (*E*)-hotrienol and (2*S*, 4*R*) and (2*R*, 4*S*) forms for (*Z*)-rose oxide, respectively). The abundance of one or the other enantiomer may contribute to modulating the strength of these odorous compounds in grape juice and wine, as well as aromatic expression. *R*-(−)-linalool, with an odor threshold of $0.8\,\mu g\,l^{-1}$, is described as having floral notes and overtones of woody lavender. It is more odoriferous than *S*-(+)-linalool, which presents a floral, sweet scent with a detection threshold of $17\,\mu g\,l^{-1}$ [8.76]. However, the proportions of enantiomeric forms may change over the fermentation process, the most fragrant enantiomer, cis-(2*S*, 4*R*)-rose oxide or (*Z*)-(−)-rose oxide, being for example, between 38 and 76% in wine [8.76]. Other than rose oxide, oxides present in grapes, such as the oxides of linalool and nerol, have little olfactory impact (i. e., high-perception thresholds of $1–5\,mg\,l^{-1}$). However, the presence of linalool oxide contributes to the increased perception of linalool [8.77, 80].

Botrytis cinerea development on grapes can also alter the composition of grape monoterpenes by degrading the main monoterpene alcohols and their oxides into generally less odorous components [8.73, 81]. Also, fermentation significantly alters the monoterpene composition of grapes through chemical and microbiological processes. The most pronounced transformation concerns the degradation of nerol and geraniol by the yeast *S. cerevisiae* via an enzymatic reduction to form citronellol, alpha-terpineol, and linalool [8.82, 83]. The proportion of the above compounds depends on the yeast strain and grape juice composition [8.84]. Also, during fermentation, the enzymatic reduction of 3,7-

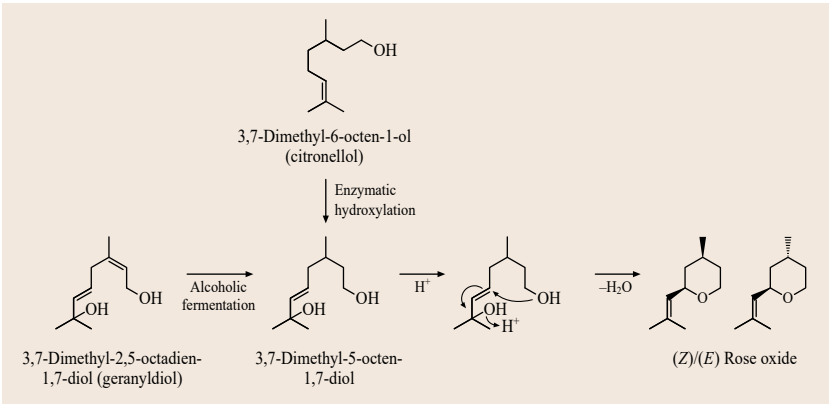


Fig. 8.1 Formation of rose oxide from various precursors in grape and during vinification (after [8.80])

dimethyl-2,5-octadien-1,7-diol or geranyldiol leads to the formation of a diendiol 3,7-dimethyl-5-octen-1,7-diol which is a precursor of rose oxide [8.80]. This diol is also derived from the enzymatic hydroxylation of citronellol in grapes [8.80]. These results illustrate the deep changes in the flavor of grapes that can result from the combination of fermentative metabolism of yeasts and wine acidic conditions (Fig. 8.1). Then during wine aging, the terpenols themselves may undergo rearrangements in acid to produce other monoterpene alcohols [8.85]. This is a classic chemical reaction involving the dehydration of alcohols in an acid medium. Thus, the alcohol dehydration of 3,7-dimethylocta-1,5-dien-3,7-diol, in the wine acidic medium, yields (*E*)-hotrienol, while (*E*)-2,6-dimethyl-6-hydroxyocta-2,7-dienoic acid has recently been identified as the precursor of the *wine-lactone* via stereoselective cyclization (Fig. 8.2) [8.86]. On the other hand, the concentrations of geraniol and nerol, which constitute part of the aroma of young wines, can rapidly decrease after 2–3 years of bottle aging (for instance in Muscat), and then no longer contribute in the same way to wine aroma. Linalool is more stable. Concentrations of this compound may even actually increase at the beginning of aging since it is formed from geraniol and nerol [8.87]. More specifically, cyclizing nerol produces α-terpineol; nerol is transformed into α-terpineol and linalool. Linalool is also converted into terpene hydrate over time [8.87]. These results account, at least partially, for the fact that the very intense young character of Muscat wine disappears during aging, acquiring a resinous odor.

Sesquiterpenoids

Sesquiterpenoids ((+)-aromadendrene, α-humulene, α-bisabolol, dehydro-aromadendrene, etc.) are secondary metabolites of grapes [8.88, 89] that do not generally contribute directly to wine flavor as their concentrations are usually in the $\mu g\,l^{-1}$ range, below the olfactory perception threshold of these compounds. Nevertheless, the characterization of pepper nuances in Syrah wines led to the successful identification of (−)-rotundone, a powerful sesquiterpene with an olfactory perception threshold in the $ng\,l^{-1}$ range (Table 8.3). Concentrations in Syrah range from 50 to $600\,ng\,l^{-1}$, and it is assumed that this compound can contribute to the black pepper flavor of this variety [8.88, 89].

C_{13}-Norisoprenoid Derivatives

The oxidative degradation of carotenoids, which belong to the family of terpenes with 40 carbon atoms (tetraterpenes), leads to many derivatives, including norisoprenoids with 13 carbon atoms (C_{13}-norisoprenoids) that may contribute to the aroma of wines. Three major groups each containing various volatile odoriferous compounds are concerned. The oxygenated megastigmane group includes powerful compounds, such as beta-damascenone [8.93, 94]. The smell of β-damascenone is reminiscent of apple sauce and tropical fruit. β-Damascenone has a very low-odor threshold in water ($2\,ng\,l^{-1}$) and a threshold close to wine in model solution ($60\,ng\,l^{-1}$) (Table 8.4). This compound, initially identified in grape juice from the Riesling and Scheurebe varieties by Schreier [8.93, 94] and then in many other varieties [8.93, 94] has maintained the myth of a major contribution to wine aromas given its very low olfactory threshold in water ($2\,ng\,l^{-1}$). In fact, the perception threshold of β-damascenone in wine is between 2 and $7\,\mu g\,l^{-1}$, although this com-

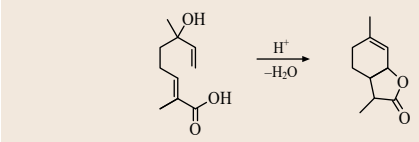


Fig. 8.2 Formation of wine lactone from (*E*)-2,6-dimethyl-6-hydroxyocta-2,7-dienoic acid (after [8.86])

Table 8.3 (−)-Rotundone a powerful sesquiterpenoid in wine (after [8.88, 89])

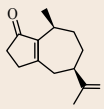
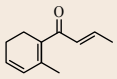
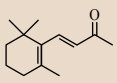
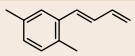
Name	Structure	Detection threshold ($\mu g\,l^{-1}$)	Concentrations in wines ($\mu g\,l^{-1}$)
(−)-Rotundone		0.016/0.008	0.05−0.6

Table 8.4 Characteristics of some C_{13}-norisoprenoids identified in wines

Name	Structure	Descriptors	Detection threshold[a] (ng/L)	Concentrations in wines (Min–Max)[b] (ng/L)
β-Damascenone		Applesauce rose	60/2[c]	100−2500
β-Ionone		Violet	800/120	nd-2415
TPB		Geranium leaf	24/20	nd-120
TDN		Kerosene-like	2300/1000	nd-30000

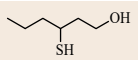
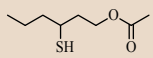
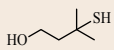
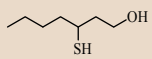
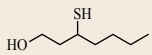
[a] Determined at ISVV, University of Bordeaux
[b] Concentrations (after [8.59, 90–92])
[c] Detection threshold in wine model solution and water, respectively

pound, usually found in concentrations of between 700 $ng\,l^{-1}$ and 2.5 $\mu g\,l^{-1}$ [8.93, 94], is rarely the only one involved in the aromatic composition of wines. However, β-damascenone can contribute by synergistic phenomena (Sect. 8.2). β-Ionone, with a distinctive smell of violet, has a perception threshold of 120 $ng\,l^{-1}$ in water, 800 $ng\,l^{-1}$ in model solution and 4 $\mu g\,l^{-1}$ in white wine, and its influence has been demonstrated in various grapes and wine varieties [8.90, 93, 94]. Other C_{13}-oxygenated norisoprenoids, such as 3-oxo-α-ionol (tobacco), 3-hydroxy-β-damascone (tea, tobacco), and β-damascone (tobacco, fruit) can provide only a very weak potential contribution to wine aroma. The second group consists of non-oxygenated megastigmane compounds, with 1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB) as a major representative. The detection threshold of this compound, presenting a typical geranium leaf odor, is 40 $ng\,l^{-1}$ in wine and 20 $ng\,l^{-1}$ in water. Concentration ranges in some old Sémillon wines can reach 200 $ng\,l^{-1}$ [8.93, 94]. The third group, composed of nonmegastigmanes, includes some odorous compounds as 1,1,6-trimethyl-1,2-dihydronaphtalene (TDN), which smells like kerosene and has a detection threshold of 1 $\mu g\,l^{-1}$ [8.91, 93, 94], (*E*) and (*Z*)-vitispirane which have camphor/woody nuances, riesling acetal (fruity descriptor), and actinidol (woody). TDN is considered to account in large part for the *petroleum* aromas of aged Riesling wines [8.93, 94] while (*E*) and (*Z*)-vitispirane, riesling acetal, actinidol are considered to have a limited contribution to wines aroma, particularly Riesling, as their concentration are usually much lower than their detection threshold [8.93, 94]. While megastigmane compounds from the first group can be detected in grape must and are present in the young wine, the representatives of the two other groups are only formed during wine aging. All these compounds originate from carotenoids present in grape, through enzymatic oxidative cleavage leading to hydroxylated C_{13}-norisoprenoid which then are submitted to several dehydration reactions in wine acidic media.

Impact of Volatile Thiols

In enology, many sulfur compounds in the thiol (sulfanyl) family are held responsible for olfactory defects. However, during the 1990s, several of these compounds were detected in wine and their positive contribution to wine flavor, particularly the varietal aroma of Sauvignon blanc wines and other white and red varietals, is now well documented. The three most important thiols in Sauvignon blanc aroma are 3-sulfanylhexanol (3SH), reminiscent of grapefruit flavor, 3-sulfanylhexyl acetate (3SHA), and 4-methyl-4-sulfanylpentan-2-one (4MSP) with box tree and broom aromas (Table 8.5) [8.95–98]. Descriptors, such as box tree and broom for 4MSP and grapefruit/passion fruit for 3SH match the occurrence of these compounds in box tree, broom, grapefruit, and yellow passion fruit, respectively. Several other odoriferous volatile thiols have also been

Table 8.5 Characteristics of some volatile thiols (sulfanyls) identified in wines

Name	Structure	Descriptors	Detection threshold[a] ($ng\,l^{-1}$)	Concentration in wines[b] ($ng\,l^{-1}$)
3-Sulfanylhexanol 3-SH		Passion fruit, grapefruit	60	100–10 000
3-Sulfanylhexylacetate 3SHA		Boxwood, passion fruit	4	0–1000
4-Methyl-4-sulfanylpentan-2-one 4MSP		Boxwood, broom	0.8	0–120
4-Methyl-4-sulfanylpentan-2-ol 4MSPOH		Citrus zest	55	0–150
3-Methyl-3-sulfanyl butan-1-ol 3MSB		Cooked leeks	1500	0–1500
3-Sulfanylpentan-1-ol 3SP		Citrus	900	0–400
3-Sulfanylheptan-1-ol $3SH_P$		Grapefruit	35	0–80
2-Methyl-3-sulfanyl butan-1-ol 2M3SB		Raw onion	nd	10–70

[a] Threshold determined in wine model solution at ISVV, University of Bordeaux

identified in Sauvignon blanc wine, such as 4-methyl-4-sulfanylpentan-2-ol, with a grapefruit zest flavor, and 3-methyl-3-sulfanylbutan-1-ol, smelling of leeks [8.97, 98]. Although these varietal thiols were first identified in Sauvignon blanc wine, they have also been found to contribute to the varietal aroma of wines made from other *Vitis vinifera* varieties, both red and white, such as Gewürztraminer, Riesling, Sémillon, Manseng, and Arvine [8.99, 100], as well as Merlot and Cabernet Sauvignon [8.101, 102].

More recently, 3-sulfanylpentan-1-ol (3SP), 3-sulfanylheptan-1-ol (3SHp), 2-methyl-3-sulfanylbutan-1-ol (2M3SB), and 2-methyl-3-sulfanylpentan-1-ol (2M3SP) were identified in Bordeaux dessert wines [8.103] and additive effects between them have been reported (Sect. 8.2). Due to their functional SH-group, thiol compounds sometimes occur in (*R*)- and (*S*)-enantiomer form. The enantiomeric distribution of 3SH, which contains one chiral center, was initially studied in passion fruit [8.97, 98], and later investigated in wines made from Sauvignon blanc and Sémillon grapes by *Tominaga* et al. [8.104]. The (*R*)- and (*S*)-enantiomer ratios of these two thiols in dry white Sauvignon blanc and Sémillon wines are approximately 30 : 70 for 3SHA and 50 : 50 for 3-sulfanylhexanol. However, in white dessert wines made from botrytized grapes, the proportion of the *R* and *S* forms of 3SH is in the vicinity of 30 : 70. The aroma descriptors of the two enantiomers of 3SH and 3SHA are quite different, although their perception thresholds are similar. Therefore, the enantiomeric distribution of 3SH and 3SHA in wine may have an impact on the perception and complexity of dry and sweet white wine aromas [8.104]. These varietal thiols originate from nonvolatile grape precursors, i. e., S-cysteine or glutathione conjugates, released during alcoholic fermentation [8.105]. 3-Sulfanylhexyl acetate (3SHA) is produced by enzymatic esterification of 3SH by *S. cerevisiae* yeast [8.106]. Accordingly, the formation of these compounds (thiols and esters) depends both on the yeast strain in alcoholic fermentation and the must matrix. Also, as thiols (sulfanyls) are highly reactive, they need to be preserved during winemaking and aging by protecting them from oxygen.

Lactones

Both γ- and δ-lactones are important aroma compounds, present in a wide variety of foods, beverages, and fruits. Many of them have been detected in wines, but one of the main cited is γ-nonalactone (Table 8.6). This compound has been identified in many different types of wine, including dry red and white, as well as botrytized and fortified wines [8.39, 107, 108]. Concentrations are low in white wines ($\approx 5.9\,\mu g\,l^{-1}$) and higher in botrytized and old red wines ($27–40\,\mu g\,l^{-1}$) [8.109, 110]. This compound, smelling of cooked peach when diluted, may con-

tribute to the characteristic prune flavor of these old red wines [8.111]. However, this substance is unlikely to contribute individually and directly to the aroma of most red wines, but probably has a greater impact in synergy with other γ- and δ-lactones. As γ-nonalactone is also found in oak wood, higher levels are found in wines aged in barrel [8.112].

This lactone is also a chiral compound, with enantiomers that differ slightly in their odor descriptors and strongly in their odor thresholds. Analysis of its enantiomeric distribution in wines revealed that, with few exceptions, the (*R*) enantiomer was more prevalent than its (*S*) counterpart in all the botrytized white wines analyzed, whereas neither isomer was overwhelming predominant in red wines [8.113].

This lactone is detected at low levels in grapes, depending on maturity or the development of noble rot (*B. cinerea*) [8.114], but is mainly produced by yeast (*S. cerevisiae*) during alcoholic fermentation [8.115]. *S. cerevisiae* yeast has been shown to produce γ-nonalactone from linoleic acid by two biosynthetic pathways [8.116]. The first features 13-lipoxygenation of linoleic acid to (*S*)-13-hydroxyoctadecadienoic acid, followed by four β-oxidation cycles and finally α-oxidation to (*S*)-γ-nonalactone (40–80% ee). The second features 9-lipoxygenation of linoleic acid to (*R*)-9-hydroxyoctadecadienoic acid, followed by a Baeyer-Villiger type oxidation to 3-(*Z*)-nonen-1-ol, which is further metabolized to the (*R*)-enantiomer (40–60% ee). Finally, this enzymatic mechanism exhibits an unsteady optical purity. Another odoriferous lactone, 2-nonen-4-olide, implicated in perceptual interaction phenomena, was recently identified in dessert wines [8.117].

Furanones

The flavor of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (Furaneol, HDMF) is influenced by its concentration; the pure compound is reminiscent of caramel, while it is described as cooked strawberry at low concentrations. This compound has a pK_a of 8,56 (20 °C) and, like many structurally related compounds, 3(2*H*) furanone is highly soluble in water (0.315 g mL^{-1}, 25 °C) [8.118]. It was first identified in grapes and wines made with certain fungus-resistant cultivars obtained by cross-breeding wild American vines (*Vitis labrusca*) with European cultivars (*Vitis vinifera*), marked by an intense strawberry flavor. For example, furaneol levels as high as 10 mg l^{-1} have been measured in wines made from the Castor cultivar [8.119]. Since then, it has frequently been reported as contributing to the complexity and quality of red wines, while the lowest levels were found in dry white wines (10–40 µg l^{-1}). On the contrary, concentrations may reach 60 µg l^{-1} in young Cabernet Sauvignon wines and even 150 µg l^{-1} in Merlot. These values are well correlated with the caramel note found in certain Merlot wines [8.120]. High concentrations have also been reported in sweet fortified wines, reaching more than 620 µg l^{-1} in young wines but decreasing during aging suggesting that this compound is unstable at wine pH, degrading to form acetylformoin [8.121], buta-2,3-dione, and acetoin [8.122]. In wines, this furanone exists in free and odorous form, as well as bound to sulfur dioxide and catechols (mainly nonpolymerized forms). According to *Ferreira* et al. [8.123], this finding may explain the low free furaneol levels found in white wines.

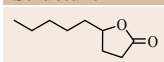
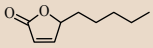
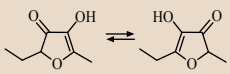
As demonstrated by *Guedes de Pinho* et al. [8.124], furaneol levels can be increased by using pectolytic enzymes with β-glucosidic secondary activities, suggesting the existence of glycosylated precursors. Also, while it is absent from grapes at maturity, high levels have been found in overripe grapes. As furaneol may be formed from pentose or hexose by a Maillard reaction, its presence in grapes may also be enhanced by high temperatures around maturity [8.125]. Besides these aspects, *Sarrazin* et al. [8.39] highlighted the fact that most of the furaneol found in wine is released or produced thanks to the yeast metabolism during alcoholic fermentation [8.39]. The use of toasted oak wood may also provide an additional source of this furanone.

Homofuraneol, HEMF (5 (or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3-(2*H*)-furanone), like furaneol, has a planar enol-oxo-configuration, but, due to the asymmetry of the molecule, both tautomeric forms have been identified and separated by GC on polar phase [8.126]. This compound was detected at the end of the 1990s in wines [8.77] and, like furaneol, is reminiscent of caramel and cooked strawberries. Concentrations in wines are lower compared to furaneol, ranging from 10 to 70 µg l^{-1} in dry red and white wines, and thus rarely exceed its perception threshold (Table 8.6) [8.39]. The lowest levels (a few µg l^{-1}) were found in sweet fortified wines, whereas the highest values, around 320 µg l^{-1}, were determined in botrytized wines. Moreover, owing to a strong synergistic effect between HDMF and homofuraneol, the fruity caramel note was increased, as evidenced in some rosé wines [8.127].

C_6 Aldehydes and Alcohols

Unsaturated fatty acids with 18 carbon atoms, such as linoleic acid and linolenic acid, are converted during prefermentation operations into the C_6 aldehydes hexanal and 2- and 3-hexenal with the aid of grape lipoxygenase [8.128, 129], and then further reduced to alcohol during fermentation. These C_6 aldehydes and

Table 8.6 Main furanones and lactones in wine

Name	Structure	Descriptors	Detection threshold
Racemic γ-nonalactone		Coconut, cooked peach	30 $\mu g\,l^{-1}$ [b]
(*R*)-form soft coconut with fatty-milky aspects strong, sweet[c]			284 $\mu g\,l^{-1}$ [a,c]
(*S*)-form fatty, moldy, weak coconut note[c]			91 $\mu g\,l^{-1}$ [a,c]
2-Nonen-4-olide		Minty and fruity	4.3 $\mu g\,l^{-1}$ [b]
Furaneol		Cooked strawberry, caramel	60 $\mu g\,l^{-1}$ [b]
Homofuraneol		Cooked strawberry, caramel	10–40 $\mu g\,l^{-1}$ [b]

[a] Wine, [b] Wine model solution determined at ISVV, University of Bordeaux, [c] *Cooke* et al. [8.113]

alcohols smell like freshly cut grass, but, considering their detection thresholds in the mg l^{-1} range, when taken separately, they rarely contribute directly to the herbaceous character of musts and wines.

8.1.4 Aging Aroma and Oak Related Flavor Compounds

Aging of wine is an important aspect of wine connoisseurship and one which distinguishes wine from almost every other drink. Already, in Antiquity, Falernian, and Surrentine wines required 15–20 years aging before they were considered at their best and were sometimes kept for decades in sealed earthenware jars or amphorae. Moreover, the Greek physician Galen was probably the first to note that an *aged* wine need not necessarily be old, but might simply have the characteristics of age. In other words, it was possible to age wines prematurely by heating or smoking them.

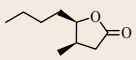
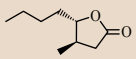
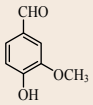
Nowadays, when we age wine, we hope for changes that will cause the wine to mature well by gaining a complex mix of complementary flavors. This is why the reputation of white and red wines is always strongly associated with their aging potential. These wines are able to retain the flavor nuances of young wines while developing specific varietal nuances. This kind of aging results in what is known as a reduction *bouquet*. While this specific organoleptic character is highly prized by connoisseurs, ideal aging does not occur in every wine. Most of the time, wine flavor develops quickly, resulting in a loss of complexity and personality, i. e., the flavor found in every oxidized wine. This phenomenon, known as premature aging, is well known in white wines and has more recently been found in red wines. Prematurely aged (Premox) white wines are reminiscent of honey, cider, and in certain cases, cooked vegetables, while red wines develop several aromatic nuances reminiscent of prunes and figs. In our experience of red wine tasting, the presence of these overriding odors affects the quality and subtlety of the wine flavor and may shorten its shelf life.

Wine aging generally consists of two phases: maturation (oxidative aging) and bottle aging (reductive aging). During maturation, wine is often stored in oak barrels. Continued aging in bottle is known as reductive aging, due to the small amount of oxygen inside the bottles.

Oak Wood Volatile Compounds in Wine Aroma

Oak (*Quercus* sp.) is currently the only wood used in making barrels for fermenting and aging quality wines. A fundamental aspect of aging wines in oak concerns the aromatic compounds extracted from the wood. When these compounds marry perfectly with a wine's intrinsic aromas, they make a significant contribution to the richness and complexity of the bouquet, as well as improving the flavor. Volatile extractive compounds from oak wood are responsible for important olfactory notes, such as coconut, wood, vanilla, caramel, and spice that play an essential role in wine aging in oak casks. They generally have low-aroma thresholds and may thus be detected by tasters in mature beverages at very low concentrations. Volatile content is strongly affected by natural factors, as well as botanical species (*Quercus robur*, *Quercus petraea*, *Quercus alba*), geographical origins, and cooperage techniques, such as seasoning and toasting (also known as hydrothermolysis). Hundreds of oak-derived volatile compounds have

Table 8.7 Oak volatile compounds

Name	Structure	Descriptors	Detection threshold[a]
(*Z*)-Oak lactone		Coconut, earthy	20 $\mu g\,l^{-1}$
(*E*)-Oak lactone		Spicy, coconut	110 $\mu g\,l^{-1}$
Vanillin		Vanilla	65 $\mu g\,l^{-1}$

[a] In wine model solution determined at ISVV, University of Bordeaux

been identified in alcoholic beverages. One group of compounds is formed by the degradation of oak lignin. This releases a range of phenolic odorants, the most important being eugenol, guaiacol, and 4-methylguaiacol, which give wine smoky and spicy aromas. Many other odorants are also directly or indirectly associated with wood aging, increasing the complexity of wines aged in oak barrels [8.130–133].

Of all the volatile compounds in oak wood, oak lactones, (*E*)/(*Z*)-β-methyl-γ-octalactone, particularly the cis-isomer, are considered the most important oak-derived compounds in wine. The structure of oak lactone, which is reminiscent of coconut, features two stereocenters, giving a total of four possible stereoisomers and two enantiomeric pairs of diastereoisomers. However, it has been established that oak wood contains only the (4*S*, 5*S*)-cis-1 and (4*S*, 5*R*)-trans-2 isomers of oak lactone [8.134]. The concentration of oak lactone (*cis*+*trans*) in untoasted wood ranges from 30 to 150 $\mu g\,g^{-1}$. In wines, the concentration of *cis*-oak lactone (the most odorous compound, Table 8.7), was found to be greater (under 20 $\mu g\,l^{-1}$ to over 1000 $\mu g\,l^{-1}$) than that of the trans-isomer (under 20 $\mu g\,l^{-1}$ to over 400 $\mu g\,l^{-1}$). The two isomers may be present at levels considerably higher than their perception thresholds [8.134]. Above a certain concentration, excessive amounts of this lactone may have a negative effect on wine aroma, giving it a strong woody or even resinous odor. Even when alcoholic fermentation takes place in barrels, the yeast metabolism has no impact on concentrations in wine. By analyzing the ratio of cis- and trans-isomers, it is possible to determine the source of wood used for barrel aging. This finding has been supported by results showing that American oak (*Quercus alba*) has only approximately 10% of the trans-isomer, while French oak (*Quercus robur, Quercus sessilis*) was found to have an almost equal amount of both stereoisomers. Moreover, this lactone is specific to oak wood. Only trace levels have been found in acacia, cherry, and chestnut woods, other species potentially used by coopers [8.135]. Detected in fresh oak woods, the two isomers of β-methyl-γ-octalactone are also formed during the air-drying process and during toasting at high temperature (150–200 °C) through dehydration of several precursors naturally present in oak wood. The precursors identified in oak wood are *cis*-3-methyl-4-galloyloxyoctanoic acid (ring-opened *cis*-oak lactone gallate, $R=4$), (3*S*,4*S*)- and (3*S*,4*R*)-3-methyl-4-*O*-β-D-glucopyranosyloctanoic acid (ring-opened *cis*- and *trans*-oak lactone glucoside $R=1$) [8.136], (3*S*,4*S*)-3-methyl-4-*O*-(6′-O-galloyl)-β-D-glucopyranosyloctanoic acid (ring-opened *cis*-oak lactone galloylglucoside, $R=2$) and 3-methyl-4-*O*-(6′-*O*-α-*L*-rhamnosyl)-β-D-glucopyranosyloctanoic acid ($R=3$) [8.137, 138] (Fig. 8.3). Concerning the glycoconjugate precursors, *Wilkinson* et al. [8.138] reported concentrations of the galloylglucoside ($R=2$) in French oak woods ten times higher (110–354 $\mu g\,g^{-1}$) than those of the others forms ($R=1$, $R=3$). Finally, knowledge of precursors indicated that the formation of oak lactone from oak lactone precursors could occur in two steps: liberation of the ring-opened oak lactone from oak lactone precursor by either pyrolysis (toasting) or enzymic activity and acid hydrolysis, followed by ring closure (lactonization) to yield oak lactone [8.139].

Vanillin plays an active part in the oaky and vanilla odors that barrels release into wine. However, its organoleptic contribution is a matter of debate. It may be direct or indirect according to its concentra-

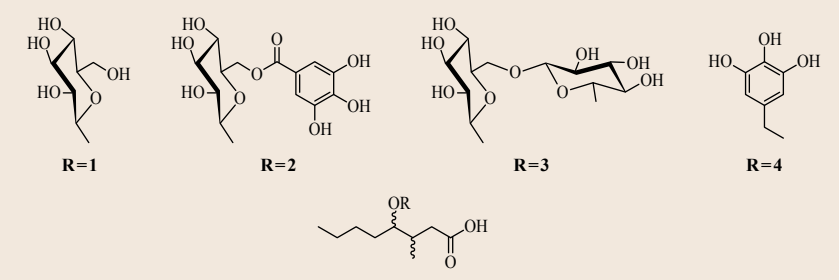


Fig. 8.3 Structure of the precursors of oak lactone identified in oak wood

tion and, more importantly, the type of wine (white or red) [8.140]. Only traces of vanillin are detected in grapes and wines, but much higher concentrations are released by toasted oak wood into wines during barrel aging. Though it is detected in nontoasted wood, toasting significantly increases the concentration. Vanillin accumulates quickly in wine: 70% of the final concentration of a 9 month storage period is reached during the three first months [8.141]. According to the aging period ($\approx$ 6–24 months) and the type of wood, the highest level ($\approx 5\,mg\,l^{-1}$) was systematically found in red wines. Indeed, traditionally, white wines undergo alcoholic fermentation in oak barrels to limit the organoleptic impact of wood compounds. This is mainly due to a biochemical transformation of vanillin into the barely fragrant vanillyl alcohol (perception threshold: $50\,mg\,l^{-1}$), vanillyl ethyl ester ($2.5\,mg\,l^{-1}$), and, finally, flavorless vanillyl ethyl ether. Moreover, yeast lees are capable of fixing and continuing to transform certain volatile compounds as they are released from the wood [8.142]. In general, the extraction of vanilllin from wood is greater than its conversion, so it accumulates in the wine. During malolactic fermentation (MLF) in wood barrels, an increase in vanillin is associated with the secondary metabolism of lactic acid bacteria (*O. oeni*) [8.143]. It has been demonstrated that these bacteria are able to hydrolyze glycosylated compounds released by oak wood; but, to date, specific precursors have not yet been identified. Ferulic acid was demonstrated to produce vanillin in the presence of *O. oeni*, but yields were low, resulting in amounts too small to explain the concentrations found in wines after MLF [8.144].

Reduction-Related Flavor Compounds: Reduction *Bouquet*

Empyreumatic aromas originate from greek empyreume, $\varepsilon\mu\pi\upsilon\rho\varepsilon\upsilon\mu\alpha$ (which is burned), the reminiscent of smoke and burnt wood odors, often feature in descriptors used by wine tasters. *Tominaga* et al. [8.145, 146] investigated the sensory impact of several volatile thiols on the reduction bouquet of wine aroma.

One potent thiol, 2-furanmethanethiol (2FM), develops a strong roast-coffee aroma and has been identified in Jurançon white dessert wines made from Petit Manseng grapes, as well as red Bordeaux wines (made from Merlot, Cabernet Franc, and Cabernet Sauvignon). These wines contain a few several dozen $ng\,l^{-1}$ 2FM (Table 8.8). The highest amount ($500\,ng\,l^{-1}$) was assayed in red wines at the end of 12 months aging in new oak barrels. Taking into account its very low-perception threshold ($0.4\,ng\,l^{-1}$), 2FM can therefore contribute to the roast-coffee aroma of certain barrel-aged red and white wines [8.145, 147]. This compound has also been reported in some old Champagnes, where it may be produced during bottle aging [8.148].

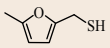
Traces of 2FM have been found in toasted oak used in barrel-making [8.149]. Furfural, released by toasted oak wood, plays a major role in 2FM formation in barrel-fermented white wines, where it is closely related to the yeast's (*S. cerevisiae*) sulfur metabolism. In barrel-aged red wines, a biochemical pathway involving furfural and a secondary metabolism of lactic bacteria (*O. oeni*) may promote a chemical route, involving furfural and H_2S. However, its formation mechanisms in bottle have not yet been elucidated.

Benzenemethanethiol (BM) contributes to the smoky/gunflint odor in several varietal wines. BM was detected systematically in white wines, including young ($20–30\,ng\,l^{-1}$) and old Champagnes ($200–400\,ng\,l^{-1}$), Sauvignon blanc ($10–20\,ng\,l^{-1}$), and Chardonnay ($20–40\,ng\,l^{-1}$). The lowest levels were found in red wines ($< 10\,ng\,l^{-1}$) [8.146]. This sulfur-containing compound has an extremely low-detection threshold ($0.3\,ng\,l^{-1}$) and makes a significant sensory contribution to the aroma of white wine. Together with 2-furanmethanthiol, these thiols contribute to the empyreumatic nuances in the bouquet of old Champagne wines [8.148]. The precursors of this thiol have not yet been identified.

2-Methyl-3-furanthiol (2M3F), the reminiscent of cooked meat, was identified for the first time in wine by *Bouchilloux* et al. [8.150]. Despite its instability, assaying 2M3F demonstrated its ubiquitous properties in young, as well as older wines (3–8 years old). It was detected systematically in red and white wines from different appellations at concentrations up to $100\,ng\,l^{-1}$, i. e., significantly above the olfactory perception threshold for this compound in model dilute alcohol solution ($2–8\,ng\,l^{-1}$). The highest values (reaching $200\,ng\,l^{-1}$) were found in Champagnes. At this level, it contributes to the overall *toasty* aroma in these wines [8.147]. The formation mechanisms of this compound, produced by Maillard reactions when food is cooked, have been studied in food, as well as in a model medium containing cysteine and pentose [8.151, 152]. In wine, its origin remains unclear. It has not been detected in must and is probably produced by yeast during alcoholic fermentation or brought in by dry yeasts [8.153].

Another important sulfur compound in the flavor of aged wines is dimethyl sulfide (DMS), identified by *Marais* [8.154]. It elicits odors described as *asparagus*, truffles, and molasses and plays an ambiguous role in wine aroma. Indeed, its influence on wine aroma was perceived either positively or negatively, depending on the DMS content and type of wine [8.155]. For example, it produces synergistic effects for fruity odors at subthreshold concentrations

Table 8.8 Volatile sulfur compounds related to aging aroma

Name	Structure	Descriptors	Detection threshold[a]
Dimethyl sulfide		Quince, truffle	25 $\mu g\,l^{-1}$ (white wine) and 65 $\mu g\,l^{-1}$ (red wine)
Benzenemethanethiol		Gunflint, smoky	0.3 $ng\,l^{-1}$
2-Furanmethanethiol or 2-Furfurylthiol		Roasted coffee	0.4 $ng\,l^{-1}$
2-Methyl-3-furanthiol		Cooked meat	2–8 $ng\,l^{-1}$

[a] In wine model solution determined at ISVV, University of Bordeaux

($10\,\mu g\,l^{-1}$) in mixtures containing fruity esters, C_{13}-norisoprenoids, and linalool [8.156, 157]. Moreover, DMS is considered a beneficial compound at low concentrations, contributing to the aroma of bottle-aged wines. DMS concentrations found in wine range from a few $\mu g\,l^{-1}$ to $480\,\mu g\,l^{-1}$, well above the sensory threshold ($27\,\mu g\,l^{-1}$) [8.158]: levels in freshly bottled wines are low and increase during aging, depending on storage temperature [8.159]. The DMS formation mechanism in wine is not clear. It may be formed in a similar way to other mercaptans. It appears during aging, via a yeast mechanism, by cleavage of *S*-methyl-*L*-methionine to form homoserine and dimethyl sulfide, and is also variously linked to the cysteine, cysteine, or glutathione metabolism in yeast.

Recently a strong correlation was established between the appreciation of reduction bouquet in aged great Bordeaux wines with the level of abundance of 2-methanefuranethiol and DMS [8.160]. Also, other thiol (sulfanyl) derivatives associated with ageing bouquet have been identified as vanillylthiol resulting from vanillin transformation and several thiopyrroles in aged Chardonnay wines from Burgundy [8.161].

Oxidation-Related Flavor Compounds

Sotolon (4,5-dimethyl-3-hydroxy-2(5)H-furanone) is an important five-branched lactone belonging to the isotetronic acid family (Table 8.9). The pure compound possesses an intense odor of curry, but develops honey and nutty flavors when diluted. NMR revealed that only the enolic tautomer of sotolon was observed in aqueous solution [8.162]. It is stable at the pH of wine, but unstable under basic conditions and in apolar organic solvent. Furthermore, this thermolabile, highly polar compound is susceptible to irreversible adsorption in GC-MS and is difficult to extract from wine samples (poor yields). However, methodologies have been optimized for its quantification and SIDA approaches (^{13}C label) gave the best results [8.162, 163].

Sotolon contributes to the aromas of *vins jaunes* from the Jura and sherries [8.164, 165], as well the *dried fig* and *rancio* nuances in French fortified wines (Vins doux naturels – VDN) and port [8.166, 167]. Concentrations in white wines made from grape varieties like Savagnin increase during aging with yeast *flor*, as well as during the barrel aging of sweet wines [8.167–169]. Wines of this type, over 20 years old, may contain up to $1\,mg\,l^{-1}$ sotolon that contributes to the typicality of these wines, made under oxidative conditions.

This furanone has also been detected in white wines made from botrytized grapes [8.170] but is rarely found in red wines. More recently, several authors determined the contribution of sotolon to the oxidation aromas of prematurely aged dry white wines [8.171–173]. The highest concentration found in these dry wines was $15–20\,\mu g\,l^{-1}$. Oxidation phenomena are involved in generating sotolon in wine. In wines that are made traditionally under reductive conditions, sotolon is considered an off-flavor.

Both enantiomers are detected in white wines, but due to its low-perception threshold, only (*S*)-sotolon contributes to the characteristic aroma of prematurely aged (premox) dry white wines. According to the wines, the maximum enantiomer excess (ee) ranged from 56 to 50 for the *R* and *S* forms, respectively [8.174, 175].

According to several studies, amino acids and, more precisely, threonine constitute a potential source of this furanone in wines. Its enzymatic or chemical deamination to 2-ketobutyric acid, followed by an aldol condensation with acetaldehyde, led to the formation of racemic sotolon (Fig. 8.4) [8.177]. Acetaldehyde and 2-ketobutyric acid are found in every wine as by-products of *S. cerevisiae*. 2-Ketobutyric acid has also been identified as an oxidative degradation product of ascorbic acid, a natural antioxidant present in small quantities in grapes and added during white wine vinification as well as at bottling (from 50 to $100\,mg\,l^{-1}$) to protect the wine from oxidative phenomena. A stereoselective pathway producing enantiopure sotolon has

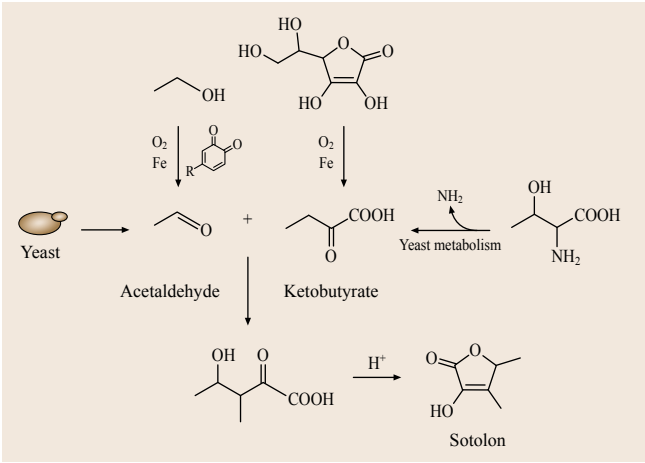


Fig. 8.4 Chemical and biochemical production of sotolon in wines (after [8.166, 176, 177])

not yet been identified. However, such a mechanism is not ruled out; low enantiomeric excess (ee) may be partly explained by the slow racemization kinetics (20 months) of optically active sotolon. Abhexon, an analogue of sotolon, with very similar organoleptic properties, has been identified in old Sauternes and flor sherry wines [8.178].

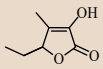
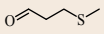
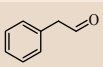
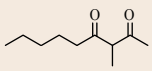
Among the oxidation-related aroma compounds, aldehydes, methional, and phenylacetaldehyde have attracted the most attention, due to their supposedly greater aroma impact and possible contribution to the aromas of red and white wines [8.179]. Reminiscent of boiled potatoes and old rose, respectively, their perception thresholds in wine model solution are 0.5 and $1\,\mu g\,l^{-1}$ [8.180]. These aldehydes are commonly found in all wines: white and red, port and sherry. Methional concentrations range from a few hundred $ng\,l^{-1}$ to $4.9\,\mu g\,l^{-1}$ in white wines and, in exceptional cases, $140\,\mu g\,l^{-1}$ [8.179], according to the oxidation level. Phenylacetaldehyde concentrations range from a few $\mu g\,l^{-1}$ to $53\,\mu g\,l^{-1}$ in white wines, sometimes exceeding $90\,\mu g\,l^{-1}$ in red [8.180] and botrytized white wines [8.39]. These compounds contribute directly to the flavors of oxidized white wines and are the reminiscent of cooked vegetables, cider, liquor, and honey. In oxidized red wines, they add nuances of figs and dried prunes and their direct contribution is not so clear. For example, *San-Juan* et al. [8.181] showed that the addition of methional to a young red wine transformed fresh fruit nuances into dried fruit aromas. According to these authors, this effect is particularly strong in the presence of C_{13} norisoprenoids.

It has been proposed that these aldehydes are formed from methionine and phenylalanine amino acids, respectively, via the Strecker reaction, involving the presence of a dicarbonyl compound. Wine contains two possible sources of dicarbonyl compounds able to induce the Strecker reaction. The first requires a reaction involving wine ortho-diphenols, forming ortho quinones [8.182] and are, in turn, able to induce the Strecker reaction with the amino acid [8.183]. Wine also contains dicarbonyls produced by microbial metabolisms. These include diacetyl, glyoxal, and methylglyoxal. The formation of aldehydes involving these carbonyls and the corresponding amino acids has been demonstrated in a wine-like medium and may play a role in the formation of aldehydes during bottle aging [8.184].

Another ketone involved in red wine flavor modifications is 3-methyl-2,4-nonanedione (MND) (Table 8.9). This β-diketone, the reminiscent of hay and anise, was recently identified in prematurely aged red wines with a marked prune flavor [8.111]. These descriptors may be concentration dependent. Indeed, at low concentrations, MND smells different from the pure compound (i. e., fruit pit, prune) and is rather reminiscent of prematurely aged (premox) red wine. Its perception threshold in wine model solution is $16\,ng\,l^{-1}$ [8.185]. MND content was shown to be lower in nonoxidized red wines than oxidized red wines, where it systematically exceeded the perception threshold ($62\,ng\,l^{-1}$). The keto-enol equilibrium, in which tautomers with the same odor and perception threshold in air [8.186] are only partially interconverted according to the temperature and polarity of the solvent, was first described by *Guth* [8.187].

Concentrations up to $340\,ng\,l^{-1}$ were determined in most oxidized red wines. High concentrations of this compound can be found in various wines i. e., rosé, botrytized wines and fortified wines made with overripe grapes. The lowest levels ($2.9\,ng\,l^{-1}$) were found in nonoxidized white wines and the highest concentrations in oxidized botrytized wines ($293.8\,ng\,l^{-1}$). From a sensory standpoint, the presence of MND alone significantly modifies the flavor of a red wine. The fresh fruit flavor in unadulterated red wine contrasts with the aromatic expression of *rancio* (old walnut flavor characteristic of oxidative aged Port wine) in wines spiked with high concentrations of MND ($308.9\,ng\,l^{-1}$). Oxidation mechanisms are responsible for its presence in wines. Guth found that certain furan fatty acids (10,13-epoxy-11,12-dimethyloctadeca-10,12-dienoic acid and 12,15-epoxy-13,14-dimethyleicosa-12,14-dienoic acid) were precursors of this compound in soybean oil [8.188]. These fatty acids have not previously been identified in wine, so the origin of 3-methyl-2,4-nonanedione in red wine is still unknown.

Table 8.9 Characteristics of volatile compounds involved in oxidation-related wine flavors

Name	Structure	Descriptors	Detection threshold[a]
Sotolon		Curry	Racemic 2 µg l^{-1}. (*S*) 0.8 µg l^{-1} (*R*) 89 µg l^{-1}
Abhexon		Curry	4.5 µg l^{-1}
Methional		Boiled potatoes	0.5 µg l^{-1}
Phenylacetaldehyde		Honey, old rose	1 µg l^{-1}
3-Methyl-2,4-nonanedione		Dried parsley, fruit pit, anise	16 ng l^{-1}

[a] In wine model solution determined at ISVV, University of Bordeaux

Other Age-Related Flavor Compounds

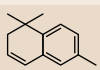
TDN (1,1,6-trimethyl-1,2-dihydronaphtalene), previously described (Sect. 8.1.2) as grape-derived C_{13}-norisoprenoid, sometimes imparts an undesirable *petroleum* or *kerosene* note to Riesling wine during aging (Table 8.10). Despite that, TDN is found in several red and white varietals, e.g., Cabernet Sauvignon, Cabernet Franc, and Sauvignon blanc, and is considered to have a strong, specific impact on Riesling wines. *Sponholtz* et al. [8.189] reported that TDN concentrations below 20 µg l^{-1} were considered to make a positive contribution to the varietal aroma of young Riesling wines and Riesling wine quality in general, up to a maximum of 4 µg l^{-1}, but recent work by *Schüttler* et al. [8.92] did not find any link between TDN concentrations in Riesling wines and the appreciation of their aroma uniqueness. TDN may be formed during alcoholic fermentation, but develops mainly during bottle storage. TDN is chemically stable in wine. Lutein, a carotenoid was shown to be a possible precursor for TDN via chemical thermal degradation in a sulfuric acidic medium. It is also generated from megastigm-4-ene-3,6,9-triol and megastigm-4,7-diene-3,6,8-triol, possibly bound as glycosides, by hydrolytic cleavage in acidic media [8.190, 191]. More recently, *Daniel* [8.192] demonstrated that Riesling acetal is a precursor of TDN in wine.

2-Aminoacetophenone is known as the aroma impact compound responsible for *untypical aging off-flavor* (UTA) in *Vitis Vinifera* wines [8.193]. This off-flavor may develop in bottle or in barrel within a few months after fermentation. UTA is associated with descriptors such as furniture polish, mothballs, or acacia blossom, combined with a loss of varietal character. Below its odor threshold in grape berries, must, and wine after alcoholic fermentation [8.194], 2-AAP concentrations increase significantly during storage. It has been detected in all wines irrespective of the grape variety, at concentrations ranging from 0.02 to 10 µg l^{-1} [8.195]. Concentrations in Sauvignon blanc range from 0.05 to 0.9 µg l^{-1}, rarely exceeding its sensory threshold, between 0.7 and 1 µg l^{-1}. Red wines contain the lowest concentrations. The 2-AAP content in wines without UTA is below 0.3 µg l^{-1}. Indole-3-acetic acid (IAA), a naturally occurring grape phyto-hormone, is considered a potential precursor of 2-AAP in wines [8.196]. A nonenzymatic mechanism is involved in its formation, when IAA reacts with sulfite, which requires superoxide radicals [8.197], generated by the aerobic oxidation of sulfured wine during storage. UTA has not yet been detected in red wines; the degradation of IAA to 2-AAP is probably blocked due to the presence of phenolic radical scavengers (Table 8.10).

8.1.5 Off Flavors in Wine

Many examples show how wine tasting can be affected by volatile compounds associated with organoleptic deviations. Also, it is important to characterize the com-

Table 8.10 Characteristics of volatile compounds involved in undesirable aging-related flavors in wine

Name	Structure	Descriptors	Detection threshold[a]
2-Amino-aceto-phenone		Mothball, acacia blossom	0.7–1 µg l^{-1}
TDN		Kerosene-like	2.3 µg l^{-1}

[a] In wine model solution determined at ISVV, University of Bordeaux

pounds responsible for off-odors in order to develop control strategies, as well as protocols to address the problem and improve wine quality. Acetic spoilage, resulting in the production of acetic acid and ethyl acetate, constitutes an historical example where deviation due to sugars being metabolized by acetic bacteria, converts wine into an unpleasant-tasting vinegary beverage [8.11]. Other off-odors in wine aroma can be traced back to the pollution of wine by contaminated materials, such as a cork stoppers (cork taint due to the release of 2,4,6-trichloranisole and 2,3,4,6-tetrachloroanisole, formed by *Ascomycetes* fungi (*Penicillium* sp., and *Trichoderma* sp.)) [8.11] or bushfire smoke [8.198], as well as undesirable metabolic by-products of grape and wine microorganisms, and, finally, chemical mechanisms responsible for transforming non-odorous compounds into defects. These off-odors may originate from grapes, as well as during winemaking and aging in tanks, oak barrels, and bottles.

Concerning the contribution of each compound to the off-odor, it is necessary to bear the following three points in mind:

1. Firstly, the perception of an off-odor depends on both the concentration of the compound responsible and its olfactory detection (or recognition) threshold. However, at a given concentration, the perception of an off-odor is highly dependent on individual sensitivity.
2. Secondly, the perception of an off-odor may be modulated by the presence of other volatile compounds.
3. Thirdly, the recognition of an off-odor depends on the wine tasters' capacity, depending on their level of experience, to distinguish a particular odor among the complex aromas of wine (*Tempere* et al. [8.199]).

Off-Odors Related to the Metabolism of Wine Microorganisms

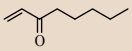
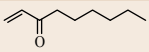
Many unpleasant odors in wine result from the development of unwanted microorganisms or are related to metabolic modifications in wine microorganisms. Among the microorganisms concerned, over the past 20 years, spoilage due to *Brettanomyces sp* contaminant yeast has increasingly deteriorated the aromatic appreciation of red wines [8.200]. This off-odor is due to volatile phenolic compounds, mainly 4-ethylphenol and 4-ethylgaiacol, which originate from the decarboxylation of hydroxycinnamic acids in grapes, ferulic, and p-coumaric acid, respectively, by *Brettanomyces sp*. [8.201, 202]. The development of *Brettanomyces sp*. is favored, particularly in oak barrels, when wine is not sufficiently protected by sulfur dioxide due to high pH.

4-Ethylphenol and 4-ethylgaiacol present a heavy phenolic flavor likely to spoil red wine aroma, particularly attenuating their fruity character, with horse sweat, leather, and phenolic descriptors, all recognized as *Brett* character [8.181, 201–203]. In model solution similar to wine, the olfactory detection thresholds of 4-ethylphenol and 4-ethylguaiacol are $440\,\mu g\,l^{-1}$ and $135\,\mu g\,l^{-1}$, respectively. They are usually present in a ratio of around 10 : 1. As *Brett* character is not always clearly detected at concentrations above the detection threshold of ethylphenols in red wines, several studies have reconsidered the sensory perception implication of these compounds in red wine aroma [8.6]. A psychophysical study with 134 wine professionals highlighted that the distribution of individual olfactory detection thresholds for a mixture of 4-ethylphenol and 4-ethylgaiacol (in a 10 : 1 ratio) varied by a factor of 100 between the persons with the lowest and highest sensitivity. Moreover, the detection threshold of these compounds in red wine may be modulated by the presence of other wine volatile constituents (Sect. 8.2). In recent studies, *Tempère* et al. [8.199] investigated the analysis of perceptual skills and socio-professional parameters implicated in the recognition of *Brett* character. An initial experiment tested the hypothesis that expertise in terms of age and qualifications could affect the assessment of *Brett* character.

Vinyl phenols also affect the *finesse* of dry white and rosé wines [8.204]. These compounds originate from the decarboxylation of hydroxycinnamic acids by *S. cerevisiae*. 4-Vinylphenol has a strong odor, described as phenol, pharmaceutical, or gouache, that is, heavy odor of painting used by painters-, while 4-vinyguaiacol elicits the smell of cloves. Various enological parameters influence vinylphenol formation in white and rosé wines, particularly the strain of *S. cerevisiae* yeast used for alcoholic fermentation.

Lactic acid bacteria belonging to *O. oeni* and *Lactobocillus sp* contribute to wine stabilization by fermenting malic acid during malolactic fermentation, a key step for numerous premium red and white wines. However, the development of these bacteria in wines may sometimes result in off odors detrimental to the aromatic quality of the wine. This is the case when sorbic acid (2,4-hexadienoic acid), a fungistatic agent used in some dessert wines to reduce the risk of refermentation, is degraded by lactic bacteria to form 2-ethoxyhexa-3,5-diene, a strongly odoriferous compound reminiscent of geranium leaves (*Pelargonium sp* [8.205]. Also, *L. hilgardii* and *L. brevis* may be implicated in the production of potent nitrogen-heterocylic compounds (2-acetyltetrahydropyridine, 2-acetyl-1-pyrroline, or 2-ethyltetrahydropyridine) that contribute to an off odor in red wines identified as mousy taint [8.206–208].

Table 8.11 Characteristics of some compounds contributing to fungal and mushroom off-odors in wines

Name	Structure	Descriptors	Detection threshold[a]
(−)Geosmin		Table beet, earth	10 ng l^{-1}
1-Octen-3-one		Fresh mushroom	30 ng l^{-1}
1-Nonen-3-one		Fresh mushroom	8 ng l^{-1}

[a] In wine model solution determined at ISVV, University of Bordeaux

Fungal, Mushroom, and Herbaceous Off-Odors

Some of these off-odors in wines are related to fungal notes, the reminiscent of damp earth, camphor, mold, and fresh mushrooms (Table 8.11). The presence of these aromatic nuances is sometimes attributed to contact between must or wine and materials (tanks, oak-barrels, or stoppers) that have been polluted during vinification, or barrel or bottle aging. These off-odors may originate from grape bunch rot when the harvest is spoiled by poorly timed rain, persistent morning mists, or hail. Analysis of these off-odors has led to the identification of several compounds with detection thresholds in the ng l^{-1} range, including 1-octen-3-one and 1-nonen-3-one (fresh mushroom odors), (−)-geosmin (powerful *damp earth* and *beetroot* odor) and the less common 2-methylisoborneol, with its camphor and earthy off-odors [8.209, 210]. These fungal off-flavors are related to grapes with bunch rot complexes involving *Botrytis cinerea* and secondary saprophytic invaders belonging to various species especially the *Penicillium* genus [8.211, 212]. Due to the potent odor of (−)-geosmin (perception threshold in water: 10 ng l^{-1}), less than 1% infection of a plot in the vineyard is enough to contaminate the entire harvest [8.209]. Particular care in the prevention and control of gray mold caused by *B. cinerea* in the vineyards concerned, together with careful sorting and removal of affected grapes, is recommended, in view of this compound's strong sensory impact [8.209]. Herbaceous off-odors have been shown to originate from the presence of high concentrations of 2-methoxy-3-isopropylpyrazine, due to the contamination of grapes during harvesting by the Asian Lady Beetle, *H. axyridis*, an insect introduced for the biological control of aphids [8.213].

Reduction Defects Caused by Volatile Sulfur Compounds

The impact of trace amounts of highly odorous volatile thiols on a wine's varietal aroma was established in the early 1990s (Sect. 8.1.3, *Impact of Volatile Thiols*), and in the wine aging bouquet. However, in enology, sulfur compounds are first considered to be responsible for organoleptic defects due to the irritating odor of sulfur dioxide or the *reduced* character related to the presence of some thiol or sulfide compounds. Winemakers have, therefore, always sought to minimize the impact of these compounds.

Historically, the first sulfur compound used in the elaboration of wine likely to cause a deterioration in quality is sulfur dioxide. This compound, naturally produced during alcoholic fermentation by *S. cerevisiae* at concentrations around 10 mg l^{-1}, is added to grape must and wine after fermentation and during aging, to prevent the development of undesirable microorganisms (bacteria and yeasts) and limit oxidative phenomena. Sulfur dioxide has been used systematically for its protective antioxidant and antimicrobial properties since at least the early 17th century. Its irritating odor, rarely perceived today in commercial wine, is considered detrimental to wine quality.

Off-odors known as *reduction* defects, evocative of rotten egg, garlic, onion, rubbery, or sometimes metallic character, are associated with volatile sulfur compounds:

1. Sulfides
2. Thiols
3. Thiophenes
4. Thiazoles.

Two groups of compounds have been distinguished according to their volatility: Highly volatile sulfur compounds (VSC) (boiling point $< 90\,^{\circ}C$), including hydrogen sulfide (H_2S), methanethiol (MeSH), ethanethiol (EtSH), dimethyl sulfide (DMS), diethyl sulfide (DES), dimethyl disulfide (DMDS) and *heavy* sulfur compounds, with boiling points above 90 °C. VSC compounds have a strong impact on wine aroma, due to their high volatility and low-odor thresholds. The main *light* sulfur compounds hydrogen sulfide, methyl mercaptans, and, less frequently, ethanethiol, dimethyl disulfide, and carbon disulfide, play a key role in reduction defects. In *reduced* wines, levels of these compounds are always above the olfactory detection threshold [8.214, 215]. The origins of these compounds are diverse, depending on vinification and aging conditions, as well as grape and wine contaminants. However, on the other hand, the absence of a small amount of oxygen, either enclosed at bottling or as a result of oxygen seeping through the closures, results in undesirable *reduction* aromas. Nitrogen deficiency in grape must and the presence of sulfur residues from pesticide treatments may also have a significant effect on the formation of these compounds

by *S. cerevisiae* yeast, as well as photochemical degradation during the wine aging (degradation of vitamin B2) [8.11].

Among the many *heavy* VSCs identified in wines, only a few contribute significantly to reduction defects. The most important of these is 3-methythiopropan-1-ol or methionol, which smells strongly of fermented cabbage (odor threshold $1200\,\mu g\,l^{-1}$). The 2-mercaptoethanol content in some *reduced* wines may also approach the odor threshold. Thiophene and 2-methyl-tetrahydro-thiophenone, act rather as odor-masking substances at concentrations exceeding $90\,\mu g\,l^{-1}$, thereby masking other aroma compounds with positive impact on the global wine aroma [8.216]. Recently, a malodorous sulfur metabolite perceived as *baked beans* and *Fritillaria sp. bulbs*, affecting dry white and rosé wine aroma, was identified as ethyl 2-sulfanylacetate [8.217]. This compound is not associated with the sulfur metabolites produced during alcoholic fermentation of grape juice containing solids, nor excessive addition of sulfur dioxide in the must. It is produced during alcoholic fermentation, particularly in wines made from hard-pressed juices and oxygenated musts. Depending on the wine matrix, concentration at which aroma is affected in dry Sauvignon blanc wines doted with this substance varies from 300 to $500\,ng\,l^{-1}$, while the detection thresholds of ethyl 2-sulfanylacetate in water and hydro-alcoholic solution are $70\,ng\,l^{-1}$ and $200\,ng\,l^{-1}$, respectively. Moreover, concentrations of this compound increase during bottle-aging of white wine. The knowledge of this compound facilitates informed choice of pressing and juice selection, as well as white wine vinification techniques that preserve aroma. Particular care is recommended in situations where this malodorous compound is likely to be produced, with grape pressing under a nitrogen atmosphere as a key strategy for minimizing the oxidation of the grape juice [8.217].

8.2 Perceptual Interaction Phenomena

The uniqueness and complexity of wine aroma depends on the presence of key volatile compounds. Characterization of these volatile compounds constitutes the heart of research into wine aroma. Indeed, the chemical characterization of impact aroma compounds and analysis of their contribution to wine aroma provides an opportunity to consider various aspects of winemaking technology, including wine microorganisms and grape ripening conditions. Despite substantial progress toward identifying unique varietal aromas, the construction of olfactory *images* in the brain is so complex that the key compounds alone are often unable to explain all the nuances perceived in wine aromas. Well-known experiments by the *Laing* group [8.218, 219] revealed the limited capacity of human beings to identify odors correctly in a mixture consisting of only three or four compounds. In fact, in considering wine aroma perception, at least two main types of perception should be considered: analytical and elemental perception, where the odors of specific compounds are clearly perceived in the wine among other volatile odoriferous compounds and synthetic or configural perception, involving perceptual interaction phenomena, where the perceived odors result from a combination of several volatile compounds, modified by masking phenomena and additive or synergistic effects, possibly resulting in new aromas.

8.2.1 Masking Effects

In enology, research into wine volatile compounds, conducted mainly in the last decade, has shown the importance of such sensory phenomena. Thus, it seems that the detection of off-odors in wine is likely to be related to analytical perception, while specific wine aromas more generally result from combinatorial effects involving several volatile compounds. In fact, empirical observations concerning the perception of *Brett* off-odor have indicated an indirect relation between the ethylphenol content in wines and the perception of this defect [8.199, 202]. For example, *Romano* et al. [8.220] showed that the detection threshold of a mixture of 4-ethylphenol and 4-ethylguaiacol (in a 10 : 1 ratio) in wine was higher (i. e., reduced perception) when other volatile compounds related to *Brettanomyces* sp. metabolism, such as isobutyric and isovaleric acids, were present, indicating that a masking phenomenon was involved.

8.2.2 Additive and Synergistic Effects

In contrast, as early as 1970, *Ribéreau-Gayon* and *Boidron* demonstrated that a mixture of the main terpenols had a significantly lower odor-detection threshold than each one taken separately, thereby highlighting a synergistic action [8.74]. This effect was also observed for wine γ-lactones by *Jarauta* et al. [8.221], who reported that the detection threshold of a mixture of wine lactones was four times lower than that expected on the basis of their individual thresholds. *Sarrazin* et al. [8.103] also noticed that chemically similar sulfanylalcohols, for example, 3-sulfanylpentan-1-ol, and 3-sulfanylheptan-1-ol, which have a similar cit-

rus aroma to 3-sulfanylhexan-1-ol, may contribute to dessert wine aromas at concentrations lower than their olfactory detection thresholds through additive phenomena.

More recently, the additive properties of some ethyl esters of fatty acids and fusel alcohol acetates in the fresh, fruity aromas of red wine have been extensively studied by several authors [8.22, 23, 26, 222, 223]. In combination, these compounds make a significant contribution to red wine fruity aroma, even at concentrations below their odor-detection thresholds, while each one added separately has no effect [8.13, 23, 23]. Ethyl butyrate, ethyl hexanoate, and ethyl octanoate were found to enhance the expression of *fresh fruit* and *red fruit* aromas, while ethyl propionate was implicated in the notion of *candied fruit* and *black fruit* [8.13, 23, 23]. By way of example, ethyl 2-hydroxy-4-methylpentanoate, also known as ethyl leucate, was recently demonstrated to enhance the fruity aroma of red wine [8.25, 26].

Both ethyl esters of fatty acids and fusel alcohol acetates are considered to impact the typical fruity character of red wines, through synergistic effects and perceptive interactions [8.13, 23, 23]. Using omission tests in model media (dilute alcohol solutions), *Lytra* et al. [8.223] highlighted the importance of ethyl propanoate, ethyl-3-hydroxybutanoate, butyl acetate, and 2-methylpropyl acetate in a fruity mixture containing 12-ethyl esters of fatty acids and fusel alcohol acetate, although these compounds were only present at subthreshold concentrations. Also, ethyl esters of fatty acids, such as butanoic, hexanoic, octanoic, decanoic, and, to a lesser extent, dodecanoic acids are involved in perceptive interactions with other aromatic compounds, including β-damascenone [8.13, 23, 23], β-ionone [8.13, 23, 23], and dimethylsulfide [8.13, 155, 157] even at concentrations below their odor thresholds.

Synergistic effects have also been revealed between monoterpenes and β-damascenone; for example, the orthonasal detection thresholds of linalool and geraniol are lowered by the presence of β-damascenone. This phenomenon has also been identified and measured with lab mice in the Pasteur Institute through psychophysic tests using a specific behavioral methodology [8.224]. Moreover, β-damascenone contributes, via synergistic phenomena, to reducing the detection thresholds of ethyl hexanoate and ethyl cinnamate and raising the detection threshold of 2-methoxy-3-isobutylpyrazine [8.59].

8.2.3 Perceptual Blending

Not many examples in the literature report the situation where a mixture of compounds triggers the perception of a different aroma from those of the compounds taken separately. A 30/70 mixture of ethyl isobutyrate (strawberry flavor) and ethylmaltol (caramel) induced the perception of pineapple [8.225]. In another situation, a mixture of six compounds (β-damascenone, β-ionone, frambinone, vanillin, isoamyl acetate, and ethyl acetate) had a flavor reminiscent of red cordial (grenadine), unlike any of the compounds taken individually [8.226].

Another example of perceptual interaction phenomena was revealed during a study on compounds involved in the *candied citrus* and *orange* aromas of dessert wines [8.117]. Emphasis was placed on the study of the phenomenon of perceptual interactions; consequently, *aromatic recovery* was an important research approach: while fractionating an extract of premium Sauternes dessert wine, one fraction was recognized as having these characteristic citrus aromas. This fraction was not present in lower quality wines. Analytical chemistry coupled with sensory analysis identified the compounds present in the fraction associated with this odor as whisky lactone (*coconut*), eugenol (*clove*), and a newly identified lactone, 2-nonen-4-olide, associated with noble rot, which has *minty* and *fruity* odors. None of these compounds considered alone at concentrations close to those assayed in wines produced an orange aroma. Subsequent omission and reconstitution tests demonstrated that these four compounds together, particularly the two lactones (one originating from oak wood and the other from botrytized grapes), generate the perception of *orange* aroma. Moreover, the importance of enantioselective distribution of 2-nonen-4-olide on this perceptual phenomena was recently evidenced (*Stamatopoulos* et al. [8.227]) The correlation of whisky lactone with wine fruitiness has also been reported [8.228, 229]. This sensory phenomenon, well known in perfumery, appears to contribute substantially to the uniqueness and complexity of wine and opens up new perspectives for understanding wine aroma perception.

8.2.4 Interactions Between the Wine Matrix and Aroma Compounds

The wine matrix contains numerous nonvolatile compounds, at concentrations in the $g\,l^{-1}$ range, that may have a significant impact on aroma perception during wine tasting. Those compounds include acids (tartaric, malic, citric, and acetic acids), ethanol (at concentrations ranging from 70 to 160 $g\,l^{-1}$), polysaccharides, proteins, and polyphenols (pigments, such as anthocyanins, and flavonoids). One aspect of this subject concerns the impact of pigments (anthocyanins) on the

appreciation of aromatic nuances. Briefly, when nonexpert wine tasters were asked to assess a dry white wine colored with anthocyanins, they used red wine descriptors [8.7].

Another aspect of the impact of wine matrix composition on aroma compounds concerns physico-chemical interactions between volatile compounds and other key compounds in wine (glucose or fructose, polyphenols, polysaccharides, proteins, etc.) and in vivo retronasal perception in the mouth. Specifically, several authors have reported the impact of interactions between nonvolatile and volatile compounds in wine on the partitioning of volatile compounds [8.10, 15, 230, 231]. First, *Dufour* et al. described the impact of (+)-catechin ($2-10\,g\,l^{-1}$) on reducing the volatility of several fermentation esters:

1. Isoamyl acetate (banana flavor)
2. Ethyl hexanoate
3. Benzaldehyde.

The sensory effect of such phenomena, studied more recently [8.14] regarding other esters (ethyl butyrate and ethyl octanoate), indicated that their olfactory detection threshold increased (at least doubled) in the presence of (+)-catechin ($2\,g\,l^{-1}$), but not in the presence of another phenolic compound, gallic acid ($2\,g\,l^{-1}$) [8.14]. Also, the attenuating impact of ethanol on the volatility and fruitiness of several wine components has been illustrated by several authors [8.13, 15]. Considering a range of volatile compounds in wine [monoterpenes (linalool, nerol), C_{13}-norisoprenoids (β-damascenone, β-ionone), and ethyl esters], *Robinson* et al. [8.15] reported an increase in volatility in the presence of glucose ($160-320\,g\,l^{-1}$) and the reverse for ethanol (> 10% vol.), whereas proline had no effect. Also, the presence of ethanol affected perceptual interaction phenomena in binary odor mixtures of isoamyl acetate and whisky lactone, [8.232]. The effect of these phenomena on the aromatic profile of wines has not yet been investigated in detail. The impact of (+)-catechin supplementation at concentrations ranging from 25 to $200\,mg\,l^{-1}$ on the aromatic profiles of three Sauvignon blanc wines was studied through triangular tests. The experiment revealed significant differences between supplemented and non-supplemented wines at concentrations between $50\,mg\,l^{-1}$ and $200\,mg\,l^{-1}$ but not at lower levels [8.233].

The perception of volatile compounds during wine tasting is not only orthonasal but also retronasal. One recent study used an in vivo approach to measure aroma compound concentrations directly in exhaled breath after wine consumption, revealing the importance of the wine matrix, particularly the presence of polyphenols, on the volatility of odoriferous compounds, such as isoamyl acetate, ethylhexanoate and linalool and also the impact of oral mucosa [8.234–236]. Further research is required to elucidate the importance of these phenomena during wine consumption.

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9. Fruits

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In a botanical sense, fruits are the developed part of the seed-containing ovary. Evolutionarily speaking, plants have developed fruits presumably to attract insects, birds, reptiles, and mammals and hence to spread the seeds. Fruits can be dry such as the pod of a pea, or fleshy such as a peach. As humans, we enjoy fleshy fruits for their flavor and nutritional value. In this chapter, we will review the common volatiles that are produced by the major fruits with commercial value: tomato, citrus, apples, and strawberries. Some volatile compounds are commonly produced by all crops, simply by the fact of common biosynthetic pathways, while other compounds are specific to certain fruit species. Fruit-specific aroma depends on species, cultivar, growing conditions, and developmental and maturity stage. In the end, however, what gives a fruit its specific flavor is the combination of volatile and nonvolatile compounds, including sugars, acids, and other water-soluble and insoluble compounds.

9.1 Fruit Volatiles

Because volatiles are derived from primary metabolites from common pathways, as described in this book, Chap. 2, many odorants are common to many fruits. However, some pathways are more active in some plant species than others, resulting in different flavor profiles. Apples and pears are characterized by a dominance of aliphatic esters, alcohols, and aldehydes, while berries (strawberries, blackberries, and raspberries) may have similar volatiles, with additional ketones or lactones that give them their characteristic flavor. Citrus fruits are usually characterized by large amounts of terpenes, but different citrus species differentiate from each other by producing more or less of other compounds: esters and sesquiterpenes are predominant in oranges relative to mandarins, sulfur compounds are found in large amounts in grapefruit, the aldehydes neral and geranial (both isomers of citral) are characteristic of lemon flavor. Tropical fruits, with a wide spectrum of genera and species, have in common sulfur compounds that give a fruity/sweet aroma when in low concentrations and combined in a matrix with high sugars/low acids such as in mango, papaya, passion fruit, durian, or guava.

9.1.1 Common Volatiles Found in Fruit

Esters

Most temperate fruits owe their fruity flavor to straight- or branched-chained esters, derived from fatty acid and amino-acid pathways, respectively (this book, Chap. 2). Esters have been reported in almost all fruits, including muskmelon [9.1], apple [9.2, 3], strawberry [9.4, 5], pineapple [9.6], oranges [9.7], to name a few. As a matter of fact, when flavorists need to add a *fruity* note to a flavor, they would first turn to esters [9.8]. Most esters have a fruity quality [9.2, 9–11], although higher concentrations may be perceived as *solvent* or *ether*, for example in the case of gas chromatography-olfactometry (GC-O) [9.11, 12], or impart a *fermented* or *spoiled fruit* and musty flavor to a fruit or juice [9.10, 13]. In

general, branched esters have a much lower threshold than their straight chain counterpart, as determined by classical odor thresholds in water [9.14] or observed by GC-O [9.15–17]. Thereby, threshold concentrations have been used by the flavor industry as indicators of the relative contribution of a compound to a food [9.8, 18, 19]. Branched chain esters with a mercapto- or hydroxyl-group were found to have higher odor thresholds than branched chain esters with methyl and ethyl groups in the acid part of the ester [9.14]; a methyl group at the 1-position of the alcohol did not have a consistent effect in increasing or decreasing thresholds [9.20]. Ester thresholds in water vary as low as 0.008 ppb (ethyl 3-methylpentanoate) to 2500 ppb (ethyl 3-hydroxybutanoate), and up to 13 500 ppb (ethyl acetate), and they all have a fruity odor [9.14, 20].

Lactones are cyclic esters with characteristic coconut or peach flavor [9.21], and were found in peach [9.22], mango [9.23], apricot [9.24, 25], and strawberries [9.26, 27]. Their odor thresholds are usually very low, and often more easily detected by GC-O than by GC-MS (gas chromatography-mass spectrometry) or GC-FID (flame ionization detector) [9.26, 27]. Wine lactone (3a,4,5,7a-tetrahydro-3,6-dimethyl-2(3H)-benzofuranone) and furaneol (2,5-dimethyl-4-hydroxy-3(2H)-furanone) are two potent lactones with a sugary/sweet odor and odor thresholds of 0.008 and 60 ppb, respectively [9.9, 28]. Wine lactone was found in fresh oranges [9.29], while furaneol is quite ubiquitous in fruit: tomatoes [9.28], strawberries [9.30], pineapples, and mangoes [9.31], to name but a few.

Aldehydes and Alcohols

Aliphatic aldehydes and alcohols are produced upon cutting or chewing plant tissue from the lipoxygenase pathway (this book, Chap. 2). Most common compounds produced are hexanal, 1-hexanol, (2*E*)-hexenal, (2*E*)-hexenol, (3*Z*)-hexenal, (3*Z*)-hexenol, all having a *green* odor, like cut grass, leaf, or woody, and with odor thresholds varying from 0.25 ppb ((3*Z*)-hexenal) to 500 ppb (1-hexanol), with the most common aldehyde, hexanal, having a threshold of 4.5 ppb [9.32]. These compounds tend to be in greater amount in unripe fruit [9.1, 33]. Straight- and longer chain aldehydes such as octanal, nonanal and decanal are present in citrus oil more than in fruit vesicles [9.34], and have typical citrus aroma [9.7]. Longer chain aldehydes, whether saturated or not, are present in citrus and have fatty or soapy characters [9.34]. Terpene aldehydes and alcohols such as neral (nerol) and geranial (geraniol) (both isomers of citral), linalool, sinensal, and citronellal (citronellol) occur in citrus juice with characteristic citrus flavors. The phenolic aldehyde, benzaldehyde, has a typical cherry or almond flavor. It is found in many fruits, including peaches [9.22, 35], strawberry [9.36], oranges [9.34], tomato [9.37], blueberry [9.38], and of course in cherries [9.39–41].

Ketones

Like alcohols and aldehydes, ketones may belong to various chemical groups. Some ketones have characteristic odors and are character impact compounds in some fruits. An example is the raspberry ketone, 4-(4-hydroxyphenyl)-butan-2-one, with an odor threshold of 0.001–0.01 ppb [9.42]. Others have very low odor thresholds and are ubiquitous in the fruit kingdom: for example, β-damascenone, α- and β-ionone are derived from carotenoid degradation when fruit ripens. β-Damascenone, with a low odor threshold, is found in mostly all fruit extracts by GC-O, without often being detected by classical GC methods. It is described as sweet, honey, tobacco, and is believed to contribute to the general fruitiness of a fruit product [9.24, 43–47]. α- and β-Ionones are found in berries [9.48], oranges [9.45], tomato [9.49], and lychee [9.50].

1-Octen-3-one and 1-octen-3-ol are two other compounds that are found in many fruits by GC-O, but instead of having a fruity or green odor, have a very characteristic mushroom odor. Their odor thresholds are 0.005 ppb and 1 ppb, respectively [9.9], and they are often detected by GC-O but not by the FID or MS detectors. Nootkatone is a terpene ketone and has a characteristic grapefruit odor [9.51, 52].

Terpenes

When talking about chemistry of terpenes, two plant groups come to mind: Labiateae, containing the mint species, and Rutaceae, containing the citrus species. Terpene biosynthesis has been studied in both groups, as they have important economic values [9.53, 54]. Terpenes occur not only in citrus, but are found in many tropical fruits and berries. Terpenes were detected in blueberries [9.55], blackberries [9.56], strawberries [9.36], and mango [9.57, 58]. In general, the high molecular weight sesquiterpenes have little odor in comparison with the low molecular weight and highly volatile monoterpenes [9.59]. Depending on their oxygenation level, their odor threshold and quality might vary considerably. Limonene is the main volatile produced by citrus fruit, but only accounts for a fraction of the odor activity. On the contrary, the oxygenated linalool has a characteristic citrus and floral odor. Odor thresholds in a deodorized orange juice matrix for both compounds are 13 700 and 113 ppb, respectively [9.60].

Table 9.1 Odor-active sulfur volatile compounds detected by gas GC-O in selected tropical and subtropical fruit

Fruit	Compound	Odor characteristic	Reference
Lychee	Hydrogen sulfide	Sulfur, fetid	[9.50]
	Dimethyl sulfide	Cabbage	
	Diethyl disulfide	Moldy, sulfur	
	2-Methyl thiazole	Garlic	
	2,4-Dithiopentane	Burning tire, cabbage	
	Dimethyl trisulfide	Cabbage, sulfur	
	Methional	Cooked potato	
	2-Acetyl-2-thiazoline	Dry fruit, nutty	
Mango	2-Methyl-3-furanthiol	Nutty, medicinal, oily	[9.61]
	4-Mercapto-4-methyl penten-2-ol	Green, grapefruit	
Guava	Methanethiol	Sulfury	[9.61]
	2-Methyl-3-furanthiol	Nutty, roasted	
	Mercaptomethylbutyl formate	Cat urine, sweet	
	4-Mercapto-4-methylpentan-2-ol	Grapefruit	
	3-Mercapto hexanol	Grapefruit, sulfury, tropical	
Strawberry	Methyl thioacetate	Sulfurous, cheesy	[9.62]
	Methyl thiobutanoate	Sulfury, cheesy, cabbage	
Melon	Methyl 2-(methylthio)acetate	Baked potato, pungent, rancid	[9.17]
	Ethyl 3-(methylthio)propanoate	Clean, fresh, melon	
Muskmelon	Ethyl (methylthio)acetate	Cucumber	[9.63]
	Ethyl 3-(methylthio)propanoate	Fruity, green	
Orange	Methional	Cooked potato	[9.29]
	p-Menth-1-ene-8-thiol	Grapefruit-like	

Sulfur Compounds

Sulfur-containing compounds are usually very potent with low odor thresholds, their odor quality varies with concentration [9.64, 65], and they are very labile and difficult to separate from other compounds for analysis [9.34]. Some tropical fruits, such as durian, are well-known for their sulfury, garlic, or onion-like odors, combined with sweet flavor; indeed, 24 sulfur-like compounds were detected by GC-O in three durian cultivars [9.66]. *Mahattanatawee* et al. [9.61] detected seven and two sulfur-like components in guava and mango, respectively, and 11 compounds in lychee [9.50] (Table 9.1). *Du* et al. [9.67] identified 19 volatile sulfur compounds in 12 Florida strawberry cultivars, with methanethiol and methyl thioacetate being the most abundant and with the presumably highest odor activity. Methyl thioacetate and methyl thiobutanoate, produced in the largest amount in Strawberry Festival [9.67], were detected by GC-O in this cultivar and in Florida Radiance [9.62] (both are strawberry cultivars) (Table 9.1). Thioesters are the type of sulfur compounds produced in melons [9.68–70] and some were found to be important contributors to the characteristic melon note [9.17, 63, 71] (Table 9.1). In citrus, most sulfur compounds occur in grapefruit [9.72], with 4-mercapto-4-methylpentan-2-one and 1-*p*-menthene-8-thiol contributing to the typical sulfurous, grapefruit-like odor [9.73]. However, a few sulfur compounds were reported in fresh squeezed oranges [9.29, 74] (Table 9.1) and in tangerine [9.59], although none were identified in the latter research. Even though consumed as a vegetable, tomato is a fruit and its flavor has been widely studied. Isobutylthiazole is a sulfur compound with important contribution to tomato flavor [9.75] and contributes to the *viney*, *green*, *earthy*, and *musty* descriptors [9.37].

9.1.2 Effect of Genetic and Environmental Factors

While each fruit species has its own flavor signature, flavor types may greatly vary within a species. For instance, within the sweet orange species, *Citrus sinensis*, the blood orange types are very different from blond oranges commercially used in orange juice (*Valencia*, *Hamlin*) [9.76] (Fig. 9.1). Not only the volatile profile is different, but also blood oranges are the only types of oranges that produce anthocyanins. Generations of breeding for growing fruit adapted to certain climatic zones have brought about commercial strawberries (*Fragaria* × *ananassa* Duch.) that have quite different volatile profiles between those grown in subtropical Florida and those grown in temperate European climates [9.62]. To further complicate the matter, interspecific breeding has resulted in new species and continues to be a source of diversity for modern breed-

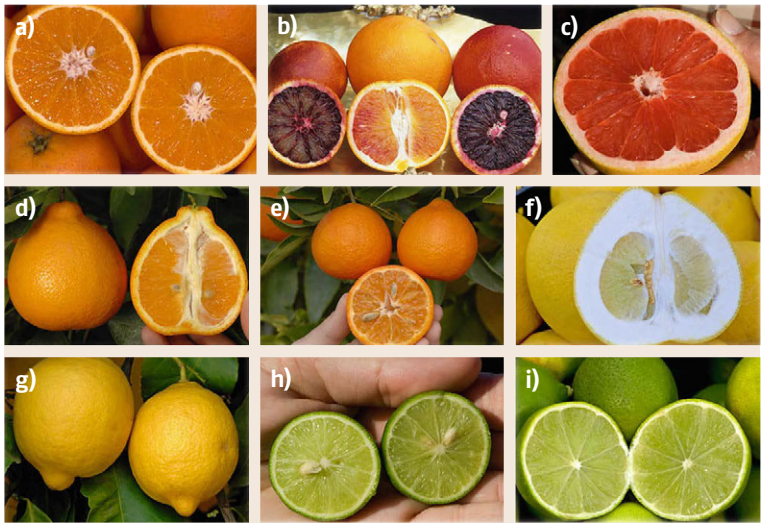

Fig. 9.1a–i Diversity in the genus *Citrus*: **(a)** Valencia orange (*Citrus sinensis* [L.] Osbeck); **(b)** Blood oranges (*Citrus sinensis* [L.] Osbeck); **(c)** Star Ruby grapefruit (*Citrus paradisi* Macf.); **(d)** Minneola tangelo (hybrid of Duncan grapefruit and Dancy mandarin); **(e)** Mandarin, *Algerian clementine* (*Citrus clementina* hort. Ex Tanaka); **(f)** Reinking pummelo (*Citrus maxima* (Burm.) Merr.); **(g)** Foothill Lisbon lemon (*Citrus limon* [L.] Burm. f.); **(h)** *Mexican* lime (or *Key* lime) (*Citrus aurantiifolia* [Christm.]); **(i)** *Bearss* lime (*Citrus latifolia* Tan.), Photos courtesy of David Karp, University of California, Riverside

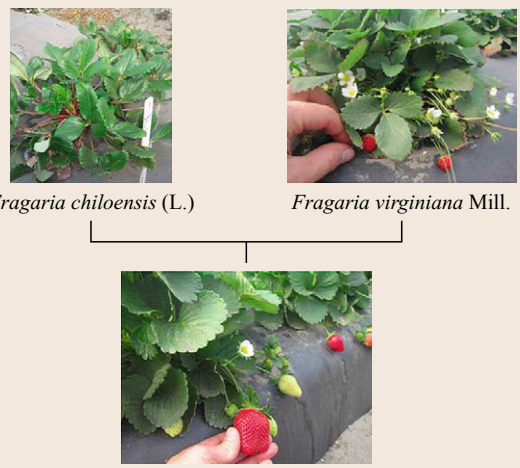

Fig. 9.2 Modern commercial strawberry, *Fragaria* × *ananassa* Duch., and its parents, *Fragaria chiloensis* (L.) Mill. and *Fragaria virginiana* Mill. Photos courtesy of Vance Whitaker, University of Florida

ers. For example, the modern cultivated strawberry, *Fragaria* × *ananassa* Duch., is the result of spontaneous hybridization between *Fragaria chiloensis* (L.) Mill. and *Fragaria virginiana* Mill. [9.77] (Fig. 9.2). *F.* × *ananassa* inherited its large size from *F. chiloensis* and its bright red color from *F. virginiana*. However, breeding for commercial production has emphasized traits such as fruit size, disease resistance, and ability to withstand handling and transportation, and as a result, flavor diversity has narrowed. This effect is observed over and over in many crops, including tomatoes, apples, and strawberries. For strawberries, current research interest is in reintroducing methyl anthranilate, a sweet and grape-like volatile component, in new cultivars. Methyl anthranilate is only produced in wild-type species of *F. vesca* and *F. moschata* [9.77]. New varieties (or cultivars) are released when the traits remain stable over several years. For example, the strawberry selection *FL-00-51* was always sweeter and produced more esters and lactones than other selections, regardless of weather pattern [9.36]. That selection has been released with the name of *Florida Elyana* [9.78].

Once new varieties are released and adopted commercially, growers learn how to optimize their growing conditions. Maturity is an important factor affecting fruit quality, and harvest maturity is crucial in determining the storage potential of some fruit such as tomatoes, apples, and pears. Fruit accumulate sugars and acids as they develop, and acids usually decrease upon ripening. Volatiles also change accordingly. Volatile changes with fruit ripening, together with sugars and acids, were documented in tomatoes [9.79, 80], oranges [9.81], apples [9.33, 82], and strawberries [9.67, 83, 84].

Handling and storage also affect fruit volatile production. In apples and tomatoes, reducing fruit respiration rate after harvest maintains fruit firmness and acidity, but lower volatile production ensues. Lower respiration rate is traditionally achieved with cold storage, with the risk however to induce chilling injury, especially in tomatoes that are susceptible to temperatures below 10–13 °C. One of the symptoms of chilling injury in tomatoes is reduction of volatile production [9.37, 75]. Reducing metabolic activity has commercially been done using controlled atmosphere storage in apples, where atmospheric oxygen is reduced

to 1–2% and carbon dioxide increased to 1–2%. Nowadays, the ripening inhibitor 1-methylcyclopropene (1-MCP) has accomplished the same purpose. For each technique, there is a compromise between extending fruit visual qualities in storage and optimizing eating quality [9.85].

Processing also induces changes in fruit volatile profile. Disrupting fruit cells release enzymes that are otherwise membrane bound. Further heating for pasteurizing may release volatiles that are glycosylated. C6 aldehydes and alcohols are typical volatiles that are released upon disruption of the plant cell. β-Damascenone is an example of compound released upon heating and only found in processed tomatoes [9.86]. Reviews of the effect of processing on tomato and orange flavor have been published [9.87, 88].

9.2 Impact of Volatiles on Fruit Flavor

Flavor is an important component of internal fruit quality and volatiles are responsible for specific fruity characters. However, it is the interaction of volatiles with nonvolatile compounds that truly contributes to the sensory perception of a food. *Baldwin* et al. [9.37] showed how some volatiles could increase or decrease the perception of sweet and, sour taste in fresh tomatoes. Sweetness and sourness in fruit are due to the presence of sugars and acids, with minerals that may affect buffering capacity and modulate perception of sourness [9.89]. Phenolic compounds, anthocyanins, alkaloids, and amino acids may elicit other sensory responses such as astringency, bitterness, and umami. The presence of long-chain polysaccharides, such as pectin and other cell wall polysaccharides, can affect the mouth feel of a fruit or fruit beverage. All these nonvolatile compounds interact with aroma volatile compounds within the intact fruit, and in the mouth upon chewing, which determines the overall sensation of aroma and taste, which comprise flavor.

9.2.1 Volatile Analysis of Fruit Commodities

Fresh produce, both fruits and vegetables, once removed from the plant, continue to undergo metabolic activities. Choosing the appropriate time for sampling is critical for flavor analyses: volatiles fluctuate during maturation, new volatiles are produced while others are metabolized, and they interact with nonvolatile components (sugars, acids, phenolics) which also change upon fruit maturation. Further, fruit-to-fruit variation makes flavor studies complicated, and sampling criteria must be rigorous to minimize this variation [9.90]. Sample preparation is also critical, as once a fruit is cut, homogenized, or macerated, enzymatic reactions occur that can affect flavor components, and need to be controlled, depending on the purpose of the study [9.91]. One well-known chain of enzymatic reactions that occur upon cutting, macerating or blending plant tissue results in C-6 aldehydes formed from oxidation of lipids (this book, Chap. 2). *Buttery* and co-workers [9.87] proposed adding an equal volume of saturated salt (sodium chloride or calcium chloride) to the homogenate in order to denature enzymes. In our laboratory, we found that sodium chloride was a better denaturant than calcium chloride for some fruit that contained high levels of pectin such as mango, apple or strawberry. The calcium ion from calcium chloride bonded with pectin, making a gel, and retarded the release of volatiles into the headspace to varying degrees, depending on the amount of pectin in the fruit [9.58]. Other methods, such as using solvent extraction, vacuum distillation, or heat would achieve the same purpose of stopping enzyme activity, each method having its advantages and disadvantages. Many reviews and book chapters are written about sampling and analytical techniques for volatile analysis [9.92, 93]. When using solvent extraction, it is usually best to compare the odor of the extract after removing the solvent, with the initial sample [9.94]. Direct GC-O, where the sample extract does not go through an analytical column and is directly sniffed by a panel and compared to the original sample, has been useful to optimize extraction techniques of headspace volatiles by direct headspace or solid phase microextraction (SPME) [9.95–98].

Once volatiles are identified analytically, contribution of compounds to the food flavor has traditionally been determined by two approaches: threshold-based and GC-O. Correlations and models to predict sensory quality from volatile compounds can be further developed using sensory evaluation.

9.2.2 Specific Sensory Tests

Thresholds

Contribution of volatile compounds to a food flavor can be estimated after calculating the compound concentration in the food divided by its odor threshold to determine its odor activity value (OAV) or odor unit [9.18, 19]. Compounds that are present in a food at a concentration greater than their threshold are deemed to contribute to that food flavor. Volatile thresh-

olds have been compiled in several databases [9.9, 99], and have been helpful to flavorists and food scientists to formulate new flavors [9.8]. By using the same methodology [9.100], researchers at the United States Departement of Agriculture (USDA), Agriculture Research Service (ARS), Western Regional Research Center have measured thresholds of hundreds of pure compounds [9.101], which have been extensively used to determine volatile contributions to fruit and food products from GC data. However, odor thresholds vary with the dilutant in which the compound is prepared [9.13, 60, 102]. Due to interactions with larger nonvolatile molecules, odorant thresholds are usually higher in complex matrices than in water: they were about twice as much and up to 600 times higher in a deodorized apple juice or orange juice matrix [9.13, 60]. While knowing the threshold value in a matrix has helped reassess the contribution of certain odor volatiles in orange juice [9.10, 60], measuring thresholds of flavor compounds is time consuming. Also, data are more robust when they are obtained with the same panelists, using the same method. Therefore, *Cliff* et al. [9.13] proposed a model to estimate thresholds in apple or orange juice matrices based on known thresholds in water, and current knowledge of thresholds in the juice matrices. These data and approach would be useful to flavorists and food scientists in quality control and research and development for the beverage industry including orange and apple juice.

GC-O

Researchers have smelled the effluent of the gas chromatograph since the inception of gas chromatography. However, very soon, two approaches considering the human factor were developed: (1) using thresholds, where the sample is diluted and sniffed repeatedly until no odor is perceived (aroma extraction dilution analysis AEDA, [9.103]; and CharmAnalysis analysis, [9.104]), and (2) using the dose–response characteristics of each compound (*Osme* the Greek word for smell, [9.105]). Having criticized these techniques for the small number of panelists used to determine odors in a GC run (one to four panelists), other authors developed the detection frequency analysis, using up to 10 panelists [9.106, 107]. Studies comparing different methods showed similarities between them in the overall results [9.108–110], with some differences observed in detection reproducibility or discrimination between levels of compounds sniffed [9.108, 110]. An excellent critical review of the methods is given by *Delahunty* et al. [9.111]. In the end, *Grosch* [9.112] demonstrates the necessity of reconstituting the sample with the odorants having odor activity in order to confirm their contribution to the food. For the study of fruit flavors, AEDA followed by the reconstitution model was published for strawberries [9.5], grapefruit [9.73], oranges [9.29], and apricot [9.24]. Reconstitution of fruit flavor based on thresholds and odor activity of volatiles in the food of interest were published for apples [9.15], orange juice [9.113], and guava [9.114].

Another way to interpret GC-O data is by comparing statistical analyses of both GC-O and sensory data, when GC-O data are amenable to statistical analysis (for instance as in the OSME or the frequency detection methods). *Miyazaki* et al. [9.59] compared OSME profiles of five tangerine juices with their sensory characteristics by a trained panel: they found that the hybrid with the highest amount of esters and β-damascenone, all compounds having fruity odors, was characterized as having an orange-like flavor by a taste panel. Conversely, a hybrid with the most GC-O descriptors with citrus/fruity, terpeney, and metallic/rubber categories had a typical tangerine flavor with sulfury and woody notes, as described by a taste panel. More complex statistical analyses may be used for interpreting GC-O data. Partial least square (PLS) regressions [9.115, 116] with sensory as a dependent variable and GC-O peak intensity as explanatory variables were used to model strawberry [9.27] or wine [9.117] flavor. Alternatively, PLS regressions could be performed using the quantified volatiles with olfactory qualities to explain sensory data [9.118–120].

9.2.3 Effect of Nonvolatile Compounds

Eating a fruit involves the experience of texture and taste. While biting into a fruit gives the first mouthfeel impression (juiciness, crunchiness, hardness, softness), the volatile components are released upon biting, then chewing the pulp. Besides the physico-chemical interactions between volatile and nonvolatile components in a fruit or fruit juice, interactions exist at the cognitive level; the brain processes information from stimuli received through the mouth and the olfactory bulb (retronasally) to produce flavor [9.121]. Studies pairing taste and odor stimuli showed that the perception of sweetness from a sweet tastant (sucrose or saccharine) could be enhanced by the presence of a volatile with fruity or sweet odor [9.122–124]. In a series of studies, it was shown that volatile perception in the mouth could be enhanced in the presence of a congruent taste stimulus (such as a volatile associated with fruitiness presented together with sugar) [9.124–126]. When the same volatile was presented without any tastant, it was mostly perceived in the nasal cavity. Studies with complex mixtures of fruit drinks or fruit puree also showed that increasing sugar (sucrose and/or glucose) generally increased the perception of aromatics associated with

fruity/ripe fruit flavor [9.127–129]. However, increasing acidity (with citric acid) had inconsistent results, depending on the study: citric acid increased orange peel flavor in a mango puree [9.127]; it increased *apple cider* but decreased *pear-candy* flavors in an apple drink, and increased grapefruit and lemon flavor in an orange drink [9.128]; and it increased citrus flavor and decreased fruity flavors in a tomato puree [9.129].

Sugars (sucrose, glucose, fructose) and acids (citric in citrus, malic in apples, tartaric in grape) are the main nonvolatiles driving fruit eating quality, with the proper balance of sugars and acids indicating the ripeness level. But also bitter components play an important role in citrus. For example, the flavanone glucoside, naringin, is the main bitter component in grapefruit. Limonin is a bitter limonoid known to increase in some cultivars, such as *Navel*, upon disrupting the vesicles in the juicing process [9.130], and has recently become a problem in juice made from oranges affected by the greening disease due to *Candidatus* Liberibacter asiaticus [9.131–133]. Bitterness was not perceived in juice made from *Valencia* oranges because the limonin level was low, but also likely because the total content of volatiles in that cultivar is much greater than in earlier season cultivars, possibly helping to mask the bitterness. Juice processors are well aware of the effects of volatiles on the taste perception of drinks, and may be able to adjust flavors to modulate taste perception. Finally, polysaccharides such as pectin have an effect on volatile release, and effect flavor perception. This was shown with mango puree [9.127] and orange juice [9.134].

9.3 Flavor of Specific Fruits

Tomato is by far the most produced fruit crop worldwide, followed by citrus, all species combined. World tomato and citrus fruit annual productions were 140−160 and 115−118 million tons, respectively, between 2008 and 2012 [9.135]. Both crops are consumed fresh or processed in can, paste, juice, and concentrate. On the other hand, the most popular fruit flavors for use in food or pharmaceutical preparations are strawberry, citrus (orange and lemon) and apple. The flavor of these specific fruits is reviewed in this section.

9.3.1 Tomato

Fresh market tomatoes sold in supermarkets garner much criticism concerning their lack of flavor or presence of off-flavor. Consumers find that fresh tomatoes lack that *homegrown* taste [9.136] and are willing to pay a premium price for full-flavored tomatoes [9.136]. Tomatoes have a characteristic sweet-sour flavor accompanied by a complex mix of aromatic volatiles that interact with the sugars and acids [9.137]. Unfortunately, most tomatoes on the market today are bland in aroma, and the aroma present is generally green, viney, earthy, and musty, while low in fruity and floral notes [9.138]. Domestication events in Central America and Europe created genetic bottlenecks that led to a low level of diversity within the cultivated tomato [9.139, 140]. Thus, lack of flavor is likely due to the lack of genetic flavor diversity as well as the fact that it is difficult for breeders to select for this complex trait. Then environmental conditions during plant cultivation, harvest practices that do not allow the fruit to initiate ripening on the mother plant [9.80, 141], and postharvest practices that include chilling the fruit to prolong stored shelf life [9.80, 142, 143] all can contribute to poor or bland flavor. Genetics are the most important as this holds the flavor potential for a variety; however, the full flavor potential inherent in the genetics of a tomato may not be realized due to less than optimal environmental conditions, harvest maturity, and postharvest handling practices etc. Nevertheless, if the genetic potential for good flavor is not there, then no amount of careful cultivation, harvesting, and handling can make up for the lack of genetic flavor potential. For this reason, tomato breeders are adding flavor analysis to their screening programs for advanced lines to compare with standard commercial cultivars using sensory consumer panels combined with chemical analyses of flavor compounds. One success story has been the recent release of *Tasti-Lee*, now popular in supermarkets, that was bred for hot Florida conditions [9.144], the state in the US where most fresh tomatoes are grown. This tomato is also harvested with some red color to avoid the green tomato harvest that results in *immature green* tomatoes that never ripen with good quality [9.80, 141].

Of the hundreds of volatiles present in tomatoes, odor threshold studies have identified around 30 to be important contributors to fresh tomato flavor based on the odor unit concept [9.75, 87, 145] (Table 9.2). Studies spiking deodorized tomato homogenate with volatile standards with or without added sugars and acids determined the important contribution of various volatiles, known to be present in tomatoes, to overall flavor [9.37, 129]. Among them and consistent with earlier studies, β-ionone, furaneol, 6-methyl-5-hepten-2-one, and geranylacetone contributed to sweet and

fruity flavor, while C6 aldehydes, isobutylthiazole, *E*,*E*-2,4-decadienal contributed to earthy and green/viney flavor. Other approaches to determine which volatiles contribute to specific sensory descriptors or consumer liking include tasting fruit with a wide range of flavors and modeling the response with chemical components [9.49, 140, 146]. Some fruity/floral volatiles were found to enhance the perception of sweetness, whereas other volatiles associated with green notes enhanced perception of sourness [9.49]. Volatiles derived from carotenoids contributed to overall tomato flavor: geranial, geranylacetone, 6-methyl-5-hepten-2-one and β-ionone, confirmed by tasting tomatoes lacking this specific group of compounds [9.140]. In another study, spiking tomato homogenate with either sugar or acid found that, generally the sweeter the tomato, the more acceptable, whereas the acceptability of acid concentration reached a peak in acceptability and then declined, depending on the amount of sugar present [9.147].

9.3.2 Citrus

Citrus fruits are produced in most of the subtropical and tropical regions of the world and provide many uses: fresh fruit, juice, canned mandarin/orange segments, marmalade, even the peel can be used in spices and condiments. Further, citrus peel oils have their own market and are widely used in the fragrance and flavor industries, as well as having industrial applications as solvents, paint, resins, etc. Contrary to all other fleshy fruits that produce volatiles in the cell vacuoles, citrus fruits have a largely developed peel that contains the oil glands. Consumers experience citrus flavor when they peel a fresh fruit, hereby splashing the peel oil into the atmosphere and transferring it to the edible segments, and by incidentally or purposely adding peel oil to the juice. Volatiles contributing from peel oil are usually water insoluble and very rich in the monoterpene limonene: 90–96% in sweet orange (*Citrus* × *sinensis* [L.] Osbeck), grapefruit (*C.* × *paradisi* Macfayden), and bitter orange (*C.* × *aurantium* L.); 60–80% in lemon (*C.* × *limon* [L.] Burm); 49–60% in lime (*C. aurantifolia* [Christm.] Swing., and *C. Latifolia* Tan.) and 23–50% in bergamot (*C. bergamia* [Risso] Wright & Am) [9.148]. Cultivars of mandarin/tangerine (*C. reticulata* Blanco) are numerous and present a great diversity of volatile components in peel oil, some contain as high as 90–96%, and others contain as low as 60–80% of limonene [9.148]. Other abundant components include β-pinene (high in lemon, lime, and bergamot); γ-terpinene (high in lemon, lime, bergamot and mandarin); citral (mixture of isomers of geranial and neral, high in lemon and lime); linalool and linalyl acetate (both predominant in bergamot); valencene and α-sinensal (high in bitter and sweet oranges, clementine and mandarin); β-sinensal (high in bitter and sweet oranges, clementine and grapefruit); nootkatone (high in bitter orange and grapefruit, and trace in lemon and bergamot); δ-3-carene (a key component at trace level in most of the citrus oils except sweet orange); and methyl N-methylanthranilate, thymol, and its methyl ether (only in mandarin/tangerine) [9.148]. For most citrus, peel oil is a by-product of juice processing, such as in orange and grapefruit, while some citrus are cultivated exclusively for their peel oil, such as bergamot and bitter orange. Not only being used for food and drink, citrus peel oils are also widely used in cosmetics, perfumes, detergents, body care products, and pharmaceutical preparations [9.149]. The most recent compilation of citrus oil composition and analytical techniques can be found in [9.150].

In addition to sweetness and sourness, bitterness is an important component of flavor in some citrus such as grapefruit or bitter orange. Bitterness may be brought out by limonoids or flavonoids, depending on the sugar moiety attached to the aglycone structure. Naringin in grapefruit and sour orange, as well as neoeriocitrin, neohesperidin, and poncirin in sour orange are all neohesperidose flavanone glycosides [9.151, 152]. Limonoids (limonin and nomilin) are bitter compounds that impart bitterness to orange juice made with some cultivars such as *Navel* [9.130], or to juice made with fruit affected by the citrus greening disease [9.132]. Both, flavonoids and limonoids, are mainly present in the fruit peel. From the taste features, citrus can be easily divided into four types: *sweet and sour* sweet oranges, *sweet* mandarins (low acid), *mild sour with variable bitter taste* grapefruit, *acidless* sweet limes, and *sour* lemons and limes [9.153]. Volatile compounds may increase perception of sweetness or bitterness when present above threshold in a juice matrix [9.10]. However, modulating sweetness or bitterness with volatile compounds such as in tomato [9.37] has not been published, and may only be a trade secret of juice processing companies.

Fruity, citrus, tropical (mango, passion fruit), fruity-non-citrus (apricot, peach), floral, terpeney, chemical, green/grassy, fatty, soapy, metallic, herbal, and mushroom are most often used in GC-O and sensory descriptions for citrus aroma [9.10, 34, 154]. They are the result of volatile terpene hydrocarbons, aldehydes, alcohols, esters, ketones, sulfur compounds, and other minor components in the juice sacs and oil glands.

Oranges and mandarin/tangerine flavor consists of more than 200 or 300 volatiles, respectively, although only about 36–49 volatiles had aroma activity and essentially contributed to citrus odor [9.34]. There is not just one or few specific character im-

Table 9.2 Concentration and odor thresholds of major components in fresh ripe tomatoes (after [9.87])

Compound	Concentration (ppb)	Odor threshold (ppb in water)	Odor activity value[a]	Log odor units[b]
(*Z*)-3-Hexenal	12 000	0.25	48 000	4.7
β-Ionone	4	0.007	571	2.8
Hexanal	3100	4.5	689	2.8
β-Damascenone	1	0.002	500	2.7
1-Penten-3-one	520	1	520	2.7
3-Methylbutanal	27	0.2	135	2.1
(*E*)-2-Hexenal	270	17	16	1.2
2-Isobutylthiazole	36	3.5	10	1.0
1-Nitro-2-phenylethane	17	2	8.5	0.9
(*E*)-2-Heptenal	60	13	4.6	0.7
Phenylacetaldehyde	15	4	3.8	0.6
6-Methyl-5-hepten-2-one	130	50	2.6	0.4
(*Z*)-3-Hexenol	150	70	2.1	0.3
2-Phenylethanol	1900	1000	1.9	0.3
3-Methylbutanol	380	250	1.5	0.2
Methyl salicilate	48	40	1.2	0.1
Geranylacetone	57	60	0.95	0.0
β-Cyclocitral	3	5	0.60	−0.2
1-Nitro-3-methylbutane	59	150	0.39	−0.4
Geranial	12	32	0.38	−0.4
Linalool	2	6	0.33	−0.5
1-Penten-3-ol	110	400	0.28	−0.6
(*E*)-2-Pentenal	140	1500	0.093	−1.0
Neral	2	30	0.067	−1.2
Pentanol	120	4000	0.030	−1.5
Pseudoionone	10	800	0.013	−1.9
Isobutyl cyanide	13	1000	0.013	−1.9
Hexanol	7	500	0.014	−1.9
Epoxy-β-ionone	1	100	0.010	−2.0

[a]Odor activity value = compound concentration/compound odor threshold.
[b]Log odor units = log OAV (odor activity value) The exact concentration and log odor units are uncertain.

pact compound(s) imparting typical orange or mandarin/tangerine flavor, but instead a combination of multiple different volatiles [9.7, 59, 155]. In fresh *Valencia* and *Navel* oranges, the two main commercial cultivars, ethyl butanoate, ethyl 2-methylpropanoate, and (*S*)-ethyl 2-methylbutanoate were the esters with the greatest aroma activity by GC-O, together with wine lactone, (*Z*)-3-hexenal, and decanal [9.29], while in blood oranges, limonene, linalool, and nootkatone had the greatest concentration to odor threshold value (odor activity value) [9.156]. Granted that the differences between these two studies may be due to sample preparation, blood oranges have quite a different flavor than blond oranges (*Navel* and *Valencia*). In another study, methyl butanoate and ethyl octanoate were detected only in the blood orange cultivars (*Moro* and *Tarocco*) by GC-O, while linalool was only in the blond oranges (*Washington Navel* and *Valencia Late*) [9.76]. *Miyazaki*, et al.'s [9.157] data show that tangerine hybrids with sweet orange in their pedigree contain more esters and sesquiterpenes than mandarins/tangerines. Other generally recognized contributors to orange juices, are decanal (green, fatty, soapy), nonanal (floral, citrus), and (*Z*)-3-hexenal; (*S*)-ethyl-2-methylbutaboate (fruity), α-pinene (pine tree), β-myrcene (metallic, geranium-like), acetaldehyde (pungent, ethereal), and β-damascenone (cooked apple) [9.34]. Usually, commercially processed oranges contain more peel oil and, thus, more volatiles associated with peel oil flavor such as terpenes (α-pinene, myrcene, limonene, valencene) and straight chain aldehydes (octanal, nonanal and decanal) in contrast with hand squeezed juice or fresh fruit volatiles that are richer in esters, short chain aldehydes (C2 to C7) and ethanol [9.88, 158].

Volatiles reported across studies in mandarins/tangerines (easy to peel) include linalool, α-terpineol (floral), terpinen-4-ol (woody, earthy), nonanal, decanal, carvone (spearmint, caraway), limonene, α-pinene, and β-myrcene [9.159]. Although thymol and

methyl-N-methylanthranilate differentiate mandarins/tangerines from oranges and may play important roles in flavor of some mandarins/tangerines [9.160], no typical volatile production pattern was found in mandarin/tangerine flavor [9.59, 161, 162].

Unlike oranges and mandarines/tangerines, grapefruit juice consists of a few character impact compounds – nootkatone, 1-*p*-menthene-8-thiol and 4-mercapto-4-methylpentan-2-one [9.51, 73, 163], which provide typical grapefruit-like odor. Other major aroma contributors include ethyl 2-methylpropanoate, ethyl butanoate, (*S*)-ethyl 2-methylbutanoate, wine lactone (sweet note), (*Z*)-3-hexenal, and (*E*)-4, 5-epoxy-(*E*)-2-decenal [9.72].

Citral is considered as a key character impact compound of lemon odor [9.164], and other major constituents are limonene, α-terpineol, terpinen-4-ol, neral, geranial, neryl acetate, geranyl acetate, linalool, and 2-methyl-3-buten-2-ol [9.165, 166].

Harvest maturity and postharvest storage affect fruit flavors. Citrus fruit usually have wide harvest windows, from a few weeks to a few months. After reaching harvest maturity, sugars keep increasing slowly and acids decrease quite fast over the harvest season [9.81]. *Bai* and co-authors [9.81] observed that *Valencia* oranges harvested in April and May had more terpene hydrocarbons, aldehydes, and esters and total volatiles in comparison with fruits harvested in February and March. Compounds that increased dramatically were β-myrcene, γ-terpinene, valencene, ethyl butanoate, ethyl acetate, hexanal, (*Z*)-3-hexenal, octanal, nonanal, geranial, and terpinen-4-ol. Meanwhile linalool, (*E*)-2-octenal, and β-ionone decreased in late harvested fruit. Total and important volatiles decreased after reaching a peak in April [9.81].

Numerous storage experiments of mandarins show that flavor quality of fruit decline for the following reasons: decrease in acidity and unbalanced sugar:acid ratio, decrease in typical aroma components, and accumulation of fermentation related off-flavor [9.34]. According to *Tietel* et al. [9.167] and *Obenland* et al. [9.168], many volatiles except acetaldehyde, ethanol, and ethyl esters, especially terpene hydrocarbons, terpene alcohols, and aldehydes which impart pleasant, desirable, fruity and citrus-like notes, decrease during storage. In contrast, volatiles derived from the ethanol fermentation metabolism and amino acid and fatty acid catabolism pathways, significantly increased during storage.

9.3.3 Apples

Apple is the third fruit produced worldwide (70–75 million metric tons, 2008–2012) after tomato and banana, and is in par with orange and table grape production [9.135]. Apple flavor has been extensively studied in the 1960s and 1970s. Initial studies aimed at identifying volatile compounds and their contribution to apple flavor [9.3, 44, 169–171]. Later, volatile profiling according to cultivar, or volatile production in fresh fruit as affected by maturity and storage were the emphasis of apple research [9.12, 33, 172–176], together with understanding the biochemical control of production of specific volatiles or volatile classes [9.177–182]. Current research aims at understanding the genetic control of volatile production [9.183–187] (Fig. 9.3).

Apple flavor is a typical example where the flavor is the combination of many volatiles without a specific character impact compound. Esters are particularly well represented in analysis of volatiles emitted by this fruit. In reviews of apple flavor, *Paillard* [9.2] listed 92 esters and *Yahia* [9.190] more than 100. Esters can account for 78–92% of the total volatiles adsorbed by activated charcoal [9.3, 191, 192]. However, the amount and type of esters depend on apple cultivar. *Paillard* [9.3] classi-

Fig. 9.3 Commercial apples (*Malus domestica* Borg): *Golden Delicious*, *Gala*, *Granny Smith*, *Red Delicious*. Photo courtesy of Scott Bauer, United States Department of Agriculture, Agricultural Research Service

Table 9.3 Volatile esters produced in whole apples at harvest (Granny Smith[a] [9.188]; Fuji [9.189]), two weeks after harvest (Golden Delicious Granny Smith[b] [9.187], or after 2 (Gala, [9.12] or 6 (Bisbee Delicious [9.174]) months in air storage[z].

	Bisbee Delicious	Gala	Golden Delicious	Granny Smith[b]	Granny Smith[a]	Fuji
Ethyl acetate	1.72		1.15	nd	23.27	4.78
Propyl acetate	2.82	10.60	6.42	nd	82.90	1.39
Butyl acetate	81.85	100.00	100.00	9.46	46.53	8.96
Pentyl acetate	6.68	9.15	5.31	2.70	20.03	1.49
Hexyl acetate	58.22	75.91	53.09	100.00	14.95	9.86
Ethyl propanoate			nd	1.35	100.00	
Propyl propanoate	1.02	0.47	0.38	2.70		
t-Butyl propanoate					16.80	2.39
Butyl propanoate	3.37	17.90	2.72	10.81		7.87
Hexyl propanoate	2.54	13.91	3.58	13.51		1.00
Ethyl butanoate	13.80		nd	12.16	nd	tr
Propyl butanoate		1.04		9.46		
Butyl butanoate	1.73	13.89	4.69	4.05	nd	1.49
Pentyl butanoate		0.54				
Hexyl butanoate	1.22	11.11	2.10	24.32	nd	1.00
Ethyl hexanoate	2.18		nd	2.70	nd	nd
Propyl hexanoate	4.28	4.64	0.17	14.86		
Butyl hexanoate	3.04	33.94	1.98	17.57		tr
Hexyl hexanoate	nd	41.71	0.37	1.35		
Butyl heptanoate		3.50				
Ethyl octanoate	3.63					
Hexyl octanoate		1.63				
2-Methylpropyl acetate	4.35	2.56	1.11	nd	38.21	2.39
2-Methylbutyl acetate	100.00	54.40	54.32	1.35	7.86	100.00
2-Methylpropyl propanoate						1.10
2-Methylbutyl propanoate						2.49
3-Methylbutyl propanoate		0.67				
Butyl 2-methylpropanoate		0.36				
Hexyl 2-methylpropanoate		0.65				
2-Methylbutyl butanoate		0.23				
Methyl-2-methyl butanoate		1.55		nd		
Ethyl 2-methylbutanoate	19.55	0.13	0.01	5.41	16.49	66.83
Propyl 2-methylbutanoate		2.95				
Butyl 2-methylbutanoate	2.76	18.76	4.07	2.70		3.29
Hexyl 2-methylbutanoate	1.86	22.62	3.83	2.70	12.94	1.00
2-Methylbutyl 2-methylbutanoate		0.05	2.70			
3-Methylbutyl hexanoate		0.60				

[z]In all studies, 1–2 kg of whole apple volatiles were sampled using the dynamic headspace method. Values are standardized to the major peak produced, which was given a value of 100. nd = not detected. tr = trace.

fied cultivars grown in France according to the predominant esters emitted by the fruit: acetate esters (*Calville blanc*, *Golden Delicious*), butanoate esters (*Canada Blanc*, *Belle de Boskoop*), while other cultivars produced an equal amount of acetate and butanoate esters. In comparing publications with similar sampling methods, *Golden Delicious* and *Gala* apples, two related cultivars (*Gala* is a hybrid of multiple crosses that have *Golden Delicious* as a parent, [9.193]), produced mainly butyl acetate, hexyl acetate, and 2-methylbutyl acetate [9.12, 187] (Table 9.3). Likewise, *Bisbee Delicious* produced, in decreasing order, 2-methylbutyl acetate, butyl acetate, and hexyl acetate (Table 9.3). In contrast, *Granny Smith* apple produces very little amounts of volatiles [9.187, 188]. Those two studies report different esters in *Granny Smith*: in the *Lavilla* et al. [9.188] study, propanol or propanoic acid esters were dominant, while in the *Zhu* et al. [9.187] study,

esters derived from hexanol (hexyl acetate and hexyl butanoate) were the main volatiles (Table 9.3). *Fuji* apple mostly produced ethyl 2-methylbutanoate and 2-methylbutyl acetate [9.189] (Table 9.3). Table 9.3 also illustrates an earlier observation by *Paillard* [9.3], that esters with even-numbered carbon chains from acetic, butanoic and hexanoic acids, and with ethyl, butyl and hexyl alcohols are more frequently found in apples than odd-numbered ones. In another study where volatiles were extracted by vacuum hydro-distillation, the main ester (and volatile) in *Golden Delicious* was butyl acetate, and in *Fuji*, it was butyl acetate and 2-methylbutyl acetate [9.82]. In that study like in others where authors are seeking the impact of odor-volatile compounds by GC-O, 15 compounds, out of a total of 36 detected, were selected as contributors to apple flavor. Out of these 15 compounds, 9 were esters, and butyl propanoate was detected only in *Braeburn* apple [9.82].

Other volatiles produced in large amount by apples include straight- and branched-chain aliphatic aldehydes and alcohols [9.2]. Alcohols include ethanol, propanol, butanol, pentanol, hexanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 2-ethyl-1-hexanol [9.174, 187, 188]. C6 aldehydes have been reported by many authors, as well as heptanal, nonanal, and decanal. Only a few ketones have been reported in apples [9.2, 190]. They are mostly straight-chain aliphatic ketones including acetone, or the hydrocarbon 6-methyl-5-hepten-2-one. 6-Methyl-5-hepten-2-one was perceived as fruity by GC-O [9.12]. Some volatiles were found only by GC-O, as they have low odor-thresholds and are produced in very small amounts. Examples are β-damascenone and 4-methoxyallylbenzene, with characteristic sweet/grape juice/cooked apple and anise/spicy odors, respectively [9.12, 44, 194]. While β-damascenone contributes to the underlying fruity/cooked note in a fruit [9.12], 4-methoxyallylbenzene is believed to impart a characteristic spicy note to *Gala* [9.12], *Cox's Orange Pippin*, *Ellison's Orange*, and *Kidd's Late Orange* apples [9.194]. An early study of apple flavor by GC-O using CharmAnalysis [9.44] clearly summarized the fact that apples do not have volatiles that qualify as character impact. Further, the variation among the 40 cultivars tested was such that no odor-active compound was found common to all samples. In that study, β-damascenone had the most intense odor, followed by esters: ethyl butanoate, hexyl hexanoate, hexyl butanoate, and ethyl-2-methyl butanoate. *Plotto* et al. [9.15] also found hexyl acetate, butyl acetate, 2-methylbutyl acetate, methyl-2-methyl butanoate, and hexanal to contribute the most to *Gala* apple aroma by mixing volatiles in a model solution.

9.3.4 Strawberries

Strawberry is a popular fruit of temperate climates, among others because it is one of the first to be on the market in the spring. In fresh strawberries, sweetness was shown to have the highest correlation with overall liking in consumer studies, much more so than other attributes such as firmness or sourness [9.195]. In general, consumers overall liking for strawberry fruit was positively correlated with total soluble solids, total sugars (sucrose, fructose, glucose), or the ratio of total soluble solids (TSS) with titratable acidity (TA) [9.36, 195, 196]. Even though total volatiles contribute to strawberry flavor, which of the volatiles affect consumer liking is less clear and depends on the studies [9.36, 195, 196]. In GC-O studies, compounds with high odor activities are esters, furanones, some lactones, alcohols, aldehydes, and acids. Most frequently cited esters are methyl and ethyl butanoate, ethyl and methyl hexanoate, and ethyl 2-methyl butanoate, all having fruity aroma [9.4, 5, 27, 62]. Interestingly, in one consumer study, the cultivar that had the lowest amount of esters was the least preferred, in spite of its highest sweetness and TSS/TA level [9.36], showing the important contribution of fruity esters to strawberry flavor. Compounds with the greatest aroma activities by GC-O are by far furaneol (2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF)) and mesifurane (2,5-dimethyl-4-methoxy-3(2H)-furanone (DMMF)) [9.5, 62, 197]. These two furanones were identified early on because of their strong sweet and pleasant odors [9.198, 199]. However, because they are highly water soluble and thermally instable, their quantification may not always be accurate when analyzed and reported together with other volatiles [9.30, 83]. It may explain why furaneol was not reported as a contributor of strawberry flavor or sweetness-enhancing volatile in two studies [9.195, 197]. Lactones (γ-decalactone, δ-decalactone, γ-dodecalactone) were identified in strawberry with high odor-activity in some cultivars [9.4, 62, 197], and positively contributing to consumer liking [9.36, 195]. In fact, furaneol and γ-decalactone were chosen as target compounds to enhance strawberry flavor in a breeding program [9.200]. Among aliphatic alcohols, aldehydes, and acids, (*Z*)-3-hexenal had the highest odor activity value in one study [9.5], but (*E*)-2-hexenal and hexanal are also reported [9.27, 62, 197]. As mentioned earlier, these volatiles are the result of sample preparation, and may vary greatly between studies [9.36]. They nevertheless contribute to green, fresh flavor when chewing the fruit. Hexanoic acid, high in *Senga*, had a negative effect on strawberry flavor in spite of high mesifurane and γ-decalactone levels in that cultivar [9.4]. Few terpenes were reported,

with linalool, geraniol, and/or nerolidol contributing to odor activity [9.27, 62, 197] and overall strawberry flavor [9.77, 195]. Sulfur compounds were also reported in strawberries, mostly methyl thioacetate and methyl thiobutanoate [9.62, 84, 195, 201]. Their contribution to strawberry flavor has not been precisely defined; however, they might enhance overall fruitiness.

Like in all horticultural crops, volatiles in strawberries depend on species, cultivar, maturity, and environmental factors. Commercial cultivars are the result of generations of crosses that have improved production yield, disease resistance, fruit size, handling and storage, to the detriment of flavor quality [9.4]. Recently breeders are trying to introduce *old* flavors (flavor from wild type species) into new cultivars. With that goal in mind, *Ulrich* and co-workers analyzed the volatiles of cultivated and wild-type strawberries, and then evaluated them by GC-O and sensory taste panels [9.4, 77]. In summary, the old European wild strawberry, *Fragaria vesca* (Fig. 9.4), is unique in that it produces high amounts of methyl anthranilate, a compound with typical grape flavor. High amounts of methyl anthranilate (greater than 1 ppm) can impart an unpleasant perfumey and soapy flavor [9.77]; however, in combination with furaneol and mesifurane, smaller amounts of methyl anthranilate produced fruit with highly desirable flavor [9.4]. Another wild species, *F. moschata* (Fig. 9.4), with pleasant aroma and taste, was characterized by large amounts of esters and terpenes, contrary to some commercial cultivars that had a much lower quantity of these fruity volatiles. These studies and others [9.202] are building blocks to understand the distribution of volatiles in different species and intra- and interspecific crosses. Modern genomic tools will identify genes that control volatile production, and together with the knowledge of genetics, make breeding for enhancing fruit flavor more efficient [9.203–205].

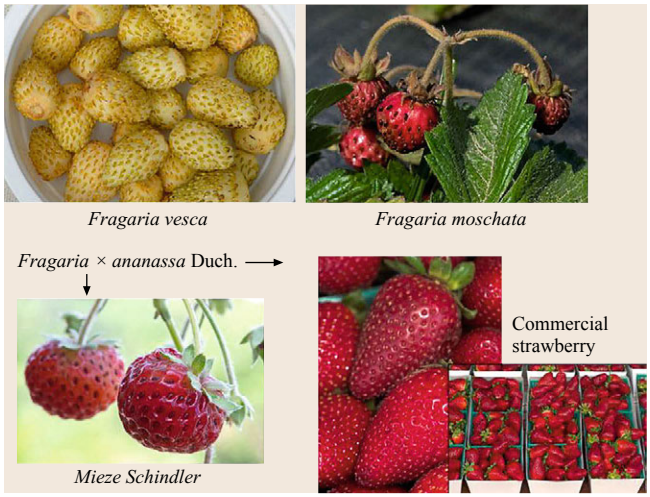

Fig. 9.4 Strawberries producing high (*Fragaria vesca*) or medium (*F. moschata* and *F.* × *ananassa* Duch., *Mieze Schindler*) amounts of methyl anthranilate, in comparison with the commercially cultivated strawberry (*F.* × *ananassa* Duch.) which produces no methyl anthranilate. Photos courtesy of David Karp, University of California, Riverside

9.4 Conclusion

Considering the diversity of fruits on earth, it is expected to find a wide array of volatile chemicals producing very diverse flavors, together with nonvolatile compounds. If there are some common volatiles across plant species, their quantities will vary with the species and variety. Further, for horticultural crops, fruit composition will be greatly dependent on cultivar, maturity, postharvest handling, and environmental factors during fruit development. The authors of this chapter hope they have succeeded in conveying the complexity of a biological commodity such as a fruit, and how fruit quality must be studied across multiple facets.

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10. Meat

Jane K. Parker

The delicious aroma of freshly cooked meat is highly attractive, stimulating the gastric juices, and giving us early indications that the meat and its eating experience are likely to be enjoyable. Consequently, there is much interest from the food industry in understanding how to control and optimize meat aroma. The aroma profiles of cooked and cured meats are extremely complex, comprising several thousand volatile compounds, of which only a few impart characteristic meaty notes. This chapter covers the characterization of meat aroma, identifying those compounds that impart meaty aromas and those that give species character, as well as those which generate off-notes. The formation pathways of these compounds are reviewed, and the role of pre- and post-slaughter conditions in altering the aroma profile of the meat is discussed. Production of optimum meat flavor involves careful selection of diet and breed, good control over pre- and post-slaughter conditions, and choice of appropriate processing conditions to maximize the formation of taste and aroma compounds.

10.1 Introduction to Meat Flavor

The aroma of meat is one of the attributes which, in conjunction with others such as color, taste, texture, juiciness, and tenderness, combine to give us the overall sensation of meat flavor, and ultimately determine the consumer enjoyment and acceptability of the meat. Meat aroma is the subject of a vast number of journal articles and patents, with several thousand published within the last decade alone. It is the topic of many reviews [10.1–5] and, although much has already been discovered about the chemistry of meat flavor, its complexity means that it continues to challenge both academics and industrialists who seek to accurately imitate, manipulate, and control meat flavor, whether it be in roast beef, soups, or snack seasonings. The challenge for the flavor chemist is to understand which compounds are important for meat aroma (Sect. 10.2), how they are formed (Sect. 10.3), and what factors are important in influencing and optimizing the flavor of meat (Sect. 10.4).

10.1.1 Sensory Aspects

The taste and flavor of meat is hard to articulate, and many groups have drawn up standard lexicons for use with sensory panels. One such standard lexicon [10.6], developed for cooked beef, included words such as brothy, umami, roast beef, juicy, browned, fatty and salty, and these descriptors were generally regarded as positive, whereas words such as barny, bitter, gamey, and grassy had negative connotations. When beef, lamb,

and pork were compared by a sensory panel [10.7], beef and lamb were described with similar terms including barny, bitter, gamey, grassy, livery, metallic and roast beef, whereas pork and turkey were described using words such as brothy, fatty, salty, sweet, and umami. A pork lexicon [10.8] contained additional words such as nutty, green and fatty as odor terms, and bloody, metallic and piggy as overall flavor terms. Inevitably, terms such as lamby or mutton, beefy, chicken-like, and pork-like are often included in sensory lexicons as sensory panels struggle to describe the differences between species using other terms.

The aroma profile of fresh raw meat is limited to a blood-like flavor [10.1] and the characteristic flavor of cooked meat only develops during thermal processing, whilst the more delicate flavor of cured meats develops during extended storage. The volatile profiles of both cured and thermally processed meats are complex, increasingly so, as technological advances produce instruments with greater and greater sensitivity. Roast beef has been reported to contain almost 5000 individual volatile compounds [10.9], but not all these have been identified and, more importantly, only a fraction contribute to the aroma of cooked roast beef.

10.1.2 Analysis of Volatile Compounds in Meat

Over 1000 compounds have been identified in thermally processed meat, and those reported are very dependent on the techniques used for extraction and analysis. Extraction of volatile compounds from the complex matrix of muscle and fat is a challenge, since most aroma compounds are present at the mg/kg level and below. Extraction techniques can be exhaustive (i. e., they strip out all (or most of) the volatile components from the entire sample) or semi-quantitative where only the headspace above the sample is analyzed. Exhaustive extraction techniques are preferable for quantitative analysis but tend to be time consuming. Solvent-assisted flavor extraction (SAFE) developed by *Engel* et al. [10.10] is currently the exhaustive extraction technique of choice, using high vacuum and low temperatures to distill the volatiles from the food, or from a solvent extract of the food. This is particularly useful for complex matrices, particularly fatty ones, where the volatiles are often difficult to separate from the fat, but the downside is loss of the highly volatile compounds during the concentration procedures. It has replaced the use of the Likens–Nickerson simultaneous distillation extraction method [10.11] which employed higher temperatures and was susceptible to the formation of artifacts. Solid phase extraction (SPE) can be used quantitatively for meat extracts, and is particularly useful for the analysis of more polar aroma compounds that are not readily extracted using headspace techniques.

Currently, a range of headspace extraction techniques is available. Solid phase microextraction (SPME) [10.12] is the most frequently used, but there are many other variants emerging. During SPME, the headspace volatiles are concentrated onto a fiber coated in a combination of adsorbing and absorbing polymers, and the fiber is then desorbed directly in the injection port of a gas chromatograph (GC). Dynamic headspace extraction (DHE) is also popular. The advantage of this technique is that more concentrated extracts can be obtained as the volatiles are continuously swept onto a trap containing an adsorbent resin. Traps can be desorbed using a solvent, or in a bespoke injection port. Both SPME and DHE are more suitable for routine analysis than SAFE, but are only semi-quantitative, unless used in conjunction with stable isotope dilution analysis (SIDA) [10.13]. These extraction techniques are reviewed by *Elmore* [10.14, 15].

After extraction, the volatile compounds are separated by gas chromatography and detected using a mass spectrometer (GC-MS) and/or an odor port (GC-olfactometry or GC-O) where the effluent is sniffed by a trained assessor. Two-dimensional GC (Chap. 17), in combination with time-of-flight MS, is a particularly powerful technique which has been used to identify trace components in meat [10.9].

10.2 Characterization of Meat Aroma

It is important to realize that only a subset of the 4700 volatiles found in the headspace of oven-roast beef [10.9] are perceived as odorous and only a fraction of those are present in cooked or cured meat at levels above their odor threshold. Those which are odor-active are generally determined using GC-O with aroma extract dilution analysis (AEDA) [10.13]. In this technique, the odor-active components of an aroma extract are identified by GC-O and then the extract is serially diluted and reanalyzed by GC-O at each dilution. Those compounds with the highest dilution factor (FD) (those being detected in the most dilute extracts) are deemed to make an important contribution to the aroma profile of the cooked meat. However, FDs are derived from a liquid extract and do not account for the relative partitioning of the components into the headspace.

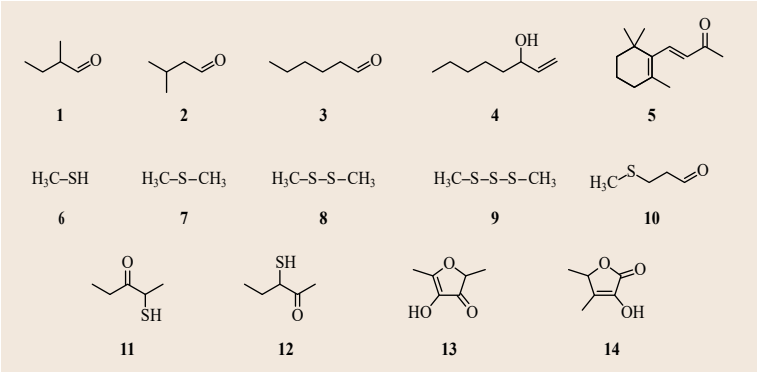

Fig. 10.1 Odor-active compounds in meat: complementary or incongruent aromas

Therefore, AEDA is often followed by quantification of the compounds with the highest FDs and formulation of a recombinate by mixing the appropriate concentrations of the most odor-active components, in the correct proportions, into a bland but representative base. The contribution of each component can be assessed by systematically removing single compounds (or pairs, or groups) from the recombinate and reassessing. Odor activity values (OAVs) can be calculated by dividing the concentration of each component in the system by the respective threshold value, and this is sometimes reported as the logarithm of the ratio. The OAV and the FD give complementary information, so those compounds with the highest FD are not necessarily the same as those with the highest OAV. Note that the result is only as good as the original extract and the proficiency of the assessors but, despite this and the natural variation in meat flavor (discussed below), there is remarkable agreement within the literature as to the compounds responsible for meat aroma.

Cerny [10.5] has compiled from the literature a list of odor-active compounds present in beef, pork, and chicken, roasted, boiled, or stewed. This list contains 100 compounds representing many classes of compounds, the majority of which are straight chain aliphatic compounds including aldehydes (C_5–C_{10}) and most of the corresponding 2-alkenals, C_9 and C_{10} alkadienals, and the branched Strecker aldehydes. The list also contains heterocyclic compounds: lactones, pyrazines, thiazoles, phenols, and sulfur compounds, all contributing a range of aromas to the overall profile which, when present in the correct proportions, gives a rounded and characteristic meaty flavor. These compounds can be classified according to their odor character, in order of increasing specificity:

1. Those compounds which are complementary or incongruent (they do not resemble meat, but are still odor-active and contribute to the overall meat aroma)
2. Those compounds which give the impression of cooked or savory aroma, but not specifically meat
3. Those which give a meaty character and
4. Those which impart species character, thus distinguishing beef from lamb, pork, or chicken.

Those in categories 3 and 4 are termed *character impact compounds* and make up only a small proportion of the odor-active compounds.

10.2.1 Complementary or Incongruent Aromas

A large proportion of odor-active compounds in meat do not, when smelt individually, resemble meat, although quantitatively they can often be major components of the volatile profile. Most of the aldehydes, alcohols, and ketones listed by *Cerny* [10.5] fall into this category, typical examples being 2,3-butanedione (buttery), the Strecker aldehydes methylpropanal, 2-methylbutanal (**1**) and 3-methylbutanal (**2**, malty, bitter cocoa), hexanal (**3**, green), phenylacetaldehyde (honey) and decanal (orange) (Fig. 10.1). These carbonyl compounds, as well as 1-octen-3-ol (**4**, mushroom), tend to be present and odor-active in most cooked foods. Other less encountered examples are 2-octanone (fruity or blue cheese) and β-ionone (**5**, floral or cooked carrots), and some are character impact compounds of other foods, for example, (*E*,*E*)-2,6-nonadienal (cucumber) and (*E*)-2-decenal (coriander), yet still appear on lists of odor-active compounds in meat.

One important contribution is made by the group of sulfur compounds that have objectionable, sulfury, rotting aromas when sniffed alone, but often play a vital role in recombinates [10.9]. These are methanethiol (**6**), dimethyl sulfide (**7**), dimethyl disulfide (**8**) and dimethyl trisulfide (**9**), as well as 3-methylthiopropanal (methional, **10**) which imparts a strong baked or dried potato note. Mercaptoketones impart a sulfury, hydrogen sulfide note, yet both 2-mer-

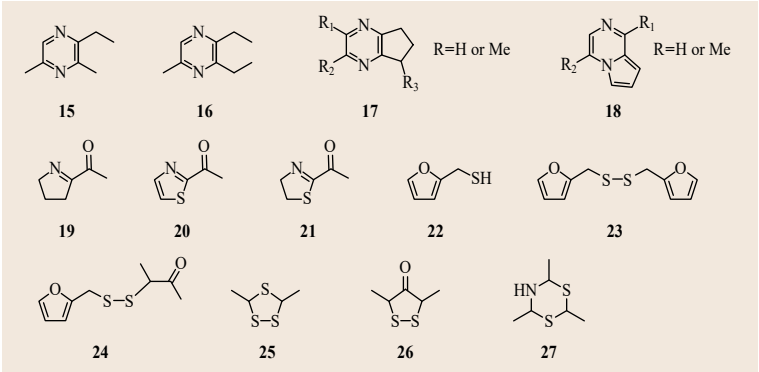

Fig. 10.2 Odor-active compounds in meat: savory and cooked aromas

capto-3-pentanone (**11**) and 3-mercapto-2-pentanone (**12**) play a significant role in the odor profile of beef, pork, and chicken [10.5].

Another exceptionally important contributor to meat aroma is 2,5-dimethyl-4-hydroxy-3[2*H*]furanone (furaneol, **13**). This compound has been found to be essential in volatile recombinates of a range of cooked and uncooked foods, particularly strawberries [10.16], pineapple [10.17], tomatoes [10.18], tea [10.19], and beer [10.20]. Alone it imparts a burnt sugar, candy-floss aroma, but it has been found to be one of the most powerful odorants in beef extracts [10.21]. Its isomer, 3-hydroxy-4,5-dimethyl-5*H*-furan-2-one (sotolone, **14**), is also important for meat flavor, imparting savory, curry and spicy notes, but also turning caramellic and maple on dilution.

Although it is not immediately obvious why these compounds are important for meat flavor, some have been shown to be vital. It is likely that these compounds are involved in interactions which may suppress or alter the more objectionable or incongruent aromas, or enhance the positive aromas. The basis for these interactions is not fully understood and is likely to be multimodal. The interactions could be physical (changes in flavor release), chemical (reactions occurring in the food), biochemical (enzymatic changes in the mouth or mucosal surfaces), physiochemical (interactions at receptor level), or psychological constructs [10.22]. The overall effect is to provide a rounded, balanced meaty flavor.

10.2.2 Savory and Cooked Aromas

Many compounds impart generic roasty, toasted, cooked (and burnt) notes, not only to thermally processed meat, but also to a range of cooked foods such as roasted nuts, french fries, potato snacks, popcorn, bread crust, biscuits, and so on. These compounds are predominantly derived from the Maillard reaction (Sect. 10.3.1), a complex process involving the reaction between reducing sugars and an amino nitrogen, and they are usually heterocyclic, belonging to chemical classes such as furans, oxazoles, pyrroles, pyrazines, thiophenes, thiazoles, thiazolines, and sulfides (Fig. 10.2). Pyrazines play an important role in cooked aromas, particularly the tri-substituted pyrazines such as 2-ethyl-3,5-dimethylpyrazine (**15**, roasty, nutty, cocoa) and 2,3-diethyl-5-methylpyrazine (**16**, potato-like, roasty) which are present in meat at low levels compared to the less substituted pyrazines, but have much lower odor thresholds (1 and 0.05 µg/kg, respectively [10.23, 24]). Two series of bicyclic pyrazines, one based on the 6,7-dihydro-5(*H*)-cyclopentapyrazine structure (**17**) and the other a group of pyrrolopyrazines (**18**), have been found in grilled, roasted, and burnt meat, imparting the characteristic roast note to their flavor profile [10.25, 26]. 2-Acetyl-1-pyrroline (**19**), which is a character impact compound in bread crust, is often found to be odor-active in meat products.

The role of sulfur compounds is of paramount importance in roasted flavors. 2-Acetylthiazole (**20**, nutty, popcorn note) and 2-acetyl-2-thiazoline (**21**, biscuit cracker note) are crucial for bakery products like biscuits, but are also found to contribute to meaty aromas, particularly beef.

2-Furanmethanethiol (**22**) is incredibly important in meat flavor and imparts a slightly meaty or roasted coffee note depending on the concentration. *Kerscher* and *Grosch* [10.27] have reported this compound in meat at 2–40 µg/kg, well above its reported threshold value of 0.005 µg/kg [10.28]. Related disulfides also give savory aromas, e.g., bis(2-furanmethyl) disulfide (**23**) which gives a roast, nutty, and baked aroma, and has been found to be odor-active in boiled chicken, and 3-[2-(furanmethyl)dithio]-2-butanone (**24**) and homologues which are reported to impart onion and burnt rubber aromas [10.29]. This last reference lists the odor properties of 30 sulfur compounds formed in a model system, mostly mer-

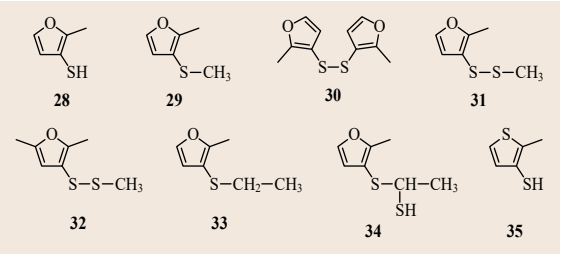

Fig. 10.3 Odor-active compounds in meat: meaty character impact compounds

captoketones, many of which impart sulfury, savory, or cooked aromas.

A series of cyclic sulfides, either thiolanes or thiolanones, was extracted from meat using Likens–Nickerson extraction [10.1]. They are not as potent as some of the compounds discussed above, but their characteristic cooked aroma (roasty, vegetable or nutty) with meaty tendencies makes them potentially important in meat flavor. Of these, 3,5-dimethyl-1,2,4-trithiolane (**25**) is the most frequently encountered, imparting a fatty, oniony, and nutty aroma which has been described as slightly meaty. A series of related disulfides, for example, 3,5-dimethyl-1,2-dithiolan-4-one (**26**) was synthesized [10.30] and most were found to have spicy, roasty, and savory character. *Werkhoff* also discusses the role of 1,3,5-dithiazines (e.g., thialdine **27**) which he believes are important for meat flavor and which tend to impart roasty, oniony, nutty, and occasionally meaty aromas [10.1]. Although these cyclic compounds have been found in model meat reactions [10.31], they are rarely found in meat, other than in SDE or Likens–Nickerson extracts. More recent studies have shown that they are formed during these extraction techniques, so their quantities can be overestimated [10.32]. Although this suggests that their contribution to meat aroma is perhaps minimal, their formation in model systems is important for the generation of meaty aroma in process reactions.

10.2.3 Meaty Character Impact Compounds

The majority of compounds that provide the characteristic meaty note are sulfur compounds (Fig. 10.3). They are also derived from the Maillard reaction, specifically from cysteine and ribose (Sect. 10.3.1), although there are alternative formation pathways from thiamine (Sect. 10.3.3) and ascorbic acid. These precursors are relatively abundant in meat, compared to cereals and vegetables. A breakthrough in the flavor chemistry of meat was made in 1976 by *Evers* et al. [10.33] who isolated, identified, and synthesized a family of meaty sulfur compounds based on the 2-methyl-3-furanthiol moiety (**28**), which included sulfides, di-, tri-, and tetrasulfides as well as analogs substituted with a methyl group on the 5 position. This was followed by the isolation of 2-methyl-3-(methylthio)furan (**29**) from cooked beef by *MacLeod* and *Ames* [10.34]. The most powerful of this family, determined by *Gasser* and *Grosch* [10.35], is the bis(2-methyl-3-furan) disulfide (**30**) that imparts a characteristic meaty flavor and is one of the most powerful aroma substances known, with an odor detection threshold in water of 0.00002 µg/kg [10.36]. The parent thiol (**28**) also has an exceptionally low threshold of 0.0004 µg/kg [10.37], whereas both the methyl sulfide (2-methyl-3-(methylthio)furan) (**29**) and the methyl disulfide (2-methyl-3-(methyldithio)furan) (**31**) impart meaty notes at low concentrations, but have odor thresholds several orders of magnitude higher (0.05 and 0.01 µg/kg, respectively [10.28, 34]). The analogs with an additional substituent on the furan ring can also impart meaty notes 2,5-dimethyl-3-(methyldithio)furan (**32**) which has an odor threshold of 0.01 µg/kg [10.28]. This family of compounds is consistently found to contribute to meat flavor, particularly the parent thiol (**28**) and the bis-disulfide (**30**) which are generally believed to be the character impact compounds of meat.

Werkhoff et al. [10.1] isolated a further 100 sulfur compounds from Likens–Nickerson extracts of cooked pork and some of these were also present in beef or lamb. Some of these are of particular interest because of their meaty aromas, although they are not

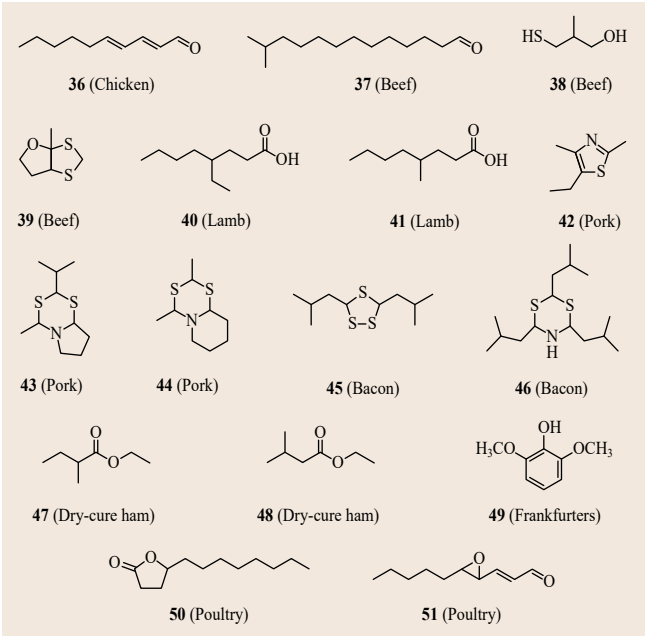

Fig. 10.4 Odor-active compounds in meat: species-specfic aromas

often detected by GC-O. The more odor-active ones are closely related to 2-methyl-3-furanthiol (**28**). For example, 2-methyl-3-(ethylthio)furan (**33**), which was found in chicken, imparts fatty and rubbery notes, but at low concentrations has a meaty, brothy aroma. This is typical of many sulfur compounds that impart meaty, vegetable or even fruity notes at concentrations close to their thresholds, but quickly develop particularly offensive sulfury, rubbery notes as the concentration increases. The ethanethiol analog (1-(2-methyl-3-furanthio)ethanethiol) (**34**) was isolated from pork and reported to have a powerful meat-like aroma, and replacement of the furan ring with a thiophene ring also produced a meaty note (2-methyl-3-thiophenethiol (**35**)).

10.2.4 Species Character

Species character is usually (but not exclusively) provided by the lipid-derived volatiles (Fig. 10.4). This was demonstrated in the 1960s by *Hornstein* and *Crowe* [10.38] who showed that aqueous extracts of lean beef and pork produced a similar meaty flavor, whereas heating beef and pork fat produced the characteristic species-specific aromas.

Beef

Beef is a popular meat, with a global consumption of over 5×10^7 t/a, typically boiled, roasted, or stewed. *Gasser* and *Grosch* [10.35] determined 40 odor-active compounds in an SDE extract of boiled beef. The most potent of these (with dilution factors of 512) were the two meaty compounds (2-methyl-3-furanthiol (**28**) and bis(2-methyl-3-furan) disulfide (**30**)), two savory compounds (methional (**10**) and one unidentified roasted compound), two fatty compounds ((*E*,*E*)-2,4-decadienal (**36**) and (*E*)-2-nonenal), and β-ionone (**5**) (violet). However, the compounds that provided the species character were bis(2-methyl-3-furan) disulfide (**30**) and 12-methyltridecanal (**37**). The disulfide contributes to the meaty aroma of all species of meat but, in beef, where it is present at higher concentrations, it imparts the characteristic aroma of aged prime-rib of beef. The aldehyde (**37**) that was found in stewed beef [10.39] is derived from plasmalogen lipids and has a distinctive beef tallow aroma with an odor- threshold of 0.01 µg/kg. The longer chain analogs have subsequently been identified in cooked beef.

The most potent aroma compounds in stewed beef were reported by *Guth* and *Grosch* [10.40]. Using AEDA on an ether extract, furaneol (**13**) was found to have the highest dilution factor, followed by butanoic acid and acetic acid. After quantification and determination of the odor thresholds, those with the highest odor activity were found to be methanethiol (**6**), followed by 12-methyltridecanal (**37**), furaneol (**13**), and acetaldehyde. 2-Methyl-3-furanthiol (**28**) was not reported as odor-active in this study, although 2-furanmethanthiol (**22**) was detected at the lower end of the odor-activity scale. Twelve of these components were required to make up a recombinate (in a base containing oil, gelatine, potassium hydrogen phosphate, lactic acid, and glutamate) which was judged by five panellists to be similar to the stewed beef juice. By systematic elimination of one single component at a time, removal of either methanethiol (**6**), furaneol (**13**) or 12-methyltridecanal (**37**) was found to have the biggest impact on the quality of the match. 12-Methyltridecanal (**37**) was found to be one of the most odor-active components of a beef broth recombinate [10.5], followed by 2-methyl-3-furanthiol (**28**), 2-furanmethanethiol (**22**), methanethiol (**6**), nonanal and furaneol (**13**). However, removal of 12-methyltridecanal (**37**) from the recombinate on this occasion did not change the aroma of the recombinate significantly and its contribution to beef aroma is debatable.

In a novel experimental set-up, *Rochat* et al. [10.9] extracted the volatiles of roast beef directly onto an SPME fiber placed in the headspace of the tubular oven as the beef was roasting, thus minimizing the formation of artifacts and capturing the more labile compounds that may not survive more exhaustive extraction procedures. The sulfur compounds that were perceived most frequently at the GC-O sniffer port (i. e., had the highest nasal impact frequency (NIF)) were dimethyl trisulfide (**9**), methional (**10**), 2-methyl-3-furanthiol (**28**), 2-methylthiophene, methanethiol (**6**), and 2-acetyl-2-thiazoline (**21**). In this study, the compound that provided the species character was 2-methyl-3-mercapto-1-propanol (**38**) which was described as beefy, but the NIF was only 11% (i. e., it was only detected in 11% of the GC-O analyses). This compound is important because it is one of few which have been described as *beefy* which have been identified in cooked beef.

Other compounds have been reported to have beef aromas. 3[(2-Methyl-3-furan)dithio]-2-pentanone is described as meaty [10.29] and, in a compilation of the sensory properties of Maillard-derived volatile compounds, *Fors* [10.41] reported boiled beef characteristics for 3,5-dimethyl-1,2,4-trithiolane (**25**), 3-methyl-1,2-dithiolane, 2,4,5-trimethyl-3-oxazoline, and 2,4,5-trimethyloxazole. 1-Methylbicyclo[3.3.0]-2,4-dithia-8-oxaoctane (**39**) has been reported by *Etiévant* [10.42] as smelling like cooked beef, but also chicken and boiled milk. Although characteristically beefy, these compounds do not appear to be particularly odor-active and it is likely that the odor thresholds are higher than for the other sulfur compounds that are consis-

tently found to be important in beef. The threshold of 3,5-dimethyl-1,2,4-trithiolane (**25**) has been reported as 10 μg/kg, which is several orders of magnitude higher than the other sulfur compounds reported, particularly the furanthiols.

Sheep and Goat Meat

Worldwide consumption of sheep meat is about a fifth of that of beef, and its unique flavor is not as universally accepted as that of beef. A recent review of sheep meat [10.43] includes a meta-analysis incorporating all recent GC-O studies. The two furanthiols (**22** and **28**) were amongst the list of 28 odor-active compounds, but the list was dominated by lipid-degradation products. The meta-analysis demonstrated that the compound contributing the most to lamb flavor was 4-ethyloctanoic acid (**40**), a compound with a fatty, mutton flavor. Branched-chain fatty acids are believed to give the characteristic mutton notes to sheep products, and the presence of both 4-ethyl- and 4-methyloctanoic acid (**41**) is unique to ovine species when compared to beef, pork, and poultry. The homologous 4-methylnonanoic acid also has a muttony note, but has been found in other species. These acids are present at relatively high concentrations in both the cooked and raw lamb fat [10.44] and do not accumulate during processing. 4-Methyloctanoic acid (**41**) was found at 3–5 mg/kg in the adipose tissue of lambs [10.45], just above the estimated threshold value.

The meta-analysis shows that for most of the other odor-active compounds, the contribution to the headspace in both lamb and beef was similar. The exceptions were (*Z*)-2-nonenal (grass/plastic), 2-acetyl-1-pyrroline (**19**, popcorn), (*E*,*E*)-2,4-hepteadienal (fried potato), and 4-methylphenol (stable, animal) which all made a far greater contribution to the aroma profile of lamb than they did to beef. (*Z*)-4-Heptenal is often considered to be very characteristic of lamb fat.

Goat meat has been compared to lamb in a recent study by *Madruga* et al. [10.46]. Although the profiles were similar, goat meat was found to contain more compounds derived from (ω-3) fatty acids such as linolenic acid (2-ethylfuran, 1-penten-3-ol, (*Z*)-6-nonenal), whereas the lamb contained much higher levels of compounds derived from (ω-6) fatty acids such as linoleic acid (pentanal, hexanal (**3**), 1-octen-3-ol (**4**) and 2-pentylfuran). It was suggested that these differences gave lamb the much stronger flavor; however, no character impact compounds have been reported for the distinctive goaty note that distinguishes goat from lamb.

Pork

The worldwide consumption of pork is almost twice that of beef, and it is the most popular meat in Europe, consumed both cooked and cured, in many different forms.

Cooked Pork. A comparison of boiled beef and pork [10.47] showed that many of the same compounds were present, with the exception of the beef-specific aldehyde (**37**) and dimethyl sulfide (**7**) which were not found in pork. The importance of the two furanthiols (**22** and **28**) in pork aroma was confirmed [10.5] and Kerscher reported a contribution from methanethiol (**6**), acetaldehyde, methylpropanal, furaneol (**13**), octanal, nonanal and 1-octen-3-one – all compounds which have also been found to be odor-active in beef. The only distinguishing compound was 2,4-dimethyl-5-ethylthiazole (**42**) which gives a roasty earthy note.

Several meaty compounds have been found in cooked pork. Many of the cyclic sulfides were found by *Werkhoff* et al. [10.1] in cooked pork: 3-methyl-4-oxo-1,2-dithiane was described as meaty, bloody, and sulfury; 3-methyl-1,2,4-trithiolane was described as oniony and meaty; and 3-methyl-5-isobutyl-1,2,4-trithiolane was described as onion, roasty, and meaty. The bicyclic dithiazines such as tetrahydro-4-methyl-2-isopropyl-4*H*-pyrrolo[2,1-d]-1,3,5-dithiazine (**43**) and tetrahydro-2,4-dimethyl-4*H*,6*H*-pyrido[2,1-d]-1,3,5-dithiazine (**44**) are unique to cooked pork and believed to be important for pork flavor, but they are described with more generic terms such as fatty, onion, roasted, and nutty. However, a typical pork-rind aroma has been reported from two compounds isolated from a model system: 3,5-di-isobutyl-1,2,4-trithiolane (**45**) and 5,6-dihydro-2,4,6-tri-isobutyl-4*H*-1,3,5-dithiazine (**46**) [10.48].

Aroma extract dilution analysis (AEDA) has been carried out on a pork and vegetable gravy prepared *a la chef* [10.49], and 50 odor-active compounds were reported. Of these, 2-methyl-3-mercapto-1-propanol (**38**), described previously as beefy, was the compound with the highest dilution factor (4096) in both pork/vegetable and beef/vegetable gravies and was described in this study as a characteristic gravy aroma. However, it was only present in the beef or pork gravy prepared with vegetables, and not with beef alone.

The volatile profile of wild boar has been compared to that of a domestic pig [10.50]. No character impact compounds of boar were identified, and the difference in flavor was attributed to changes in the concentration of a range of potent aroma compounds.

Cured Pork Products. The curing of pork meat is a tradition going back many centuries when it was a vital method of preservation. Records start as early as Cato (234–149 BC) who described the process for dry-cure ham. Cured cuts of pork, which are not subse-

quently cooked, have quite a different aroma profile to cooked pork products. Dry-cured hams are speciality products that are aged or ripened for several months to generate the delicate flavor associated with such products. Over 600 compounds were identified in Bayonne ham [10.51], using DHE, of which 29 were odor-active. Although many of the compounds detected have also been found in cooked meat, they tend to be the compounds that contributed fruity, green, and sweet notes belonging to the chemical classes of aldehydes, esters, alcohols, and acids, rather than the thermally derived heterocyclic compounds. Other authors using dynamic headspace showed that the most odor-active compounds in Iberian ham were hexanal (**3**), (*Z*)-3-hexenal, 3-methylbutanal (**2**), 1-octen-3-one, 1-octen-3-ol (**4**), and methylpropanal [10.52], but this group also found five meaty or toasty compounds amidst those which were odor-active (2-methyl-3-furanthiol (**28**), 2-furanmethanethiol (**22**), 3-mercapto-2-pentanone (**12**), 2-acetyl-1-pyrroline (**19**), and 2-propionyl-1-pyrroline). The odor-active compounds in Parma ham [10.53] were shown to include 2-acetyl-1-pyrroline (**19**), methional (**10**), 1-octen-3-one, furaneol (**13**), 4-methylphenol, and sotolone (**14**).

The odor-active compounds reported in the volatile profile of fermented sausages [10.54] were hexanal (**3**), heptanal and 1-octen-3-ol (**4**), and a salty meat, dry-cured ham aroma which was attributed to (*E*)-2-hexenal. When the volatile profile was monitored over time using SPME [10.55], the most odor-active compounds at the beginning of the process were 3-methylbutanal (**2**), 2-methylbutanal (**1**), octanal, 2,3-butanedione, and ethyl 2-methylbutanoate (**47**), whereas propanal, pentanal, hexanal (**3**), ethyl 3-methylbutanoate (**48**), 1-octen-3-ol (**4**), 3-ethylbutanoic acid, methylpropanoic acid, ethyl hexanoate, and nonanal developed during storage.

It is clear that, to date, no single compound has been identified which gives a characteristic cured meat note and the typical flavor is derived from low levels of meaty compounds and relatively high levels of short chain aldehydes, esters, and alcohols.

Other Pork Products. Bacon is a traditional product which is prepared using different methods around the world. It is both cured and cooked to generate a unique flavor. The volatile profile of green bacon (uncooked) [10.56] contains similar classes of compounds to dry-cured ham, e.g., 3-methylbutanal (**2**), and 1-octen-3-ol (**4**). An SPME extract of Chinese sweet cure bacon [10.57], which is also smoked, contained a range of odor-active compounds, including those already discussed for cooked meat, and in addition a range of phenols derived from the smoke. Bacon aroma has been generated by *Shu* et al. [10.48] in a model system containing 3-methylbutanal (**2**) and ammonium sulfide. 3,5-Di-isobutyl-1,2,4-trithiolane (**47**) and 5,6-dihydro-2,4,6-tri-isobutyl-4*H*-1,3,5-dithiazine (**48**) were identified in the model system, synthesized, and the pure compounds were described as having typical fried bacon and pork-rind aromas.

Frankfurters are a more complex product involving the addition of spices and smoking. The odor-active compounds in frankfurters prepared from a blend of pork and beef, with and without spices (mostly pepper, mint, ginger, nutmeg, and cardamon) and smoke flavoring (hickory smoke flavoring D402V, Dalgety Food Ingredients, Dublin, Ireland), have been compared [10.58]. The odor-active compounds derived from the meat component were found to be very similar to those discussed previously (furanthiols and sulfides (**22–23** and **28–31**)), whereas those derived from the spices (α-pinene (green), 1,8-cineole (medicinal) and linalool (flowery)) were found in the commercial frankfurters and those prepared with the spice mix. A group of methoxyphenols, derived from the smoke, were highly odor-active. Of these, 2-methoxyphenol, 2-methylphenol, and 2,6-dimethoxyphenol (**49**) were found to be character-impact compounds, described by the assessors as smoky and frankfurter-like.

Poultry. Poultry flavor has been reviewed by *Dawson* and *Spinelli* [10.59], *Cerny* [10.5], and most recently by *Jayasena* et al. [10.60]. Chicken aroma has been shown to involve many of the savory and meaty compounds already discussed. However, in red meat, the meaty, sulfury notes dominate, whereas in chicken, it is the lipid-derived compounds that are more odor-active and the sulfury, meaty compounds, although still present, are far less important. *Kerler* and *Grosch* [10.61] found that the compounds with the highest dilution factor in an ether extract of boiled chicken were (*E*,*E*)-2,4-decadienal (**36**) and furaneol (**13**) (FD = 1024), followed by butanoic acid (FD = 512), sotolone (**14**), 2-furanmethanethiol (**22**), 2-acetyl-2-thiazoline (**21**), and acetic acid. Although present and odor-active in most other meat aromas, (*E*,*E*)-2,4-decadienal (**36**) is present at higher concentration in chicken aroma and is considered to contribute the species character to roast chicken. It was found to be the compound with the highest dilution factor in chicken broth [10.62] followed by the furanthiols (**22, 28**) and γ-dodecalactone (**50**). 2,4-Decadienal (**36**) is derived from linoleic acid that is present in chicken fat at higher concentrations than in pork and beef, and its oxidation product ((*E*,*E*)-4,5-epoxy-2-decenal, **51**) is also believed to contribute to fried chicken aroma.

When compared to boiled beef and pork [10.47], the odor-active compounds that distinguish chicken from beef or pork were hydrogen sulfide, hexanal (**3**), (*E*,*Z*)-2,6-nonadienal, the lack of 12-methyltridecanal (**37**), and there was a much smaller contribution from the meaty furanthiols (**22**, **28**). After quantification and formulation of a recombinate to match boiled chicken, the most odor-active compounds were methanethiol (**6**), (*E*,*E*)- and (*E*,*Z*)-2,4-decadienal, (*E*,*E*)-2,4-nonadienal and 2-furanmethanethiol (**22**).

Several alkyl-substituted trithiolanes and dithiazines have been isolated from fried chicken [10.1, 63]. They are derived from the interaction between hydrogen sulfide and aldehydes generated either from the Strecker degradation or lipid oxidation. A comparison of roasted and fried chicken by *Shi* and *Ho* [10.63] showed an increase in lipid degradation volatiles with roasting, but a decrease in 2,4-decadienal (**36**), and the Maillard reaction compounds increased significantly with frying cf. roasting. The most odor-active compounds in a fried chicken skin recombinate were found to be acetaldehyde (**64**), methanethiol (**6**), methional (**10**), and the Strecker aldehydes.

The volatile components of turkey [10.64] are similar to those of chicken and no character impact compounds have been identified which distinguish the two meats. However, turkey is more susceptible to the development of lipid oxidation and warmed-over-flavor (WOF).

Similarly, duck meat contains many of the same compounds and the list of most odor-active compounds in Bejing duck [10.65] determined by AEDA contained the same compounds as chicken, although it was particularly rich in the fatty fried alkadienals. The lipid-derived volatiles in an SPME extract of Chinese Nanjiing water-boiled salted duck [10.66] were found to be very high, accelerated by the combination of curing, brining, and roasting. *Lesimple* et al. [10.67] have reported volatiles on smoked duck fillet.

Organ Meats. Organ meat, particularly liver, is highly nutritious, low in fat, and full of vitamins and minerals, but many consumers find liver unpalatable. The first of only a few papers reporting volatiles in cooked liver was published by *Mussinan* and *Walradt* in 1974 [10.68] who identified 179 volatile compounds from a distillation extract of cooked pork liver. The classes of compounds were similar to those found in cooked meat, but the range of pyrazines and furans was far greater, probably as a result of the high glucose content of liver. Interestingly, among the 23 sulfur compounds identified, derivatives of 2-furanmethanethiol (**22**) were identified, but no derivatives of 2-methyl-3-furanthiol (**28**) were reported. One explanation is the lack of ribose, which is a key precursor, but liver contains up to 1 mg/100 g thiamine, which is also an effective precursor of (**28**). *Werkhoff* et al. [10.69] isolated 120 sulfur compounds from cooked beef liver, and reported sensory properties and MS data for several mercapto- and methylthio-substituted aldehydes and ketones. Of these, 3-mercapto-2-pentanone was described as cooked liver. *Lorenz* et al. [10.70] identified 108 compounds in different fractions of a Likens–Nickerson extract of sheep liver. No furanthiols were detected in this study, and it was suggested that the thiazoles were the most odor-active compounds. AEDA on roasted beef, pork, and duck livers [10.71] found that 2-acetyl-1-pyrroline (**19**) and 2-acetyl-2-thiazoline (**21**) had high FDs in all three roasted livers, imparting the toasted notes associated with cooked liver. A third toasty note, 2-acetyltetrahydropyridine, had a high FD only in the roasted duck liver, possibly contributing to its unique aroma.

A comparison of fried beef and pork liver from both young and old animals [10.72] using several extraction methods (Tenax trapping, SPME and steam distillation) demonstrated that the significant difference between the sheep and beef liver was the presence of unidentified methyl-branched C14, C15, and C16 aldehydes that were present in the aged and fattened beef liver, but not in the more delicately flavored calf liver or the sheep liver. These aldehydes have subsequently been identified as the group of iso- or anteiso-methylbranched aldehydes, of which 12-methyltridecanal (**37**) was identified as a character-impact compound of beef. GC-O was carried out on these extracts, and a liver-like aroma was reported in the beef liver extracts, but no compound could be assigned to that aromatic region. *Parker* et al. [10.73] recently reported the volatiles present in SPME extracts of pressure-cooked beef, pork, lamb, and chicken liver, confirming the presence of the branched aldehydes in the beef liver and showing that, analytically, the species differentiation was driven by the lipid degradation products.

Other authors have focused on the off-notes and negative attributes of cooked liver. 1-Octen-3-one (metallic), hexanol (weak metallic), 1-octen-3-ol (**4**, mushroom-like), (*E*)-2-nonenal (cardboard-like), and (*E*,*E*)-2,4-decadienal (**36**, fatty, oily) were shown by *Im* et al. [10.74] to be responsible for metallic and fishy off-notes when oxidation was induced in pork liver using oleic, linoleic, linolenic, and arachidonic acids in the presence of $FeCl_2$. These compounds can be reduced by the addition of rosemary extract [10.75].

Finnish liver sausages and liver pâtés have been compared [10.76], particularly with a view to the minimization of undesirable lipid-derived volatiles. The volatile profiles were highly characteristic of the additional ingredients, particularly the commercial products that had high levels of terpenes, derived from added flavorings, that could potentially mask the off flavors due to lipid oxidation.

One major use for cooked liver is in pet food with a global annual production of over 20 million tons. There are very few publications on pet food volatiles with only one study reporting a list of volatile compounds found in dry dog food using SPME [10.77]. There was some overlap between the compounds found in pet food and those found in meat or liver, particularly the lipid-derived aldehydes, ketones, and pyrazines, but there was a noticeable lack of sulfur compounds, reflecting the low meat content and high cereal content of many of the products. Most of the literature is in the form of patents reporting the chemical composition of palatability enhancers. Notably, butanoic acid and 3-methylbutanoic acid are reported to increase the palatability of dog food [10.78], and a range of different α-cyclic enolones can be used as palatability enhancers for dry dog food [10.79]. Various combinations of Maillard and lipid-derived products have been patented [10.80–82] and the use of herbs and spices [10.83]. Several inventors have patented particular processes, based on the Maillard reaction, for the generation of meaty aromas for use in pet food [10.84] and a multistep process has been patented by *Davidek* et al. [10.85].

10.2.5 Taste Compounds

Aroma compounds do not work in isolation. The flavor of meat is also characterized and modified by the presence of nonvolatile tastants in the meat. Those which contribute to the flavor are salts, acids (sour), sugars (sweet), and both glutamic and aspartic acid contribute to the savory/umami perception of meat flavor. The role of nucleotides such as inosine 5′-monophosphate (IMP) is essential in our perception of meaty flavor, contributing the savory and umami sensation detected on the tongue and acting as a flavor enhancer. More recently, multimodal taste enhancers have been isolated from cooked meat that have been shown to be vital in recombinates to reproduce the respective meat flavor. Alapyridaine was isolated from beef bouillon [10.86] and found to enhance sweet and umami character, whilst β-alanyl peptides identified in chicken broth gave a thick white-meat orosensation [10.87] to the broth.

10.2.6 Off-Notes

Warmed-Over-Flavor

All meats are susceptible to the generation of WOF, a distinctive off-note associated with reheated meat and particularly prevalent in re-heated poultry. In freshly boiled chicken [10.61], (*E*,*E*)- and (*E*,*Z*)-2,4-decadienal, (*E*,*E*)-2,4-nonadienal, 2-furanmethanethiol (**22**), hexanal (**3**), octanal, and acetaldehyde were found to be the character impact components; however, after refrigeration and reheating of the chicken, there was a loss of sweet chicken notes and an increase in the green, cardboard aromas. These were attributed to the sevenfold increase in hexanal (**3**) and a decrease in (*E*,*E*)-2,4-decadienal (**36**) and 2-furanmethanethiol (**22**). It is the combination of accelerated lipid oxidation and the loss of desirable notes that make the WOF so striking in reheated meat, and there are clear implications for the cook-chill industry. The mechanism is discussed in Sect. 10.3.2.

In pork, WOF was characterized by words like fishy and rancid flavor [10.88], as well as linseed oil, rubbery, and cardboard aroma, which increased as the length of storage increased. An increase in the development of WOF was also observed when the animal's diet was supplemented with iron.

The susceptibility toward the development of WOF was investigated in meat from turkey and chicken, both breast and thigh meat, and from pork longissimus dorsi muscle [10.89]. Ground meat samples from these five sources were heated for 30 min in a water bath at 60, 70, or 80 °C and stored at 5 °C for up to four days. Measurement of thiobarbituric acid-reactive substances (TBARS) and sensory evaluation showed that turkey was more susceptible to WOF than chicken, thigh meat more susceptible than breast meat, and pork was the least affected.

Pastoral Aroma in Lamb

The flavor of meat from pasture-fed sheep has a pastoral aroma to it which has been attributed to 3-methylindole (skatole, **52**) and 4-methylphenol (*p*-cresol, **53**) (Fig. 10.5). The pastoral diet, particularly when it contains lucerne and clover, contains a higher proportion of protein compared to concentrates, which is readily broken down to free amino acids by the microflora in the rumen, and subsequent anaerobic metabolism of L-tryptophan results in the production of skatole (**52**), a highly potent odor compound with a faecal farmyard aroma. Similarly, *p*-cresol is formed from the metabolism of L-tyrosine and has a manure-like aroma. Both these odor-active compounds are lipophilic and tend to deposit in the fat tissue. Addition of forage

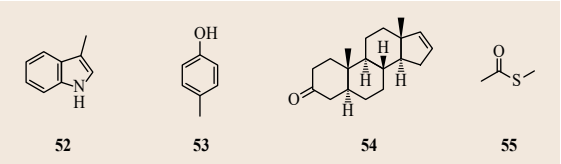

Fig. 10.5 Odor-active compounds in meat: off-notes

legumes containing high levels of polyphenols (condensed tannins) [10.90] or grape-seed extract [10.91] to the diet was found to reduce the formation of indole and skatole (**52**). In a study by *Priolo* et al. [10.92], skatole (**52**) and indole were monitored in the rumen of lambs which had been fed either herbage or concentrate with, and without, tannins. Both skatole (**52**) and indole were present at significantly higher amounts in the rumen of lambs fed on herbage, but skatole (**52**) was reduced if the feed was supplemented with tannins. The caudal fat of herbage-fed animals tended to have higher levels of indole and skatole (**52**), but this was not reflected in the sensory evaluation. Sensory evaluation did, however, show that supplementation with tannins significantly reduced the sheep meat odor.

Boar Taint

Boar taint is a problem associated with the meat from uncastrated male pigs [10.93, 94]. The compound 5α-androst-16-en-3-one (androstenone, **54**) (Fig. 10.5) is produced in the testes and transported to the sub-maxillary glands in the mouth by the blood stream. It is then released into the saliva, along with the corresponding alcohol, and volatilized into the boar's breath where it is sensed by the female who responds to the boar's advances if she is ovulating. Androstenone (**54**) accumulates in the fat of boars and this causes a taint in cooked meat. Although, amongst consumers, about 30% of males and 20% of females show anosmia to the compound, many find it extremely objectionable. In general, females are more sensitive than males, but sensitivity varies from country to country [10.95]. Those who perceive it, find the odor of androstenone (**54**) unpleasant, sweaty, urinous, and fecal. Consequently, boars for meat are slaughtered before maturity or, in some countries, they are surgically castrated usually without any pain relief. The alternative immunocastration, where vaccination against gonadotropin releasing hormone suppresses androstenone synthesis [10.96], is costly and therefore less commonly used. In the EU, due to animal welfare concerns, there has been a voluntary declaration since 2012 which, in the short term, dictates that if surgical castration is necessary, it should be carried out with prolonged analgesia and/or anaesthesia. However, in longer term (2018), it suggests that the practice of castration should be abandoned entirely and that in the meantime, research should be devoted to understanding how boar taint (and the sexual and aggressive behaviors associated with adult entire males) can be managed through breeding [10.97] and production management [10.98].

Skatole (**52**) and indole are also implicated in boar taint but, unlike androstenone (**54**) which is formed during puberty, skatole can be manipulated using the diet. Addition of fermentable carbohydrates to the feed prior to slaughter reduces the skatole in the colon [10.99]. Skatole (**54**) causes rejection at 0.2 μg/g of fat.

Others

Some off-notes in poultry are generally believed to be due to gram negative psychrotrophic bacteria and the production of H_2S [10.59] and other small sulfur molecules such as dimethyl sulfide, methyl trisulfide, and methyl thioacetate (**55**).

10.3 Mechanisms of Aroma Generation

Aroma compounds in meat are generated from the pool of precursors that exist in raw meat and develop post-slaughter. In cooked meat, the Maillard reaction is of paramount importance in generating the characteristic meaty flavor, but other pathways are also known to contribute to meaty flavor such as degradation of thiamine and ascorbic acid. Species character is generally given by the thermal degradation of lipids, and the interaction of the two can also have an impact on flavor generation. In cured products, flavor is generated via fermentation reactions which are based on the catabolism of amino acids. Again, lipid degradation is also extremely important. These pathways are discussed in the following sections.

10.3.1 The Maillard Reaction

The Maillard reaction is the basis for flavor generation in all cooked foods. It involves the reaction between a reducing sugar and an amino compound, initially forming a Schiff base, which breaks down in a cascade of parallel reactions to form not only flavor, but also color and antioxidants [10.100], as well as less desirable compounds such as acrylamide [10.101] and

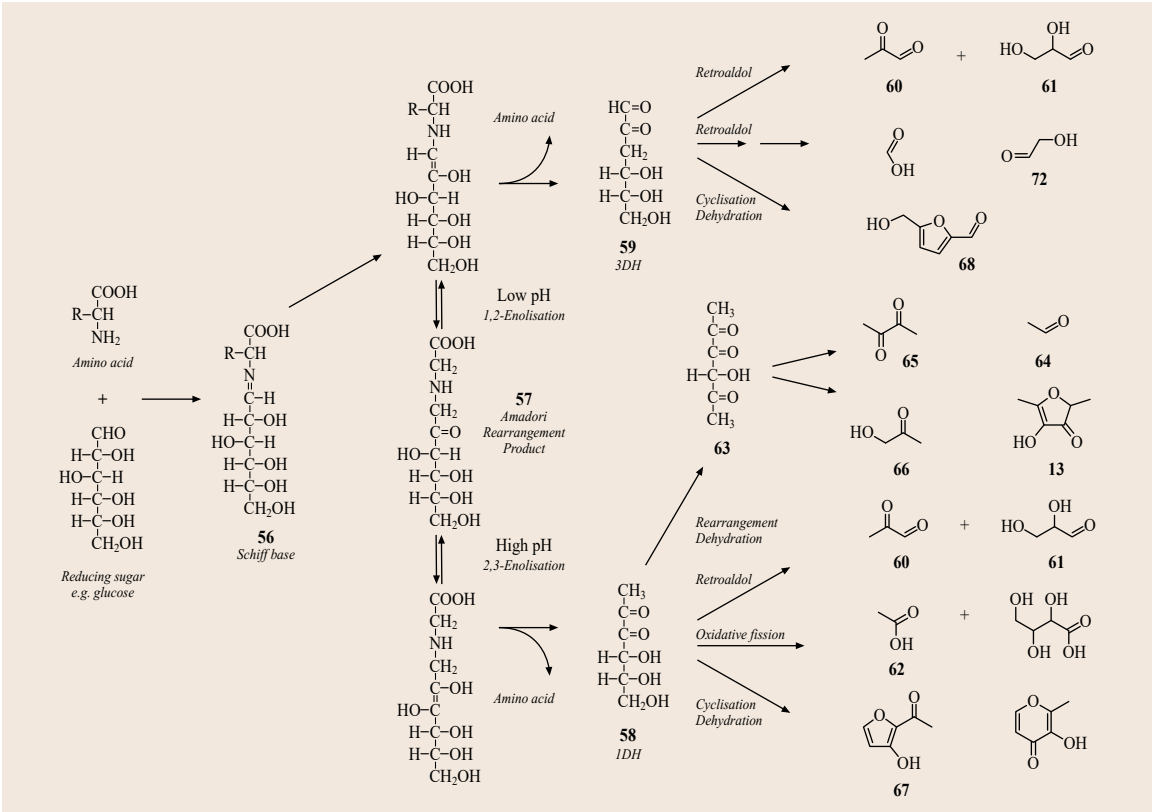

Fig. 10.6 Formation of reactive intermediates via the Maillard reaction

heterocyclic aromatic amines [10.102]. Further details of the Maillard reaction are given in Chap. 5.

The Early Stages of The Maillard Reaction

The first step involves the nucleophilic attack of the amino group on the reducing sugar to form a Schiff base (**56**) which rearranges to form the Amadori Rearrangement Product (ARP) (**57**) if the sugar is an aldose, typically ribose or glucose in meat, or a Heynes rearrangement product if the sugar is a ketose. The Maillard reaction between an amino acid and glucose is summarized in Fig. 10.6. The ARP breaks down, regenerating the amino acid and, depending on pH, forms either the 1-deoxyhexosulose (1DH, **58**) at high pH or the 3-deoxyhexosulose (3DH, **59**) at low pH [10.103]. At high pH, the deoxyhexosuloses breakdown by retroaldol or oxidative fission reactions, providing a pool of highly reactive dicarbonyl and hydroxycarbonyl compounds. For example, with glucose, retroaldolisation produces pyruvaldehyde (**60**) and glyceraldehyde (**61**), whereas oxidative fission produces acetic acid (**62**) [10.104]. Rearrangement and dehydration of the 1DH give diacetylformoin (**63**) which is the precursor for the formation of other reactive intermediates such as acetaldehyde (**64**), 2,3-butanedione (**65**), and hydroxypropanone (**66**). Some of these are odor-active in their own right (e.g., 2,3-butanedione imparts a characteristic creamy, buttery note), but they all undergo further reactions. Cyclization and dehydration of diacetylformoin give the highly odor-active 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (furaneol) (**13**). The high pH route is also important in the formation of odor-active furanones and pyranones such as 2-acetyl-3-hydroxyfuran (isomaltol) (**67**) and 4-hydroxy-5-methyl-3(2*H*)-furanone (norfuraneol) is formed by the corresponding route from pentoses. Low pH tends to favor the formation of 5-hydroxymethyl-2-furfural (HMF) (**68**) or 2-furfural (from pentoses) [10.105].

Although glucose is the major sugar present in raw meat (7–10 mmol/kg) [10.106], the aromas that it generates tend to be the cooked, roasted notes such as pyrazines and thiazoles. It is ribose, which is present at one-tenth of the concentration (0.6–1 mmol/kg), that forms the basis for the generation of characteristic meaty aromas. Pentoses can participate in the Maillard reaction, and do so faster than their hexose counterparts, certainly when color formation is considered [10.107]. When the rate constants for the first step of the Maillard

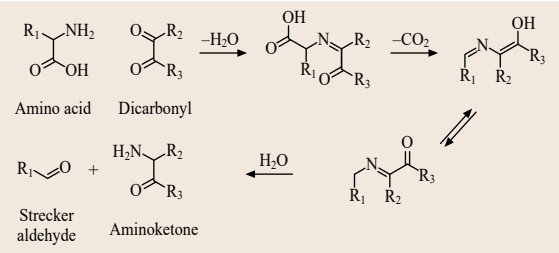

Fig. 10.7 The Strecker degradation

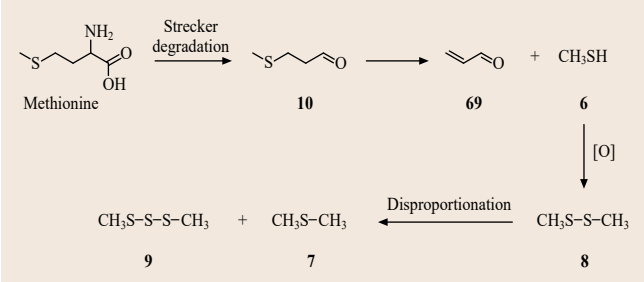

Fig. 10.8 The Strecker degradation of methionine

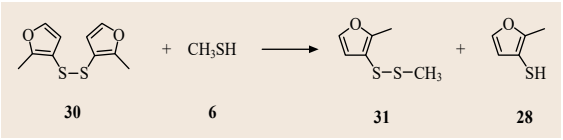

Fig. 10.9 Exchange reaction between thiols and disulfides

reaction were determined in an aqueous extract of meat heated at 130 °C, it was found that the participation of ribose was ten times faster than glucose (rate constants of 0.007 and 0.05 min^{-1}, respectively). Pentoses are less sterically hindered than hexoses and, in aqueous solution, equilibrium lies in favor of the reactive open-chain form. However, *Mottram* and *Nobrega* [10.108] showed that in unbuffered aqueous model meat systems, the major product after heating was 2-furfural, and it has been suggested from the kinetic model that 2-furfural may form directly from earlier intermediates, such as the ARP [10.109]. As a consequence, with ribose, there is a reduction in the availability of sugar fragmentation products which are critical for the Strecker degradation and for the formation of heterocyclic compounds such as pyrazines and thiazoles, and the major products are cyclization products such as 2-furfural or norfuraneol.

The Strecker Degradation

The role of the early stages of the Maillard reaction is to break down sugars into more reactive intermediates such as dicarbonyls and hydroxycarbonyls. These undergo an array of reactions which include addition reactions with H_2S and NH_3, aldol condensations, dehydration, and cyclization reactions. However, one of the most important reactions for generating flavor is the Strecker degradation, in which a dicarbonyl reacts with an amino acid to provide the corresponding aminoketone and a Strecker aldehyde, derived from the parent amino acid, but containing one carbon less (Fig. 10.7). The aminoketone, which is derived from the sugar moiety, reacts further, forming pyrazines (see below). The Strecker aldehyde, which is directly related to the amino acid, provides a characteristic aroma to the food. For example, the reaction with proline gives a toasted, bread crust aroma, whereas reaction with leucine or isoleucine gives a malty or cocoa aroma. The two most important amino acids for the generation of meat flavor are methionine and cysteine.

Methionine breaks down in the Strecker degradation to form methional (**10**) (Fig. 10.8), a compound which imparts a very characteristic baked or boiled potato aroma, but has also been found to be odor-active in beef and chicken. It subsequently breaks down to form 2-propenal (**69**) and methanethiol (**6**), and the latter readily oxidizes to dimethyl disulfide (**8**). These are all reactive intermediates: propenal (**69**) readily undergoes aldol condensation reactions and addition reactions; methanethiol (**6**) can undergo oxidation, addition reactions, and exchange reactions with disulfides such as the bis(2-methyl-3-furan) disulfide (**30**) to give the related methyl sulfide (**31**) and 2-methyl-3-furanthiol (**28**) (Fig. 10.9), thus altering the meaty profile of the sample; dimethyl disulfide (**8**) can undergo disproportionation reactions to form dimethyl trisulfide (**9**) and dimethyl disulfide (**7**) (Fig. 10.8), again altering the aroma profile.

Cysteine Degradation

Cysteine is one of the most important precursors for the generation of meat flavor. Breakdown of cysteine, via the Strecker degradation, produces a number of highly reactive intermediates with flavor generating potential (Fig. 10.10): ammonia, hydrogen sulfide, acetaldehyde (**64**), mercaptoacetaldehyde (**70**). Ammonia and the hydrogen sulfide react with the pool of reactive carbonyl compounds to form heterocyclic aroma compounds such as pyrazines, thiophenes, and thiazoles which are important for the development of cooked notes. The formation of thiazoles requires the substitution of ammonia into an aldehyde, and hydrogen sulfide into a dicarbonyl or hydroxycarbonyl (Fig. 10.11). Condensation of the resulting mercaptoketone and the imine produces a thiazoline which undergoes oxidation to form the corresponding thiazole.

Hydrogen sulfide and acetaldehyde (**64**) combine to form dimethyltrithiolanes, or when ammonia is involved, dithiazines can be formed (Fig. 10.12) [10.110].

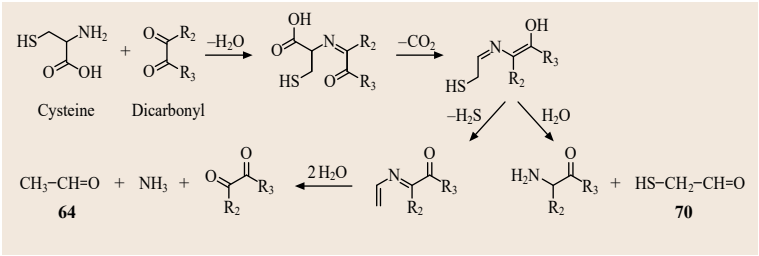

Fig. 10.10 The Strecker degradation of cysteine

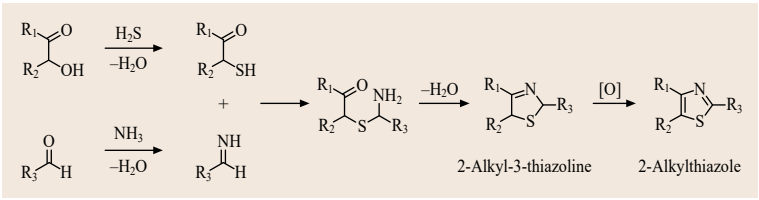

Fig. 10.11 Formation of thiazoles

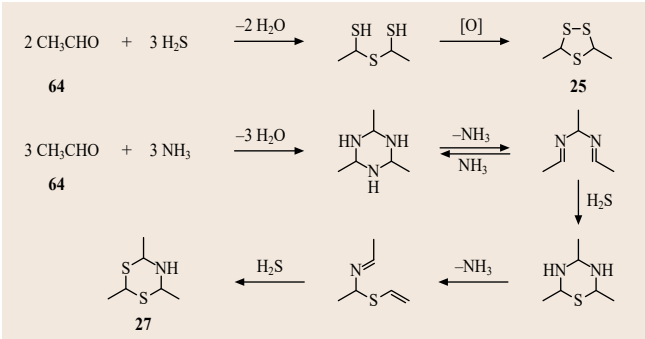

Fig. 10.12 Formation of 3,5-dimethyl-1,2,4-trithiolane and thialdine

Other aldehydes can participate in the reaction so, for example, if 3-methylbutanal is present, one or more of the methyl groups can be replaced by a methylpropyl group. Mercaptoacetaldehyde (**70**), which retains part of the carbon skeleton of the cysteine, is also highly reactive and forms thiophene, whilst the nitrogen analog cysteamine produces thiazoles [10.111].

One critical role of cysteine is to provide H_2S for the formation of the furanthiols (**22, 28**). 2-Methyl-3-furanthiol (**28**) is formed from a number of routes, and one of those is from a pentose sugar [10.112]. The early stages of the Maillard reaction give 1-deoxypentosulose, which in its tautomeric form, cyclizes losing water to form norfuraneol (**71**). *Whitfield* and *Mottram* [10.113] reported the formation of 2-methyl-3-furanthiol (**28**) in a model system containing cysteine and norfuraneol (71) at pH 4 and proposed that norfuraneol was a key intermediate (Fig. 10.13). The first step in the generation of (**28**) from norfuraneol involves a reduction and thereafter a dehydration step followed by the addition of H_2S and removal of water. However, *Cerny* and *Davidek* [10.114] carried out a similar reaction in the presence of $^{13}C_5$ ribose and demonstrated that 93% of the 2-methyl-3-furanthiol (**28**) was derived from ribose, rather than from the unlabeled norfuraneol, and proposed an alternative route via a 1,4-deoxypentosulose (**72**). Furthermore, low levels of 2-methyl-3-furanthiol (**28**) can be found in glucose/cysteine mixtures [10.112]. One possible route is via norfuraneol which can be produced from the aldol condensation of pyruvaldehyde (**60**) and hydroxyacetaldehyde, both of which can be derived from glucose. However, *Hofmann* and *Schieberle* [10.112] demonstrated in a model system that the aldol condensation between these two aldehydes preferentially formed 2-furfural rather than norfuraneol, and there was a more favorable route to (**28**) via the condensation of hydroxyacetaldehyde (**73**) and mercapto-2-propanone (**74**) which can be formed from H_2S and pyruvaldehyde (**60**). The initial step is an aldol condensation which can produce two isomeric mercaptoketones. One cyclizes and dehydrates to form 2-methyl-3-furanthiol (**28**) (Fig. 10.13), whereas the other forms 2-furanmethanethiol (**22**). In this model system at pH 5, the formation of (**28**) from thiamine was not favored.

However, reactions in meat are influenced by the other components of the matrix. Ho has looked at incorporating other sources of nitrogen such as other amino acids, urea, and carnosine. Addition of urea, which is naturally present in beef and pork at 20–40 mg/kg [10.115], releases ammonia into the system and raises the pH, thus favoring pyrazine formation and producing more roasted and buttery notes. Addition of carnosine had a similar effect [10.116], decreasing the formation of meaty sulfur compounds such as 2-methyl-3-furanthiol (**28**) and increasing pyrazine formation. Alternative sources of sugars have also

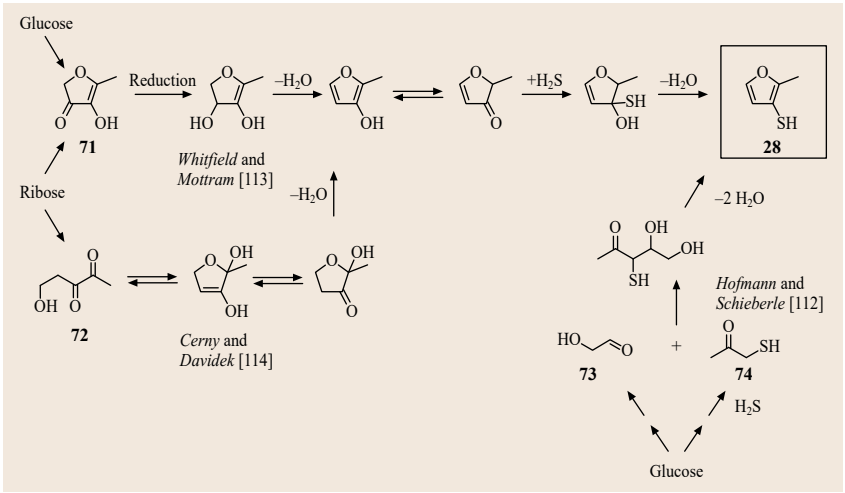

Fig. 10.13 Formation of 2-methyl-3-furanthiol from sugars

been studied. *Nobrega* and *Mottram* [10.117] included both inosine-5′-monophosphate (IMP) and ribose-5-phosphate in a cysteine-containing model system and showed that the IMP was relatively unreactive but ribose-5-phoshate contributed to the formation of the 2-methyl-3-furanthiol (**28**). Ribose phosphate provides a direct route to norfuranol, bypassing the first stage of the Maillard reaction [10.118].

Whitfield and *Mottram* [10.113] also reported the formation of 26 different disulfides, several containing the 2-methyl-3-furan moiety. These require an oxidation step, but given that the conditions in the model were highly reducing (H_2S), they proposed that the redox system involved in the formation of the disulfides was 2,3-butanedione, which could be reduced to 3-hydroxybutanone, providing the oxidation required for the formation of disulfides. However, *Baines* et al. [10.119], based on the large number of disulfides found in a cysteine/ascorbic acid model system compared to ribose/cysteine model system, suggested that it might be dehydroascorbic acid providing the oxidative step.

Pyrazine Formation

Simple pyrazines are formed from the condensation of two amino ketones (generated during the Strecker degradation) and subsequent oxidation (Fig. 10.14). However, the more substituted pyrazines, which are important in meat flavor, are more likely to be generated from the nonoxidative route in which one of the substituents comes from an aldehyde [10.120]. In the case of 2-ethyl-3,5-dimethylpyrazine (**15**), it may be that the methyl group is derived from formaldehyde or the ethyl group from acetaldehyde. One important source of these aldehydes is the Strecker degradation, with formaldehyde being derived from glycine and acetaldehyde from alanine and cysteine. Acetaldehyde can also be generated from lipid oxidation.

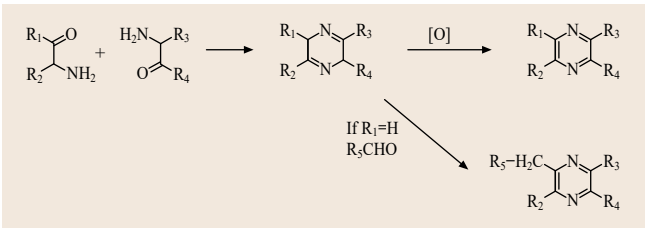

Fig. 10.14 Formation of pyrazines

Ascorbic Acid

The nonenzymic browning of ascorbic acid is well known and is partly due to Maillard-type reactions. Degradation of ascorbic acid can provide a pool of intermediates containing reactive dicarbonyls and hydroxycarbonyls – amongst these are glyoxal, pyruvaldehyde (**60**), and 2,3-butanedione [10.121]. *Vernin* et al. [10.122] has shown a pathway for the degradation of ascorbic acid to 1-deoxyopentosulose (**58**). These compounds are typical of those formed during the Maillard reaction and react further to form hundreds of odor-active compounds. Recently, *Yu* and *Zhang* [10.123] reported the volatiles generated when ascorbic acid and cysteine were heated in aqueous buffer at 141 °C at different pHs ≥ 5. Many of these were compounds that can contribute to meat flavor such as thiophenes, thiazoles, pyrazines, and cyclic sulfur compounds. However, using DHE rather than SPME, *Parker* et al. [10.124] showed that 2-methyl-3-furanthiol (**28**) and many related disulfides were formed in buffered model systems containing ascorbic acid and cysteine. She suggested that these are formed directly from ascorbic acid via 1DO, bypassing the early stages of the Maillard reac-

tion and avoiding the possible formation of the semistable 2-(1,2,3,4-tetrahydroxybutyl)thiazolidine-4-carboxylic acid which is readily formed between ribose and cysteine [10.125].

Process Reactions

Process reactions are designer Maillard reactions performed outside the food environment from highly tailored precursors, chosen to target specific compounds, groups of compounds and aromas. They are carried out using typical Maillard precursors under carefully controlled conditions of time, temperature and pH which mimic conventional cooking but have been optimized to maximize the desired flavor. They are widely used in the food industry, particularly to flavor meat products and meat-flavored soups and snacks. Meat process reaction flavors typically contain a pentose (or a source of pentose, such as ribonucleotides) and cysteine, but often contain other amino acids which catalyze the early stages of the Maillard reaction and can also modulate the flavor. The first such system was patented by *Morton* et al. [10.126] and, since then, hundreds have followed. Their complexity has developed over the years as significant advances have been made in understanding the chemistry of meat flavor and the interactions of aroma compounds with other components of the food matrix. Process reactions have become quite sophisticated with the use of phospholipids, nucleotides, thiamine, protein hydrolysates, yeast extracts, hydolyzed vegetable proteins (HVPs) and even specific Maillard intermediates, spices, flavor enhancers, and glutamic acid or monosodium glutamate (MSG). Often carried out in highly concentrated format, these give rise to hundreds of sulfur-containing compounds, many of which are highly potent odorants. *Hofmann* and *Schieberle* [10.127] used AEDA to identify the most potent odorants in a buffered ribose cysteine process reaction. The most potent in the extract were the two meaty thiols (**22**, **28**) and 3-mercapto-2-pentanone (**12**) and the remaining 25 were either sulfur compounds or one of the potent sugar degradation products (furaneol (**13**), norfuraneol, and sotolone (**14**)). Process flavors have been reviewed recently by *Cerny* [10.128].

10.3.2 Lipid Oxidation

Quantitatively, the volatile profile of cooked meat is dominated by lipid degradation products, but the odor thresholds tend to be much higher than those from compounds derived from the Maillard reaction. They do, however, play a major role in meat flavor and tend to give the species character to cooked meat. Subcutaneous fat is one obvious source of lipids, but the lean tissue also contains intra- and intermuscular triglycerides and structural phospholipids [10.129] which also contribute to flavor formation. Lipid oxidation contributes to the desirable aroma of meat, but also causes many of the off-odors associated with reheated meat (or WOF) and rancidity developed during the prolonged storage of either the raw or cooked meat.

Lipid Oxidation Mechanism

The mechanism of lipid oxidation has been reviewed by *Frankel* [10.130, 131] and more details are given in Chap. 11. It is generally believed to occur via a free radical-induced chain reaction that occurs in three phases: initiation, propagation, and termination. During the initiation phase, hydrogen is abstracted from the lipid to leave a lipid radical, which reacts with molecular oxygen to form a lipid hydroperoxide (Fig. 10.15). Hydrogen abstraction from unsaturated fatty acids forms more stable radicals due to conjugation of the radical with the double bonds, and unsaturated fatty acids are, therefore, more prone to oxidation, particularly at low temperatures. Saturated lipids are relatively stable by comparison, and their degradation becomes more important at higher temperatures, that is, during thermal processing.

The second phase involves the breakdown of the lipid hydroperoxides by the cleavage of the C–C bond on one side or other of the radical. Because there is no double bond to guide the initial abstraction of hydrogen, saturated fatty acids degrade to form series of alkanes, alkanals, and alcohols with a range of chain lengths; however, the major products from triglycerides such as tristearin are medium chain (C6–C10) aldehydes, alcohols, alkanes, carboxylic acids, and lactones.

With monounsaturated fatty acids (MUFAs), the degradation is more directed. In oleic acid, for example, the abstraction of the hydrogen can happen more readily at the position alpha to the double bond at either carbon 8 or 11 (Fig. 10.15). At carbon 8, the resultant allylic radical can tautomerize across the conjugated double bond to form the radical on C10. Likewise, both C11 and C9 radicals can be formed from abstraction at C11 and the corresponding hydroperoxides are formed at C8, C9, C10, or C11 in approximately equal amounts [10.130]. Each of these peroxides can cleave on either side of the hydroperoxide (A or B), resulting in the formation of medium chain aldehydes, 2-alkenals, alkanes, and alkenes. The dominant pathways are influenced by the reaction conditions and by the relative stability of the lipid radicals and the compounds that are formed. Model systems [10.132] showed that the major degradation product of oleate at ambient temperatures was nonanal (40–60 wt %), whereas at temperatures more relevant to roasting and frying (192 °C), nonanal (22%), 2-decenal (17%), and

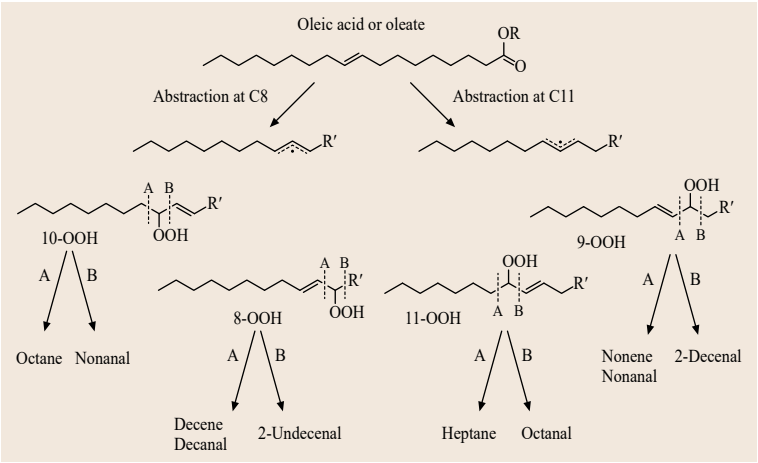

Fig. 10.15 Oxidation of oleic acid

2-undecenal (11%) were the major products which all impart fatty, but fruity notes, atypical of cooked meat. These three aldehydes are formed from the β cleavage of the 10-OOH, 9-OOH, and 8-OOH hydroperoxides, respectively (Fig. 10.15).

The most reactive fatty acids are the polyunsaturated fatty acids (PUFAs), estimated to be about 10 times more reactive than the MUFAs which, in turn, are 100 times more reactive than the fully saturated fatty acids. For PUFAs such as linoleic acid, there are more possibilities for the abstraction of the hydrogen, but abstraction at C11 is much more favored because it is alpha to two double bonds. Tautomerism of the resulting radical gives stable radicals at both C9 and C13 so this is the favored position for the formation of hydroperoxides. Subsequently, cleavage can occur at A or B, resulting in the formation of alkanals, 2-alkenals, 2,4-alkadienals, and cyclization products such as 2-alkylfurans. In the model system [10.132] at moderate temperatures, hexanal (**3**) (formed from β cleavage of the 13-OOH) was the major product quantitatively from trilinolein (50%) and linoleic acid (66%), although organoleptically the most important was the (*E*,*Z*)-2,4-decadienal (6%), which imparts a fatty fried note. However, when the methyl linoleate was irradiated under similar conditions, both (*E*,*E*)- and (*E*,*Z*)-2,4-decadienal were formed in greater amounts (9 and 19%, respectively), formed from the B-cleavage of the 9-OOH. At 192 °C, trilinolein produced similar amounts of pentane, hexanal, 2-heptenal, and 2,4-decadienal, emphasizing the point that under different processing conditions, the balance of the lipid degradation pathways changes.

Linolenic and arachidonic acids provide yet more possible pathways, leading to several unsaturated aldehydes. Under moderate conditions, the major oxidation product of linolenic acid (ω-3) was (*E*,*Z*)-2,4-heptadienal (40%) followed by (*Z*)-3-hexenal (11%) and 2,4,7-decatrienal (11%), but in the presence of haem, propanal, (*Z*)-2-hexenal, and 3,5-octadien-2-one were the major products. At 250 °C, ethyl linolenate formed 2-ethylfuran and a range of ethyl esters. The degradation products of arachidonic acid (ω-6) were similar to those of linoleic acid (hexanal, (*Z*)-2-heptenal, and (*E*,*Z*)-2,4-decadienal). Given the relative reactivity of PUFAs compared to their more saturated counterparts, and the high odor activity of many of the compounds formed, it is often degradation of the PUFAs that has the greatest impact on the volatile profile. The free fatty acid profile of the raw material, therefore, has a major role in the development of the aroma profile, and this is particularly relevant for the aroma of meat where many factors, particularly species, diet, and breed, can influence the fatty acid composition of the animal.

The Role of Phospholipids

The PUFA content of triglycerides of both the lean muscle and the fat of lamb and beef is only 1–2% [10.133] although pork is relatively rich in linoleic acid (14%) [10.134]. However, the PUFA content of phospholipids is much higher. Phospholipids contain a triglycerol backbone with just two ester linked alkyl chains, the third position being occupied by a polar phosphate group. They are an integral part of cell membranes and the increased unsaturation renders them more prone to oxidation, especially when released from the cell membrane into an aqueous environment where they come into contact with potential catalysts (iron). In pork, the phospholipids contain about 30% linoleic acid and 10% arachidonic acid (C20 : 4 (n-6)), whereas the figures are 22 and 10%, respectively, for beef and 12 and 5% for sheep. Hence, the phospholipids play a far greater role than the triglycerides in the development of flavor in cooked meat.

The role of phospholipids in flavor generation has been reviewed by *Mottram* [10.129]. He showed that removal of the polar lipid fraction containing the phospholipids resulted in a significant decrease in the aliphatic lipid degradation products, indicating that the phospholipids were a major source of lipid oxidation products – many of which are odor-active. Many studies since have confirmed the role of the phospholipids [10.135, 136].

Warmed-Over-Flavor

The phospholipids are also believed to play a role in the development of warmed-over-flavor (WOF), the distinctive off-note associated with reheated meat. In raw meat, the phospholipids are still contained within the cell membranes, and are only released as the meat is heated. Thus, when the meat is heated a second time, they can readily undergo further oxidation, catalyzed by traces of iron released from haem pigments during the initial cook [10.129].

Mitigation strategies have been reviewed by *Mielche* and *Bertelsen* [10.137]. Generally, the use of the freshest possible meat minimized oxidation processes in the raw material, and the addition of antioxidants in the form of free-radical scavengers or chelators reduced the development of WOF. Chelating agents such as polyphosphates and pyrophosphates are also effective, acting by chelating the metal ions, particularly haem-derived, that initiate the oxidation reactions. The reduction of storage time and temperature reduces the development of WOF; however, the use of high temperatures during processing seems to retard the development of WOF, and this is believed to be a result of the antioxidant properties of Maillard reaction products which are formed at higher temperatures. Excluding oxygen and light is an important factor to consider, and the addition of antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisoles (BHAs) effectively extend the induction period by absorbing free radicals. However, tighter regulations means that natural antioxidants such as ascorbate, tocopherols (Vitamin E), β-carotene, and rosemary extract are often applied. Application of rosemary extract was found to suppress both lipid oxidation and the Maillard reaction in a cooked beef extract, and this antioxidant activity was attributed to carnosic acid and rosemanol [10.138]. Addition of ground canola seeds to ground pork patties significantly inhibited WOF [10.137]. Application of natural smoke can also prevent oxidation and imparts additional flavor.

There are many instances where these natural antioxidants have been fed pre-slaughter; for example, a study carried out by *O'Sullivan* et al. showed that by incorporation of vitamin E into the feed of hogs, the freshness was maintained for longer and the development of WOF was reduced [10.88].

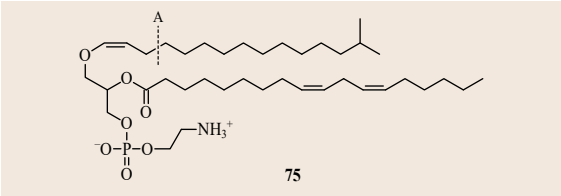

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Fig. 10.16 Structure of a beef plasmalogen

Role of Plasmalogens

Plasmalogens are also important for flavor generation. They are phospholipids that are based on phosphatidylethanolamine (rather than phosphatidylcholine which is the major component of lecithin), and contain a vinyl ether linkage in place of one of the ester linkages (**75**) (Fig. 10.16). As with the oxidation of unsaturated ester linkages, hydrogen abstraction occurs at the position alpha to the double bond and cleavage of the ensuing hydroperoxide produces acetaldehyde (**64**) and a branched aldehyde, such as 12-methyltridecanal (**37**) and the longer chain analogs. They are predominantly found in the internal organs of animals, but high levels of 12-methyltridecanal (**37**) have been found in the rumen of cattle [10.139] where it is reabsorbed by the animal and transported to the muscle tissue and incorporated into the phospholipids. In pork, the ether linkages lack the methyl branch and the corresponding straight chain aldehydes are formed.

Interactions Between Lipid Oxidation and the Maillard Reaction

Additionally, in cooked meat, the interaction between the Maillard reaction products and the lipid degradation products is important for moderating the flavor [10.140–143]. *Mottram* and *Edwards* [10.144] initially showed that when phospholipids were removed from meat before cooking, pyrazine levels increased in the cooked meat, suggesting that the products of lipid oxidation were interfering with the progress of the Maillard reaction. In model systems containing phospholipids and Maillard precursors (ribose and cysteine), fewer aldehydes were formed when the Maillard precursors were present, but there was no effect on other classes of lipid degradation products [10.145]. This reduction in aldehydes was attributed to their removal from the system by reaction with free amino groups, H_2S, or free thiol groups. Later work in model systems [10.141] confirmed that long-chain alkylthiophenes and alkylthiazoles could be generated from phospholipids, cysteine, and ribose model systems, and these compounds have been reported in cooked meat. *Elmore* et al. [10.143]

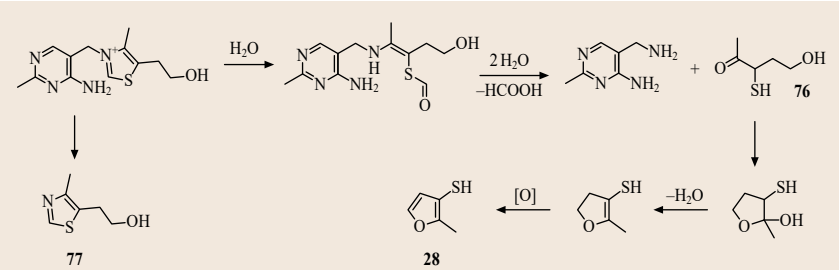

Fig. 10.17 Thermal degradation of thiamine

confirmed the role of the PUFAs in generating a series of 2-alkyl-substituted thiazoles and thiazolines, which were present at much higher levels in beef steaks from animals fed fish oil supplements compared to the control diet. Model systems confirmed that these could be formed from reactive Maillard intermediates, such as dicarbonyl and hydroxycarbonyls in the presence of ammonium sulfide and C3–C9 aldehydes. Thus, an increase in lipid oxidation could effectively remove reactive intermediates from the system which would otherwise participate on the Maillard reaction to form roasty, meaty aromas. Likewise, alkyl substituted thiophenes and thiapyrans were found in cooked beef and lamb, present at higher levels in meat from animals which had been fed a diet high in n-3PUFAs [10.146]. Model reaction confirmed that these were formed from the reaction of 2,4-alkadienals with H_2S, thus removing the H_2S from the system and reducing the amount of H_2S available to participate in the Maillard reaction, and in key flavor forming reactions. Neither the alkylthiapyrans/thiophenes nor the alkylthiazoles/thiazolines were found to be particularly odor-active.

10.3.3 Thiamine Degradation

Thiamine is present in raw meat at relatively high concentration (0.1−1 mg/100 g) and is prone to thermal degradation. Under acidic conditions (Fig. 10.17), the thiazole ring opens and 5-hydroxy-3-mercapto-2-pentanone (**76**) is released [10.147]. This is a particularly reactive molecule, but its presence in model systems was confirmed by *Cerny* and *Guntz-Dubini* [10.147]. It generates a number of low molecular weight sulfur-compounds [10.148, 149] many with rubbery, sulfury odors and some with vegetable, meaty aromas. One of the most potent is 2-methyl-3-furanthiol (**28**), the key meaty thiol, which is formed from the cyclization of 5-hydroxy-3-mercapto-2-pentanone (**76**) followed by loss of water and an oxidation step. This is a third route by which this highly potent meaty compound can be formed. *Grosch* et al. [10.150] showed the importance of the thiamine route compared to the ribose/cysteine route by directly comparing the two in model systems. An aqueous solution of L-cysteine (0.65 mM) and ribose (1.34 mM) produced only 0.2 µg/L of 2-methyl-3-furanthiol (**28**), whereas when ribose was replaced with thiamine at a very low concentration (0.06 mM), the yield increased 1500 times to 300 µg/L. *Güntert* also studied the compounds formed when meat model systems containing thiamine, ribose, cysteine, and ascorbic acid in various combinations were heated together [10.148]. Over 60 sulfur compounds were identified including thiophenes, thiols, sulfides, mercaptoketones, thianes, dithianes, thiolanes, dithiolanes, and three fused ring compounds including 1-methylbicyclo[3.3.0]-2,8-dioxa-4-thiaoctane. Of these, mercaptopropanone, 2-methyl-2-tetrahydrothiophenethiol, 3-acetyl-1,2-dithiolane, and 1H-pyrrolo[2,1,c]-1,4-thiazine were described as meaty. Details of the formation pathways have been elucidated by *Cerny* [10.151] using isotopically labeled starting materials.

Under acidic conditions, the thiazole ring remains intact, and 5-(2-hydroxyethyl)-4-methylthiazole (sulfurol, **77**) is produced [10.152]. Although this is reported to have meaty qualities, pure sulfurol has very little odor and the meaty notes are likely to be due to the presence of impurities, such as 4,5-dihydro-2-methyl-3-furanthiol [10.153].

10.3.4 Fermentation

In contrast to thermal generation of flavors in cooked meat, flavor formation during fermentation is controlled by enzymic processes [10.154–157]. The curing of meats is time consuming, and understanding the flavor formation is critical to the industry in order to shorten ripening periods in aid of more efficient processes [10.158]. The ripening or curing process is susceptible to changes in time, temperature, starter culture [10.159], position in the joint [10.160] and curing agents [10.161].

Amino Acid Catabolism

Post slaughter, the major components of muscle meat undergo proteolysis (14.4.2) generating small peptides and free amino acids. The first stage in amino acid

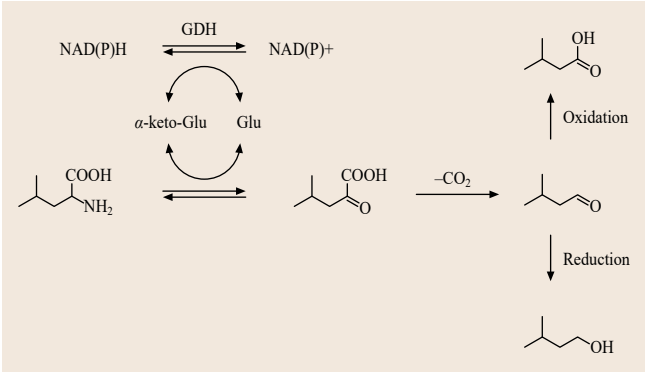

Fig. 10.18 Catabolism of leucine during fermentation

catabolism is the removal of the amino group by aminotransferases and the formation of the corresponding α-keto acids (Fig. 10.18) [10.162]. Further loss of CO_2 produces the aldehyde (which corresponds to the Strecker aldehyde) and this can be oxidized to the corresponding acid or reduced to the corresponding alcohol. During the processing of dry-cured ham [10.163], the free amino acid pool comprises predominantly alanine (Ala), leucine (Leu), valine (Val), arginine (Arg), lysine (Lys), glutamic acid (Gla), and aspartic acid (Asp). In the case of Leu and Val, catabolism gives the respective Strecker aldehyde – 3-methylbutanal or methylpropanal, compounds which contribute to the delicate aroma of cured meats. Further oxidation gives the respective acids, known more for their cheesy characteristics. However, the conversion of acids to esters gives the fruity notes which are a desirable characteristic of the final product. Catabolism of methionine produces methanethiol which can react with carboxylic acids to form potent thioesters which are desirable and fruity at low levels. Catabolism of tyrosine and tryptophan can produce the exceptionally potent compounds *p*-cresol (**53**) and skatole (**52**), respectively. At very low levels, these can be desirable but, if their formation is not halted, they can impart highly objectionable medicinal, phenolic and fecal, farmyard notes, respectively. Addition of cocktails of free amino acids prior to the fermentation of sausages was shown to increase the catabolism of amino acids and increase the concentration of the respective aldehydes. Use of just valine, isoleucine, and leucine produced a sausage with better sensorial quality [10.164].

Lipolysis and Lipid Oxidation

In subcutaneous fat and intramuscular fat, lipases are responsible for the breakdown of triglycerides into free fatty acids, and subsequent oxidation, initiated by lipoxygenases, results in the generation of aroma compounds, such as hexanal (**3**), 3-hexenal, 1-octen-3-one, and 1-octen-3-ol (**4**). The mechanism is a three-phase radical reaction, similar to those shown for the thermal degradation of lipids, but the dominant pathways will be different. The formation of the radical in the initiation stage is catalyzed by muscle lipoxygenases as well as light, moisture, and metal cations. In the structural phospholipids, a similar process is carried out by phospholipidases. Lipolysis occurs with the endogenous enzymes, but *Hierro* et al. [10.165] demonstrated in fermented sausages that the application of various starter cultures, such as *Lactobacillus plantarum* 4045 or *Staphylococcus* sp., enhanced the process. Enzymic reactions are influenced by time, temperature, pH, water activity, redox potential, salt, and ascorbic acid [10.166]. One example is the role of calcium chloride which has been shown to inhibit the formation of lipid-derived volatiles but promote the formation of branched alcohols and ethyl esters, thus reducing off-notes and increasing the desirable aroma [10.167].

10.4 Factors Affecting Meat Flavor

The factors affecting meat flavor are numerous, and this section can only begin to summarize some of the extensive literature in this area, focussing on those studies where there are volatile data to back up the sensory and consumer results.

10.4.1 Pre-Slaughter Handling – Diet and Breed

Pre- and post-slaughter factors play a major part in flavor generation and have been the subject of many papers and reviews. Pre-slaughter factors, such as diet, age, gender, slaughter weight, fat levels, stress and conditioning all have an influence on consumer acceptability and sensory evaluation [10.168] and these differences can be correlated with changes in the volatile profile of the meat.

The Evolution of Flavor in Animal Husbandry

Ever since the domestication of animals 10 000 years ago, communities have sought to improve the flavor of meat by selective breeding and a careful choice of pastureland. During the agricultural revolution of the eighteenth century, improvement focused primar-

ily on yield and other elements of efficiency (such as increasing herd survival rates and maximizing the value of by-products such as tallow). However, even at the height of the movement to use breeding to improve output, there was an interest in improving the flavor of meat. The great breeding experimenter Robert Bakewell for instance, whilst interested principally in breeding sheep able to yield more wool, was not indifferent to the claims of breeding to improve flavor [10.169]. *Young* [10.170], noting the impact of Bakewell's work on mutton quality in 1804, commented on the fact that the effect of selective breeding on flavor was understood even in the kitchens of Eton College. Later writers, such as *Low* [10.171] reflected that the most successful of the new sheep breeds of the period (the Southdown cross, which with a broader back and deeper carcass produced more meat, tallow, and wool) was conspicuously improved in meat flavor too.

It was not until the 1850s that flavor became a dominant element in breeding and husbandry [10.172], and then largely due to the declining significance of non-meat animal products in domestic trade. The science of breeding, such as it was, began to take an interest in the effect on meat quality and meat flavor, and – through the new agricultural chemistry of the 1860s – in minimization of taints and off-notes, the kinetics of cooking, and the effect of preservation techniques on flavor. By the last quarter of the nineteenth century and into the twentieth, as *Perren* has shown [10.173], selective breeding for flavor in the extensive hacienda and prairie farms of the New World was responding increasingly to flavor requirements, and flavor fashions, in European dining rooms. By the mid-twentieth century, with the development of new forms of preservation and canning alongside better transportation and logistics for shop and, ultimately, supermarket delivery, flavor became a vital element in competitive meat production systems across the world.

This prompted activity amongst the research institutes, where laborious derivatization techniques and column chromatography were used to characterize the aroma of cooked meat from different breeds of sheep [10.174] and other animals [10.175]. A review was published by *Lawrie* [10.176], and thereafter, the number of publications escalated as advances in technology provided capillary GC and an increase in the sensitivity of several orders of magnitude. The influence of diet and breed continues to challenge the scientific community and is often used as a marketing opportunity by retailers and restaurateurs to denote premium products. Grass-fed beef, corn-fed chicken, and saltmarsh lamb often feature on more exclusive menus and the flavor of the finest Iberian ham, jamón ibérico de bellota, is attributed to the acorn diet of the free-roaming pigs. In the UK, there is a return to the more flavorsome traditional breeds of pig such as Hampshires, Gloucester Old Spots, and Tamworth, and rare breeds of lamb are often selected for their superior flavor. Understanding the chemistry underpinning the differences in flavor produced by the different breeds, or diet, helps to optimize nutritional and organoleptic quality whilst maintaining efficient production systems.

Beef

Sensory and consumer trials [10.168] indicate that the breed of animal plays a part in the flavor of beef steaks, and most of this is attributed to differences in fat. The age of the animal plays a bigger role, with animals over 30 months old tending to have too intense a flavor and the acceptability decreases. Diet has a significant impact, and this is attributed to changes in the fatty acid profile. Forage-based diets, such as grass and hay are high in ω-3 fatty acids, such as linolenic acid, whereas cereals tend to have higher levels of ω-6 fatty acids (linoleic acid). Comparison of the volatile profile of grilled steaks (*M. longissimus lumborum*) from animals fed either concentrate or silage [10.177], showed that typical ω-6 lipid-derived compounds, such as hexanal (**3**), 1-octen-3-ol (**4**), and 2-octen-1-ol were four times higher in the meat from animals fed concentrate. This correlates well with the higher concentration of ω-6 fatty acids in the meat from the same concentrate-fed animals. Linoleic acid increased from about 60 to 140 mg/100 g in both Aberdeen Angus and Holstein Friesian.

Lamb

The acceptance of lamb flavor is influenced by previous exposure and the accepted norm. In Australia, where animals are pasture fed, there is a distinctive skatole (**52**) note derived from the feed, which is considered desirable in Australia but unacceptable in other countries where the normal diet is concentrates. The results of consumer studies are, therefore, not always in agreement, but descriptive sensory panels have consistently found differences between feeding regimes [10.43]. Sheep meat flavor and odor are higher in meat from pasture-fed animals compared to grain-fed, and are correlated with high levels of skatole (**52**), 2,3-octanedione, terpenes, and diterpenoids, whereas the grain-fed have higher levels of lipid-degradation products and branched-chain fatty acids, the latter formed in the rumen from the propionate-rich grain diet. A sensory study, using grilled meat from 96 castrated male Corriedale lambs [10.178] fed finishing diets with different proportions of pasture and concentrate, revealed a preference among Euro-

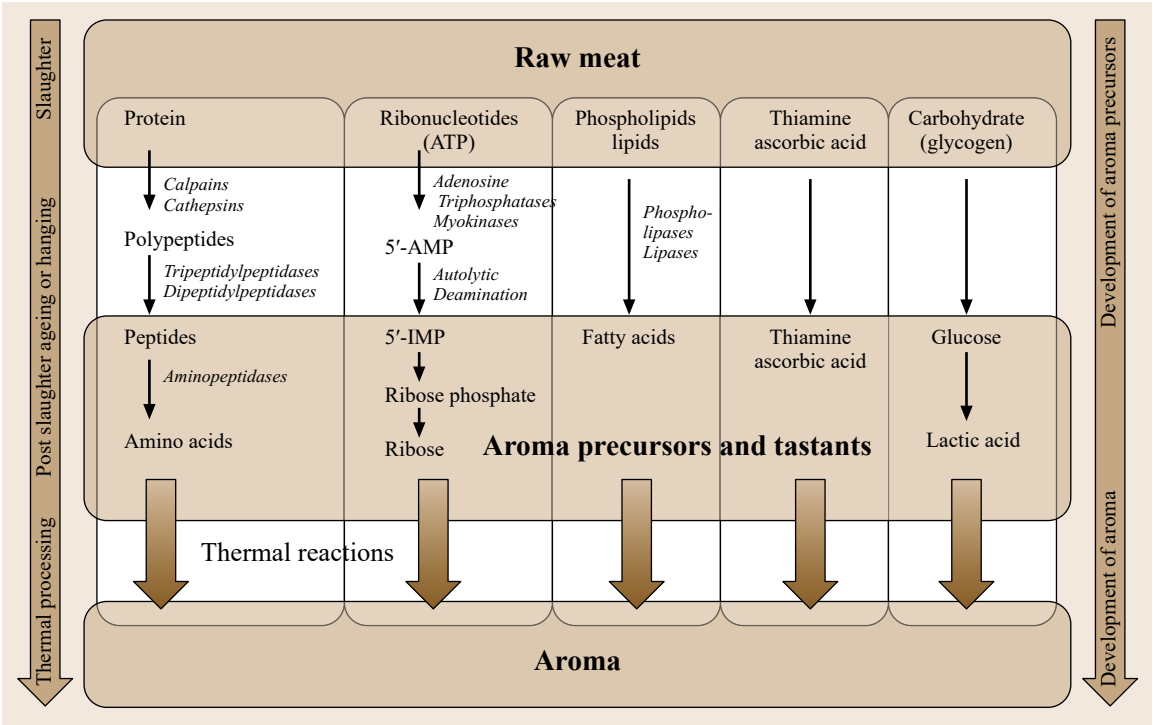

Fig. 10.19 Generation of tastants, aroma precursors, and aroma from raw meat

pean consumers, for the meat from the concentrate-finished animals. There was a corresponding reduction in rancid notes and an increase in typical lamb flavor. Volatile analysis of these samples showed an increase in lipid oxidation in the meat from the concentrate-fed animals (and, therefore, no correlation with the rancid aroma), but 2,4-heptadienal, derived from ω-3 fatty acids was higher in the meat from pasture-fed animals.

The pasture species can also influence flavor and acceptability [10.43]. Sensory panels have found stronger flavors linked to white clover and lucerne (cf. rye grass) and alfafa (cf. corn and soybean), and these flavors become objectionable at high concentrations. A strong meat odor was produced from a perennial diet of ryegrass which was acceptable only to some consumers, and a diet of green oats produced a pungent meat flavor [10.179]. Feeding brassica and rape tend to get low acceptability scores.

Dietary supplements are often incorporated in feed to enhance the nutritional properties and can have a substantial influence on the aroma profile of sheep meat. *Elmore* et al. studied the effect of five dietary supplements on aroma generation in lamb [10.180]:

1. Linseed oil
2. Fish oil
3. Protected lipid high in linoleic acid (C18 : 2 (n-6)) and α-linolenic acid (C18 : 3 (n-3))
4. Fish oil/marine algae (1 : 1), and
5. Protected lipid/marine algae (1 : 1).

The fatty acid profile of the supplements was reflected in the fatty acid composition of the meat and in the volatile profile of the grilled meat. Volatile compounds, such as (*E*)-2-heptenal, 1-octen-3-ol (**4**), and 2,3-octanedione, derived from ω-6 fatty acids, were highest in the meat from the lambs fed the protected lipid diet, whereas those derived from ω-3 fatty acids (e.g., 1-penten-3-ol, (*E*)-2-hexenal, 2-(2-pentenyl)furan) were highest in the meat from the lambs fed the fish oil/algae diet. The linseed-fed lamb was the most preferred, whereas the meat from those fed fish oil had a fishy flavor and those fed the protected lipid had a green note.

Poultry

It has long been established that corn-fed poultry has a superior flavor to those fed on barley, oats, and wheat [10.59]. Addition of beans, peanuts, and dried poultry-waste has been shown to have little effect on flavor, and the major dietary influence in poultry is the use of dietary fish meal or supplementation with fish oil, which results in meat with a fishy off-note, attributed to the oxidation of the PUFAs.

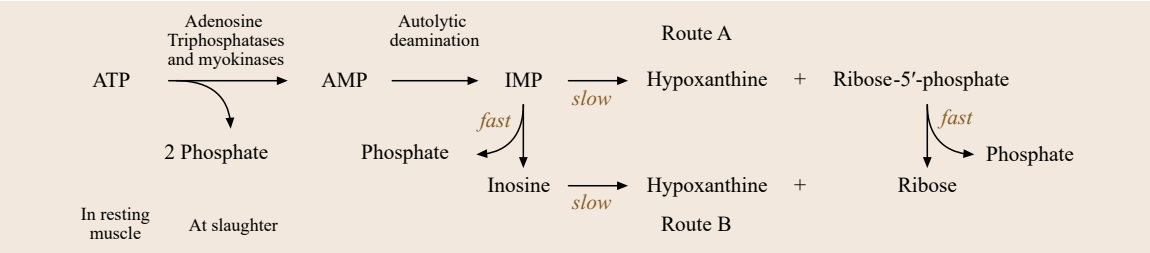

Fig. 10.20 Breakdown of ATP

Pork

Supplementation of pig feed with unsaturated fatty acids to reduce the levels of saturated fats resulted in a favorable change in the fatty acid profile with minimal change in flavor, but under less controlled conditions where lipid oxidation is allowed to proceed unhindered, a rancid off-note is readily detected.

10.4.2 Post-Slaughter Handling – Generation of Precursors

Post-slaughter conditions, such as chilling method, irradiation, storage conditions, and particularly processing or cooking techniques also exert a significant influence on the flavor of the meat. Aside from texture differences, many of the differences between breed, diet, and handling can be attributed to changes in the profile of the flavor precursors, and the challenge is to understand the origin of the precursors and how best to manipulate them for optimum flavor. Based on the knowledge of the formation pathways, sugars, amino acids, fatty acids, thiamine, and ascorbic acid have been identified as the key precursors of meat flavor. Many of these are generated post-slaughter from the breakdown of fat and muscle tissue. At slaughter [10.179], blood circulation ceases, oxygen supply fails, and under these conditions, proteolysis, lipolysis, glycolysis, and breakdown of ribonucleotides (Fig. 10.19) all start to occur, generating a pool of precursors, many of which have taste-active properties. Subsequent thermal processing is responsible for the generation of aroma.

Breakdown of Ribonucleotides

Of particular importance in the generation of meat flavor is the breakdown of adenosine 5′-triphosphate (ATP) to inosine 5′-monophosphate (IMP), and subsequent degradation to form ribose and ribose 5′-phosphate (key Maillard precursors), hypoxanthine (the constituent base in IMP), and inorganic phosphate (Fig. 10.20). In the living animal, ATP is found in resting muscle. In practice, almost invariably the animal is in a state of some exercise or stress at the point of slaughter so the labile phosphate residues are lost by the actions of the tissue adenosine triphosphatases and myokinase systems to form adenosine 5′-monophosphate (AMP). This usually happens very fast – the disappearance of ATP within a few hours post slaughter has been shown in pork [10.181] and chicken breast [10.182]. Within 24 h, AMP undergoes rapid autolytic deamination to form IMP, which is converted by the action of 5′-nucleotidase or nonspecific phosphomonoesterases to inosine and inorganic phosphate. Inosine is then converted either autolytically or under the intervention of a developing spoilage microflora to form hypoxanthine and ribose. The hypoxanthine degrades further to xanthine, uric acid, and ring cleavage products. This process provides both IMP, which is essential to the taste of meat, ribose, and ribose 5′-phosphate which are vital precursors for the development of meaty aromas (Sect. 10.1).

It has been shown that IMP, when compared to ribose and ribose 5′-phosphate, does not contribute greatly to the formation of meat aroma [10.108], unless the pH drops to 4.2 or below. Hydrolysis of the *N*-glycoside link between the sugar and the base is required for the ribose to participate in the Maillard reaction. During heat treatment, there are potentially two IMP degradation pathways as outlined in Fig. 10.20. In route A, the base is lost before the phosphate, whereas in route B, the phosphate is lost before the base. In both cases, the loss of the base is the rate-limiting step. Once formed, both ribose and ribose 5′-phosphate readily participate in the Maillard reaction and both were equally efficient (under model conditions [10.108]) at producing 2-methyl-3-furanthiol (**28**), but the more acidic conditions (pH 4.2) produced 3-4 times more than pH 6.5.

Proteolysis

Post slaughter, the muscle proteins denature and undergo proteolysis. Calcium-dependent proteases (calpains and cathepsins) act upon the myofibrillar and sarcoplasmic proteins to form polypeptide chains (≈ 30 kDa) [10.166]. Further breakdown of these by di- and tri-peptidyl peptidase releases small peptides that are broken down into free amino acids by amino pep-

tidases. The small peptides and free amino acids can provide taste characteristics to fermented meats, but they are also precursors of many of the important flavor compounds [10.183]. Amino acids, in general, catalyze the Maillard reaction, and many aroma compounds, particularly Strecker aldehydes, are generated from specific amino acids so it is both the nature and quantity of the free amino acids that are important for meat aroma.

Lipolysis

Although triglycerides can undergo oxidation, the fatty acids are far more reactive after they have been cleaved from the triglyceride backbone. This process requires the action of muscle lipases on muscle or adipose tissue, or phospholipases on membrane phospholipids. These lipolytic enzymes are sensitive to changes in pH, time, and temperature of storage or processing conditions, water activity and the presence of curing agents which are summarized by *Toldrá* [10.166]. Lipases remain active during freezing, and their activity increases greatly during the initial stages of cooking. Salt can partly inhibit some lipases, and ascorbic acid present at 500 ppm slightly inhibits all lipolytic enzymes. There is also limited evidence to show that some fatty acids are released quicker than others. Once released, the free fatty acids are the precursors for further enzymic or autolytic activity forming species characteristic aroma compounds. This process is, therefore, vital for flavor generation, but in excess leads to rancidity, off-notes, and WOF.

Glycolysis

Post slaughter, blood circulation stops, and in the absence of oxygen, muscles turn to anaerobic glycolysis, a process that breaks down glycogen without oxygen, to generate ATP and lactic acid. The intermediates, which are glucose and fructose phosphates, are precursors of the Maillard reaction. High levels of glucose and fructose have been demonstrated in beef muscle from animals fed sucrose prior to slaughter [10.184].

Precursor Studies

Koutsidis et al. investigated the effect of breed and diet on levels of precursors [10.106] and showed that diet had a greater effect than breed. Animals fed with concentrates rather than silage tended to have slightly higher levels of sugars. Changes in post-mortem conditioning were more substantial. A steady decline in IMP was observed during the 21 days post-mortem storage of meat (*M. longissimus lumborum*) from Charolais steers fed on concentrate. Ribose phosphate remained constant throughout the period, but ribose increased from 0.25 to 1.67 mmol/kg. This was concomitant with a rise in cysteine levels from 0.05 to 0.16 mmol/kg and in methionine from 0.05 to 0.35 mmol/kg. These are essential precursors for meat flavor and these changes are likely to have an impact on the final volatile profile.

In chicken breast, *Aliani* and *Farmer* [10.185] systematically increased by 2–4-fold the concentration of natural precursors (ribose, ribose 5′-phosphate, cysteine, thiamine and IMP) in raw chicken prior to cooking. Sensory evaluation showed that only ribose had an effect, and the chicken breasts with a higher concentration of ribose had an increase in cooked chicken aroma. This was attributed to the increase in 2-methyl-3-furanthiol (**28**), and possibly 2-furanmethanethiol (**22**), thus demonstrating that ribose concentration is a limiting factor in the development of chicken aroma.

10.4.3 Processing Conditions

The effect of processing or cooking conditions on any meat is significant, as any cook can testify. For example, the use of dry heat (roasting, grilling) favors the Maillard reaction and produces quite different aromas to moist heat (boiling, steaming, poaching). There are also distinct differences between roasted, grilled, pan-fried, or barbecued meat [10.59], and more complex flavors can be generated by combining two methods, or by the use of marinading and brining. The meat processing industry requires a consistent product and uses tight controls over the processing parameters.

Boiled, roasted, and stewed meats contain many of the same odor-active compounds, but they are present in very different proportions [10.5]. Boiled chicken, for example, has much higher levels of hexanal (**3**) than either roasted or stewed chicken. 2-Methyl-3-furanthiol (**28**) is reported in boiled beef and pork, but not in the corresponding roasted or stewed meats. 2-Ethyl-3,5-dimethylpyrazine (**15**) is found in roasted and stewed beef, but not in boiled where the mild conditions are not conducive to the formation of Maillard reaction products. Under boiling conditions, the contribution from the Maillard reaction is minimal, but under roasting conditions, it is the major pathway for flavor formation. A systematic sensory evaluation of cooked pork [10.186] has shown the increase in brown roasted notes and the decrease in metallic blood-like notes as the end-point temperature increased. Time, temperature profiles for thermal processing of beef muscle extract [10.109], and beef liver extract [10.187] show the progression of the Maillard reaction and the formation of 2- and 3-methylbutanals, indicating that after a certain time, levels of 3-methylbutanal start to decrease as the degradation (or condensation) pathways exceed the rate of formation. Overcooking produces high levels of Maillard reaction products which produce a burnt flavor when present at high concentrations.

The pH of the system also has an impact on the flavor. One major consequence of pH change is on texture. As lactic acid builds up in the muscle tissue post mortem, the pH drops, and if it is allowed to accumulate, the meat loses its water-binding ability and becomes pale and watery. If the acid is too low, the meat will be tough and dry. The Maillard reaction is also highly sensitive to pH changes. High pH tends to favor the formation of pyrazines giving roasty notes, whereas the meaty thiols are preferentially formed at lower pH where thiol groups exist as reactive thiolate anions.

As we move toward a predicted global population in excess of 8 billion, increased food demands, reductions in water and energy, and changes in land-use, meat production is under threat and methods to optimize meat quality or produce meat flavorings for alternative proteins become highly sought after. The production of optimum meat flavor involves the careful selection of diet and breed, good control over pre- and post-slaughter conditions and the choice of appropriate processing conditions to maximize the formation of aroma compounds.

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11. Fats and Oils

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Foods that contain fat are generally well liked by consumers. Fats and oils contain a large variety of aroma compounds. These aroma compounds are either trapped within the oil phase of the food matrix or can be formed from the lipids themselves. The analysis of aroma compounds in fat is difficult. This chapter discusses the analytical techniques used to identify the lipid-associated odorants and presents some comparisons of the results obtained from these techniques. Insights into the main families of chemicals imparting specific flavors to fat-containing foods and their formation pathways are presented, with a special emphasis on the compounds that are formed from lipids. Based on this premise, the aroma compositions of selected vegetable oils and animal fats are reviewed with a focus on the specific compounds that have been discovered during the last decade and their proven or postulated formation pathway. Finally, odor descriptions of many of the main compounds and their relative contribution to the overall flavor of fats and oils are given.

Food preferences are predominantly guided by taste, aroma, and texture, although other variables – genetic, physiological, educational, and metabolic – also play a role [11.1]. Sweet and fat tastes generally drive preferences in children and adults [11.1]. This is likely to be linked to the nutritional value of sugar and lipids in food. Despite this, however, it is still unknown whether human receptors for fat taste actually exist. In rodents, CD36 and GPR120 have been shown to be lingual receptors able to recognize free fatty acids [11.2]. Such receptors are also present in humans, and GPR120 in particular, might be involved in the perception of fat taste [11.3]. Certainly, the perception of fats in food relies primarily on smell and texture [11.4]. Thereby, there is an intricate and complex relationship between fat content, flavor release, and viscosity [11.5]. In view of this intrinsic interaction, flavor release as a function of the fat content or composition has been intensively studied, in model emulsions [11.6] and in model margarine [11.7]. Besides its physicochemical behavior and nutritional value, fat is highly desirable because it is a source of aroma compounds, formed either by the reaction of fat compounds or simply trapped within the lipid phase of the food. This chapter outlines the analytical techniques used to characterize the odorants in fats and oils and provides insights into the chemicals that impart the specific flavor to fat-containing foods and how these chemicals are formed. The aroma composition of selected vegetable oils and animal fats is then reviewed.

11.1 Analytical Techniques Used to Study Aroma Compounds in Fats and Oils

The analysis of flavor compounds in foods in general is a complex task. The aroma compounds are almost always analyzed by gas chromatography-mass spectrometry (GC-MS) (or GC-flame ionization detection (FID)) and as such they must be separated from the nonvolatile matrix prior to analysis to prevent damage to the GC column and the formation of artifacts within the GC injector. Odorant compounds in foods are typically present in low quantities, generally in the order of parts-per-million (ppm; or mg/kg) or parts-per-billion (ppb; μg/kg) levels, and even down to parts-per-trillion (ppt; ng/kg) for the most potent odorants. Therefore, food flavor analysis requires an isolation of the volatile flavor compounds from the matrix and an enrichment of the extract for subsequent analysis by GC-MS. The reader may refer to a comprehensive review by *Chaintreau* on the different techniques in flavor analysis [11.8] or Chap. 17 within this book. In particular, the analysis of aroma compounds in high-fat foods can be considered as one of the most complex and challenging tasks in flavor science. This is mainly due to the hydrophobic character that the fat flavor molecules share with the lipid molecules, thus making the odorants difficult to isolate with good recoveries. The techniques used for the isolation of odor molecules from fat can be classified on the basis of separation: distillation, headspace techniques, extraction, or size-exclusion chromatography. Overall, one needs to be aware of the fact that there is not *the* perfect isolation technique for aroma compounds, and that each procedure imparts discrimination and incomplete recoveries for some compounds, depending on the nature of each individual aroma compound and the matrix that is involved. Accordingly, it is important to know what each technique can achieve and to be familiar with the respective measures of compensation and correction for such discrepancies (e.g., Chap. 20 on stable isotope dilution analysis), especially when aiming at a precise quantification of the target odorants.

In view of the most commonly applied isolation techniques, each of these techniques is briefly described here and compared with each other by way of examples taken from the field of oil analysis. Odor compounds can be identified and their odor contribution assessed by using classical GC-MS and GC-olfactometry (GC-O) techniques, respectively.

11.1.1 Distillation

High Vacuum Transfer (HVT) and Cold Finger Molecular Distillation

The principle of the high vacuum transfer (HVT) and cold finger molecular distillation techniques is to evaporate flavor compounds from heated oil, commonly at 50 °C, by applying a high vacuum in two steps [11.9]. The two techniques are applied sequentially in the same apparatus (Fig. 11.1). The first step (HVT) takes place at 10^{-1} torr whereby the volatile compounds of the sample placed in a heated flask (3) are collected in a trap at −196 °C. A second trap (10) prevents contamination from the pump oil. After the content of this trap (6) is recovered, the second step (cold finger molecular distillation) can be carried out. The volatile odorants are collected on the surface of the cold finger (5), which is loaded with liquid N_2 and a higher vacuum of 10^{-2}–10^{-4} torr is applied. *Forss* and *Holloway* studied the recovery of aliphatic alcohols and ketones from butter oil using this technique [11.9]. They spiked butter oil with known amounts of alcohols and ketones of increasing carbon chain length. The volatile alcohols (C_2–C_5) and ketones (C_3–C_7) were recovered to more than 50% by HVT, whereas alcohols (C_6–C_{10}) and ketones (C_8–C_{10}) were recovered on the cold finger during step two. Total recoveries were

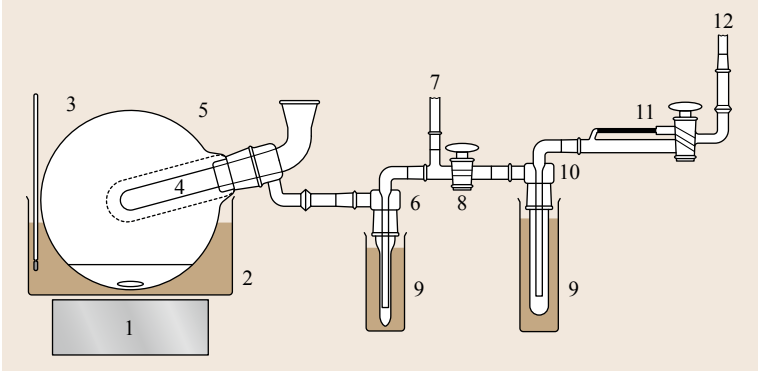


Fig. 11.1 Scheme of HVT and cold finger molecular distillation according to [11.9]

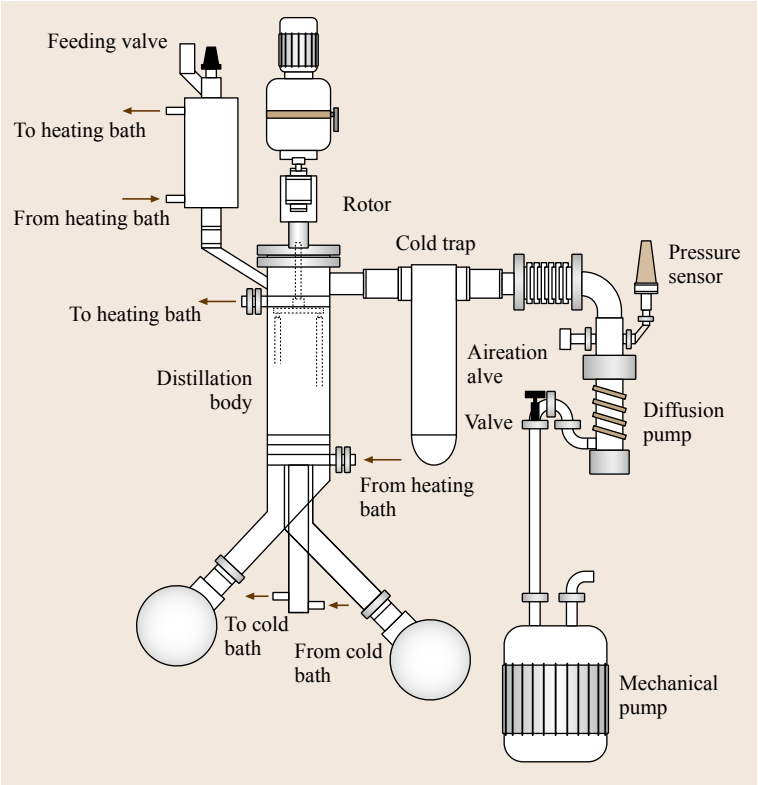


Fig. 11.2 Scheme of short-path distillation (after [11.10])

predominantly good (72–97%) or moderate for higher alcohols (C_8–C_{10}, 55–61%). Many other setups based on the same principle have been summarized by *Weurman* [11.11].

Short-Path Distillation

Also called falling-film, wiped-film, or thin-film distillation, short-path distillation is a continuous low-pressure distillation process [11.12]. In this technique, the oil is introduced drop-wise into the high-vacuum distillation body, where it forms a thin film by falling or is spread to form a thin film under the action of Teflon-coated rolling wipers (Fig. 11.2). The sample is introduced from the feeding flask (upper left) which may be heated. The falling oil forms a thin film in the distillation body. Most of the volatile compounds are collected in the cold trap. An additional cold trap (not shown) may be used to prevent contamination from the pumps. Odorants are collected on the surface of the cold finger located inside the distillation body, while the remaining oil flows to the receiving flask (bottom left). The volatile compounds are collected on the surface of a cold finger located inside the distillation body, or in the cold trap for the most volatile ones. As the residence time of the oil in the distillation chamber at 50 °C is less than a few seconds [11.12], the formation of artifacts is greatly minimized. In contrast to solvent-assisted flavor evaporation (SAFE; see below), short-path distillation is performed without the assistance of a solvent, thus yielding a more concentrated extract. At the end of the distillation, the cold finger is rinsed with a volatile solvent to recover and dilute the odorant extract and avoid it remaining in its concentrated form as it warms up. Using falling-film short-path distillation on a model mixture in synthetic oil, *Krings* et al. [11.13] measured recovery factors of many aroma compounds. For most compounds, the recoveries were in the range of 60–90% and were linearly correlated with the product of the boiling point and log P. For lactones recoveries were much poorer, for example with 57.4% for octan-4-olide and only 5.1% for dodecan-5-olide.

Reduced Pressure Steam Distillation

Steam distillation is a very old technique used for the production of essential oils. The product to be extracted is brought into contact with steam. Generally, the volatile compounds are distilled at a much lower temperature than during normal distillation because of the formation of azeotropes. After condensation, the organic compounds can be recovered from the water phase by decantation or by solvent extraction. In their

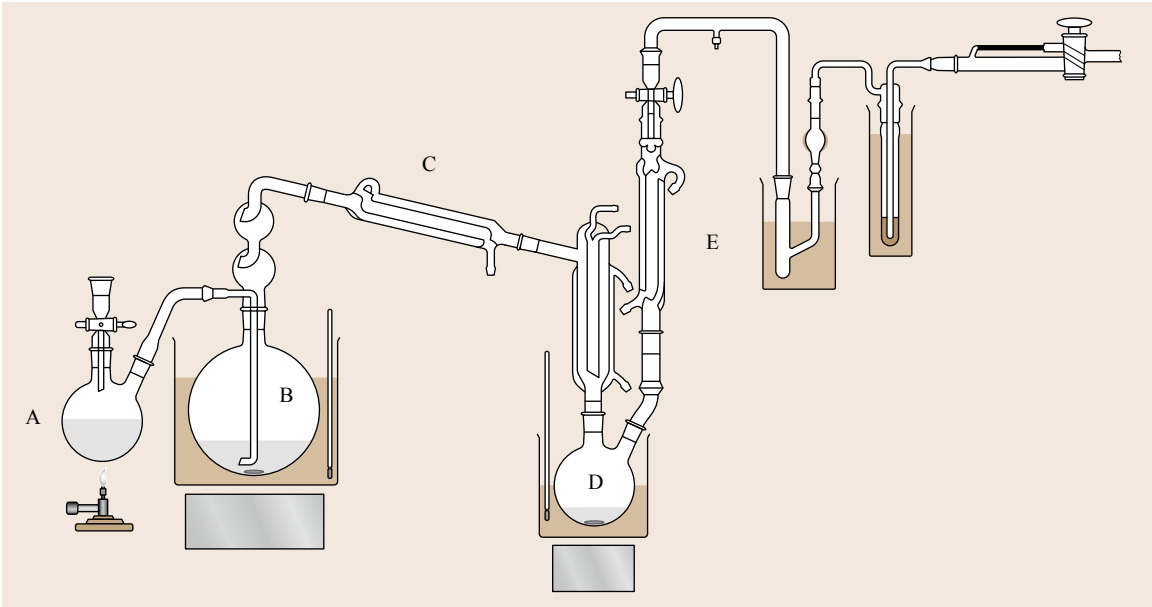


Fig. 11.3 Reduced pressure steam distillation

study on butter oil, *Forss* and *Holloway* applied this technique under reduced pressure [11.9] (Fig. 11.3). Butter oil in a heated flask (B) was percolated by steam from the steam generator (A). The aroma compounds together with water that condensed in the flask (D) could be further enriched (Fig. 11.3, E). Thereby, the recoveries of alkan-2-ones ranged from 75–90% (C_3–C_9) down to 48–12% (C_{10}–C_{12}).

As a modification of the previous setup, a simpler glassware arrangement can be used in some cases: thereby, the oil and water phases are mixed in a flask and subsequently distilled. This operation, often called hydrodistillation, can be carried out, for example, in a rotary evaporator. The flavor compounds that are soluble in the water phase can subsequently be recovered by adsorptive or solvent extraction techniques, by solid-phase extraction (SPE; Sect. 11.1.3). This combination was previously used for the analysis of sesame oil flavor and led to the identification of more than 150 compounds [11.14].

Simultaneous Distillation Extraction

In analytical scale steam distillation, the flavor compounds are extracted from the condensed water phase by using an organic solvent or by adsorptive techniques such as SPE. In simultaneous distillation extraction (SDE) the steam distillation and the continuous extraction are performed at the same time by using a Likens–Nickerson apparatus (Fig. 11.4). The solid or liquid fat product is placed in a flask attached to the left branch (A). The extractive solvent (ether, pentane) is placed in a flask attached to the right branch (B). The steam and the solvent vapors are brought into contact, decanted in the bottom loop of the device, and return to their original respective flasks. If the extractive solvent is denser than water (dichloromethane), it should be connected to the left branch (A). A comprehensive review of this technique is given in the literature [11.15].

To provide some examples, beef and lamb/mutton fat were comparatively analyzed by SDE and solid-phase microextraction (SPME; Sect. 11.1.2) of the headspace. Fewer aroma compounds were detected by SDE, but no trend could be drawn regarding the influence of the chemical class or the extraction efficiencies [11.16]. The same two techniques were compared for the analysis of dry-cured ham and gave more expected results: SPME was far better for the recovery of highly volatile compounds (acetaldehyde, dimethylsulfide, butanal), whereas compounds of lower volatility (C_{10}–C_{12} aldehydes, lactones) were more abundant in SDE samples [11.17]. In addition, SDE can lead to artifact formation not observed in SPME, such as di-unsaturated aldehydes formed by autoxidation of fatty acids [11.17]. The same observations were reported in a study of olive oil [11.18].

Solvent-Assisted Flavor Evaporation (SAFE)

Solvent-assisted flavor evaporation (SAFE) is a mild technique for the recovery of volatile flavor compounds from difficult liquid, solid, or heterogenous matrices, including high-fat foods [11.19]. The SAFE apparatus

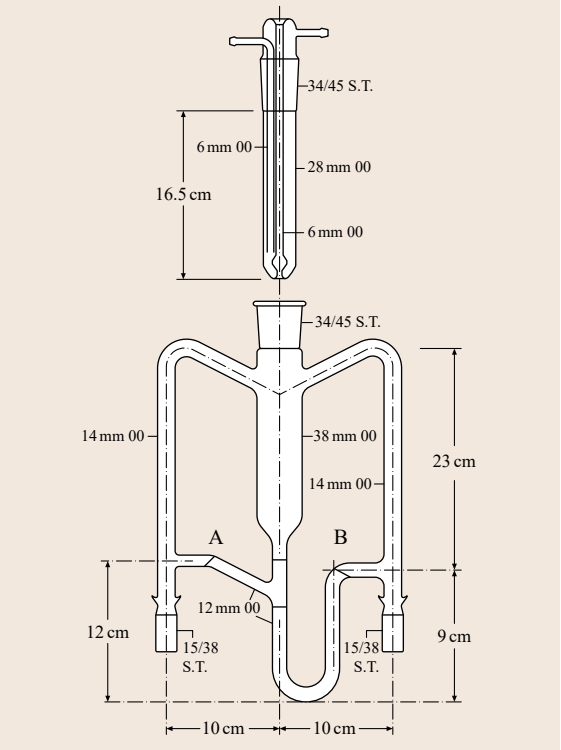


Fig. 11.4 Likens–Nickerson apparatus for SDE

consists of a complex assembly of especially constructed glassware (Fig. 11.5). The food sample is diluted or suspended in an organic solvent, usually dichloromethane or diethyl ether. This is then placed into the *addition funnel* (A) and then added drop-wise into the heated flask (B) in which a high vacuum (10^{-5}–10^{-4} torr) provided by a diffusion pump is applied. The solvent and the volatile flavor compounds are volatilized concomitantly before the vapors are trapped at −196 °C (C and D). Careful temperature regulation minimizes the freezing of material in unwanted zones of the apparatus. The use of a high vacuum allows very moderate heating (20–35 °C), thus minimizing odorant degradation during extraction. A model flavor containing seven aroma compounds with different functional groups diluted in synthetic triglycerides (TGs) demonstrated recoveries in the range of 37–13% (3-methylbutanoic acid, phenylacetaldehyde, phenylethanol) or considerably lower (Furaneol 4%, vanillin 0.5%). The recoveries were slightly higher than with HVT (Sect. 11.1.1). SAFE has the reputation of providing olfactively representative extracts. Therefore, it is one of the most often used preparative techniques in flavor research; interestingly, a few chefs who have embraced the *molecular gastronomy* trend also use SAFE [11.20].

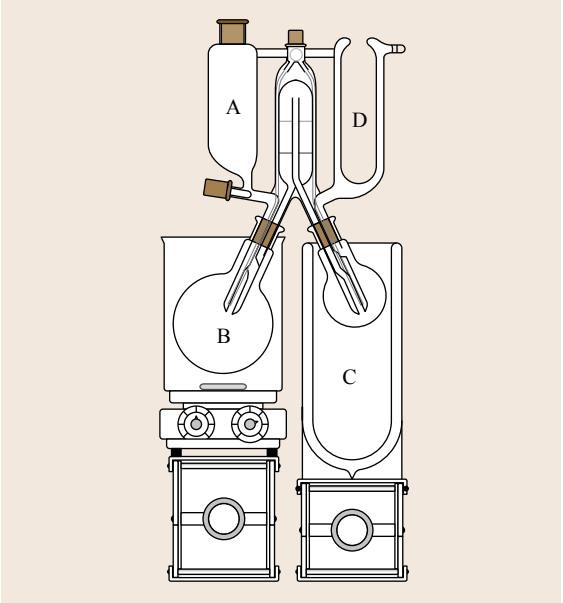


Fig. 11.5 Scheme of the SAFE apparatus (after [11.19])

11.1.2 Headspace Techniques

Headspace analysis is of great interest in flavor analysis since it requires little or no sample preparation and as odorants collected by this technique are believed to represent the initial olfactory impression of a sample, for example, when smelling a dish. The volume above the sample, called the headspace, is collected in many different ways and injected onto the GC column. Alternatively, it can be analyzed directly by online chemical ionization mass spectrometers (Chap. 18). The many variants of headspace analysis are reviewed in [11.21]. In view of fats and oils, one needs to keep in mind that, overall, the partitioning behavior of the fat-associated odorants commonly does not favor the gas phase due to the strong affinity of the odorous molecules to the rather nonpolar matrix. Another crucial step in headspace analysis, especially when working under static conditions, may be the application of incubation steps to achieve the equilibrium of odorant distribution between the sample matrix and the gas phase. In the case of sensitive fats and oils, this introduces the necessity to carefully consider and avoid potential artifact formation, for example, due to fatty acid oxidation. In such cases, working under a protective atmosphere, as well as careful adjustment of the temperature and time parameters during incubation might be required.

Static or Dynamic Headspace

In classical static headspace analysis, a certain volume above a sample is collected by using a gas-tight

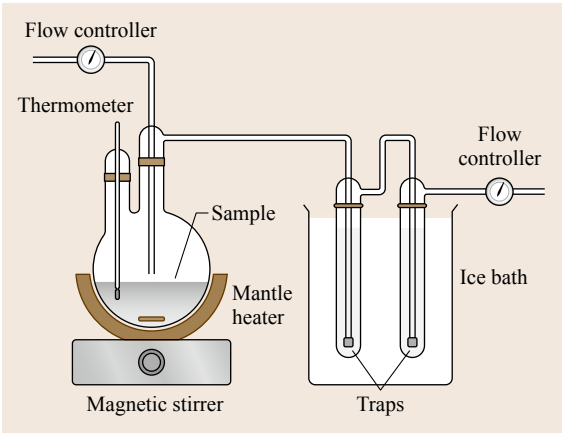


Fig. 11.6 Trapping of volatile compounds in the headspace of pork fat. Reprinted from [11.22] with permission from Elsevier

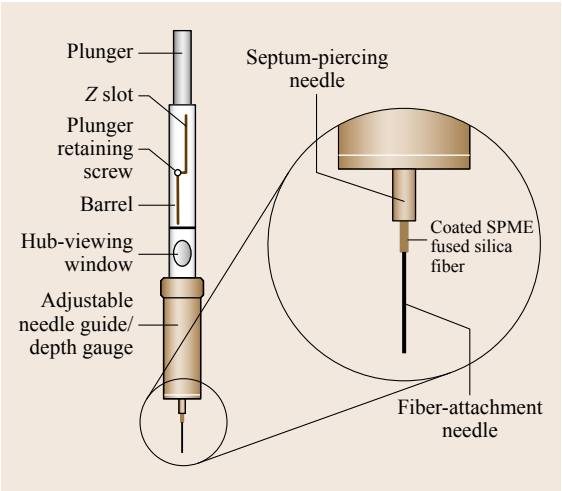


Fig. 11.7 Solid-phase microextraction (SPME) assembly

syringe and is subsequently analyzed by GC, commonly coupled with FID or MS detection. In dynamic headspace analysis, the headspace volume is continuously purged by a flow of a purified gas and the volatile compounds are collected in a cold trap, either in cold solvent or by using an adsorbent, or by pure cryo-focussing, for example, using liquid nitrogen. Dynamic headspace is often preferred over static headspace in the analysis of fats and oils because it leads to much more concentrated volatile extracts. Figure 11.6 shows the setup used for the analysis of heated pork fat [11.22] whereby the respective volatiles were collected in cold dichloromethane for 6 h. The trapping of the volatiles over adsorbent-containing cartridges, such as Tenax, has gained much popularity because it can be applied in a fully automated process. For example, blue cheese was studied by using this technique, and its simplicity, reliability, and high level of automation were highly appreciated [11.23].

Solid Phase Microextraction and Related Techniques

Solid phase microextraction (SPME) is a static headspace sampling technique introduced by Pawliszyn in 1993 that achieves a high level of volatile enrichment [11.24]. An adsorbent, polydimethylsiloxane (PDMS), polyacrylate, or Carboxen, coated onto a fused silica fiber, forms the end of a needle assembly as is shown in Fig. 11.7. The SPME fiber is exposed to the headspace of the sample and the volatile analytes are adsorbed. The amount of a given analyte adsorbed on the fiber results from its partitioning between the sample matrix, the headspace, and the adsorbent. This phenomenon creates huge discrimination between different analytes. Also, the choice of the fiber material, extraction time and temperature are of crucial importance. The accumulated volatiles are desorbed thermally into a conventional split/splitless GC injector. SPME analysis is perhaps the simplest and most easily automated technique described in this chapter, which explains its huge popularity. To increase the concentration capability of SPME, various techniques with thicker sorbent films have been developed. In headspace sorptive extraction (HSSE) the headspace volatiles are collected onto a bar that is coated with at least 100 times more sorbent, commonly PDMS. It should be noted that HSSE is a special case of stir bar sorptive extraction (SBSE) whereby the bar is just suspended in the headspace of the sample instead of being stirred in the solution. All of these sampling techniques were recently assessed using two-dimensional GC-MS [11.25]. The physicochemical properties of the adsorbent are similar to those of oils and fats. Therefore, the partitioning between the oil phase, the headspace, and the rather low amount of SPME adsorbent for the most hydrophobic and less volatile compounds is not favorable. However, high-concentration capacity sampling techniques, such as HSSE or SBSE showed good results, even for whole milk samples [11.25]. HSSE and SPME have been compared for the analysis of olive oils [11.26] and HSSE gave slightly better results at the expense of the need for a special desorption/GC-injection device. It should be noted that most of the compounds analyzed in this study were volatile, weakly hydrophobic aldehydes and alcohols (C_5–C_{10}).

11.1.3 Extraction

Solvent extraction is perhaps the oldest technique in flavor analysis. It is still used, but new techniques that are

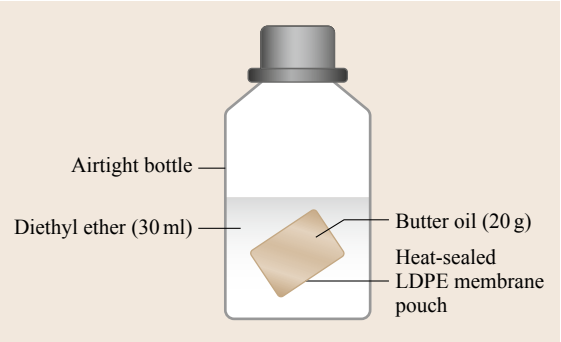


Fig. 11.8 Membrane-assisted solvent extraction (after [11.28], courtesy of John Wiley & Sons, Ltd)

claimed to be more ecological, such as SPE and supercritical fluid extraction (SFE), have recently gained popularity.

Solvent Extraction (or Liquid–Liquid Extraction (LLE))

Solvent extraction is used to extract the oil from fat-containing food samples, for example, chicken adipose fat tissue [11.27]. Since most of the solvents also dissolve TGs and other nonvolatile lipid constituents, which prevents direct GC analysis, liquid–liquid extraction of aroma volatiles from oils is never used alone. An attractive technique called membrane-assisted solvent extraction was reported on recently [11.28]. Butter oil was placed in a sealed low-density polyethylene membrane pouch in a closed bottle containing the organic solvent (Fig. 11.8). The membrane acted as a molecular sieve, allowing the volatile flavor molecules to reach the solvent side, whereas TGs did not diffuse through it. Spiking experiments were performed to determine recovery factors. These were modest (1–2% for acids, 10% for lactones), but very reproducible. Interestingly, lactones with higher molecular weights (C_{14}, C_{16}) could also be recovered by using this technique.

Solid Phase Extraction

Solid phase extraction (SPE) is extensively used for the extraction of aroma volatiles from water-based foods and drinks. The sorbent is generally composed of C_{18}-bonded silica or polymers of the same lipophilicity. SPE cannot be used for the direct analysis of fats. The polar flavor compounds of butter were previously recovered by SPE, but only after the water phase was first separated from the butter oil [11.29].

Supercritical Fluid Extraction (SFE)

Besides its *green chemistry label*, SFE, mostly performed with carbon dioxide, offers many advantages over solvent extraction. First, its selectivity toward solutes can be modulated by variation of the applied pressure. Second, CO_2 evaporates easily at ambient pressure, whereas it is difficult to separate liquid solvents from extracts without losing the volatiles. Supercritical CO_2 extraction was used in an extensive analysis of raw beef volatiles [11.30]; thereby, a lipid-like fraction was produced under high pressure of CO_2 (400 bar). By reducing the pressure to atmospheric pressure, the gaseous CO_2 was forced to pass through a cartridge that contained Tenax adsorbent. The condensed fraction remaining after CO_2 evaporation was primarily composed of lipids. Thermal desorption of the Tenax cartridge and GC-MS analysis led to the identification of more than 120 compounds [11.30].

11.1.4 Gel Permeation Chromatography (GPC)

Gel permeation chromatography (GPC) has long been used for the determination of contaminants, such as phthalates [11.31] or pesticides [11.32] in vegetable oils by GC. Despite this technique being able to separate semivolatile compounds, such as fatty acid esters from TGs [11.33] it has been rarely used for aroma compound analysis. *Fröhlich* described the separation of fat and flavor compounds by using a column of Bio-Beads SX-3 eluted with dichloromethane [11.34]. The analysis of a chocolate with coconut filling spiked with various amounts of ethylvanillin gave recoveries from 92 to 108%.

11.1.5 Comparison of Techniques

It is difficult to objectively compare all of the techniques described above because no extensive systematic study has been conducted to determine the recoveries of different aroma compounds from the same fat or oil by each approach. Table 11.1 shows the main features of each technique. Distillation techniques are seemingly best suited for in-depth analysis because a large quantity of oil can be processed. The extracts thus obtained can be further fractionated by flash chromatography. High-vacuum distillation may be considered best for semivolatile compounds, such as long-chain lactones. On the other hand, these techniques require expensive hardware, such as a high-vacuum pumps or dedicated glassware, in addition to a longer operation time. SDE may be a good technique but compounds of lower volatility or those that are heat sensitive may induce biases. Also, artifact formation from nonvolatile precursors might be an issue that needs to be considered. Solvent extraction is often difficult because the physic-

Table 11.1 Pros and cons of each sampling technique for the analysis of fat/oil volatiles

Technique	Price	Ease of use	Advantages	Drawbacks
HVT	$$$	−	Prep scale, good recoveries of volatiles and semivolatiles	Tedious, solid fat analysis not possible
Short path distillation	$$$$	−	Continuous, prep scale, good recoveries of low and high boiling chemicals	Tedious, solid fat analysis not possible, dedicated glassware, difficult to clean
Steam distillation	$	+	Prep scale	Artifacts, poor recovery for polar compounds
SDE	$	+	Prep scale, easy use, low cost	Artifacts, difficult under vacuum, dedicated glassware
SAFE	$$$$	−	Prep scale, versatile	Needs solvent, price, dedicated glassware, difficult to clean
Headspace	$	++	Easy use, sensitivity	Small scale only, semivolatiles not recovered
SPME	$	+++	Choice of fibers, easy use, sensitivity, quantification	Small scale only, semivolatiles not recovered
LLE	$	++	Prep scale, large choice of solvent	Difficult to eliminate TGs
LLE with membrane	$	+	Prep scale, easy use	Not much data in the literature
SPE	$	+++	Choice of sorbent, easy use	Difficult to separate lipophilic molecules from TGs
SFE	$$$	−	Prep scale, *green technique*	Dedicated instrument, needs skilled technician
GPC	$	+	Mild technique, no artifacts	Not much data in literature

ochemical properties of glycerides are not sufficiently different from those of certain classes of aroma compounds. Nevertheless, the complete removal of nonvolatile molecules before GC analysis can subsequently be performed by size exclusion chromatography (SEC) or with the help of membrane separators. For studies requiring a larger number of sample analyses, headspace techniques offer substantial advantages, but the downside here is that semivolatile compounds are discriminated or omitted.

11.2 The Different Chemical Classes of Aroma Compounds Found in Fats and Oils, and How They Are Formed

Aroma chemicals present in the fat phase of foods can either originate from lipids or can simply be trapped in the fat phase because of their lipophilicity. Flavors derived from fat have been reviewed extensively by Urbach and Gordon, who focused on milk fat products [11.35]. The most typical molecules are shown in the following subsections; their odor qualities and thresholds, when available, can be found in the literature [11.36]. A valuable source of information used in the present article is the Volatile Compounds in Foods database, which contains more than 7000 compounds present in 675 types of foods, together with their corresponding literature [11.37]. The data entries in this database are updated every year by the Netherlands Organisation for Applied Scientific Research. In Sect. 11.3, only the most common members of each class of aroma compounds are presented. Their described mechanisms of formation may be generalized to other aroma chemicals to understand how they are formed from lipid products.

11.2.1 Acids

Free fatty acids are formed by lipolysis of TGs. Long-chain fatty acids are not potent odorants, which is in contrast with short-chain fatty acids (C_4–C_{10}). Short-chain acids, such as **(1)–(4)** (Fig. 11.9) are more abundant in animal fats than in vegetable oils. *Frerot* et al. analyzed butter TGs by ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) [11.38]. Most of the identified TGs contained a butanoyl moiety in position 1 (or 3) of the glycerol. In contrast, none of the diglycerides present in butter oil contained a butyric acid residue. The enzymatic hydrolysis of the primary ester bond positions (1 or 3) of TGs is easier than that of the secondary ester bond (position 2). Furthermore smaller, less sterically hindered fatty acids are cleaved first. This explains why rancid butter and good cheese contain a substantial amount of short-chain fatty acids, such as **(1)** and **(2)**.

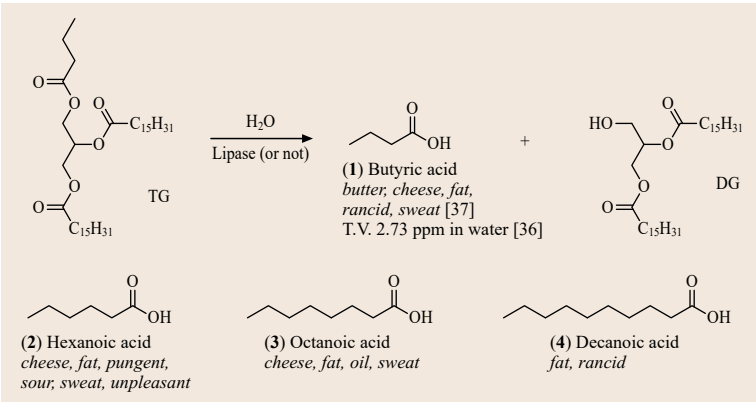


Fig. 11.9 Short-chain fatty acids from triglycerides

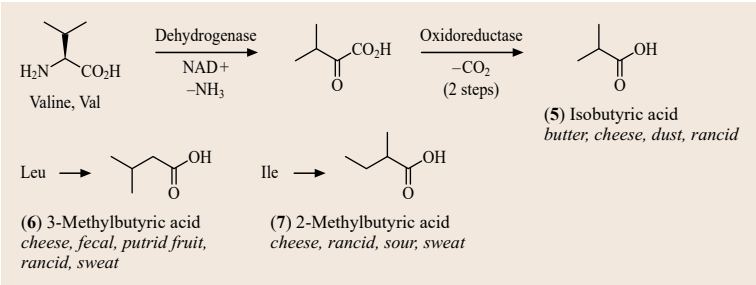


Fig. 11.10 Short branched-chain fatty acids from amino acids

Small branched-chain fatty acids do not originate from TGs, but from the degradation pathways of the amino acids valine, leucine, and isoleucine (Fig. 11.10), as can be found in the Kyoto Encyclopedia of Genes and Genomes (Kegg) database [11.39].

Short-chain fatty acids constitute a chemical class of high aroma importance in many fat-containing foods, in particular milk fat products. These acids generally impart the character of the food and its initial flavor impact, and are primarily quality markers in ripening processes but also arise due to deterioration (as in over-ripening).

Short-chain fatty acids and long-chain fatty acids are also the biochemical precursors of many other important aroma compound classes, such as aldehydes (Sect. 11.2.3), ketones (Sect. 11.2.4), and lactones (Sect. 11.2.5).

11.2.2 Esters

Esters do not constitute an important class of odorants in fat-containing foods, in contrast to their overwhelming importance in fruits. They are not abundant in fats and oils, as compared with acids or aldehydes, and they do not contribute much to their overall flavor profiles. Ethyl-2-methylbutyrate **(8)** was described as contributing to the odor of olive oil to a moderate extent [11.40]. A noticeable exception is ethyl butyrate **(9)** (Fig. 11.11), which plays an important role in the aroma profile of many cheeses (Cheddar, Pecorino, Gorgonzola), as reviewed by *Curioni* and *Bosset* [11.41].

11.2.3 Aldehydes

Aldehydes have tremendous importance in fat-related aromas and, accordingly, in the flavor industry. In particular, many aroma-impact compounds in vegetable oils and meat fats belong to this chemical class. Most of the aldehydes identified in foods are produced by the oxidation of unsaturated fatty acids. In plants, this process can be enzymatic and is called the lipoxygenase (LOX) pathway (Fig. 11.12, Chap. 2). Hydroperoxides, specifically 9-HPOT or 13-HPOT, are first formed from linolenic acid upon the combined action of a lipase and of a lipoxygenase (9- or 13-LOX). A hydroperoxide lyase, 9-HPL or 13-HPL, then cleaves the hydroperoxide to give C_9 or C_6 aldehydes, respectively. In olive

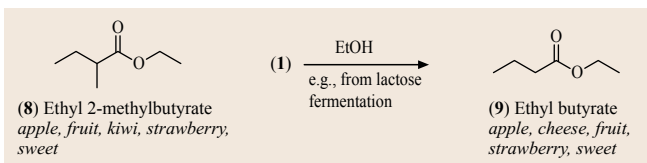


Fig. 11.11 Esters in olive oil

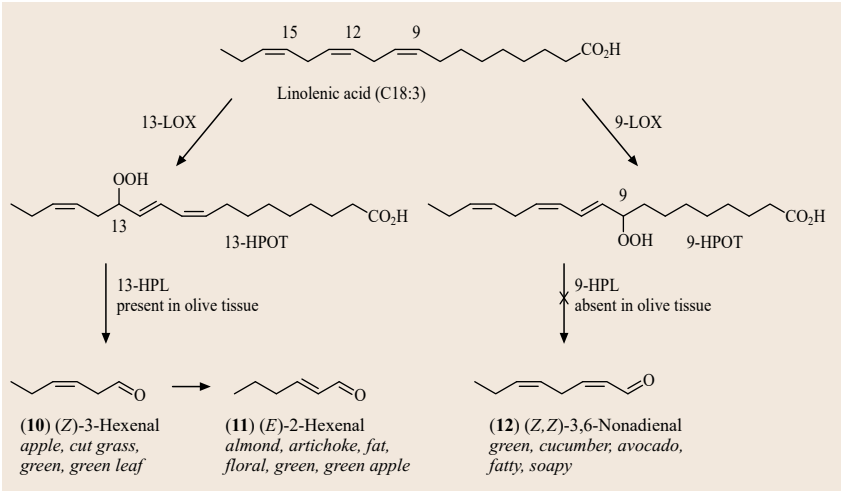


Fig. 11.12 Lipoxygenase (LOX) pathway to *green* aldehydes in olive oil

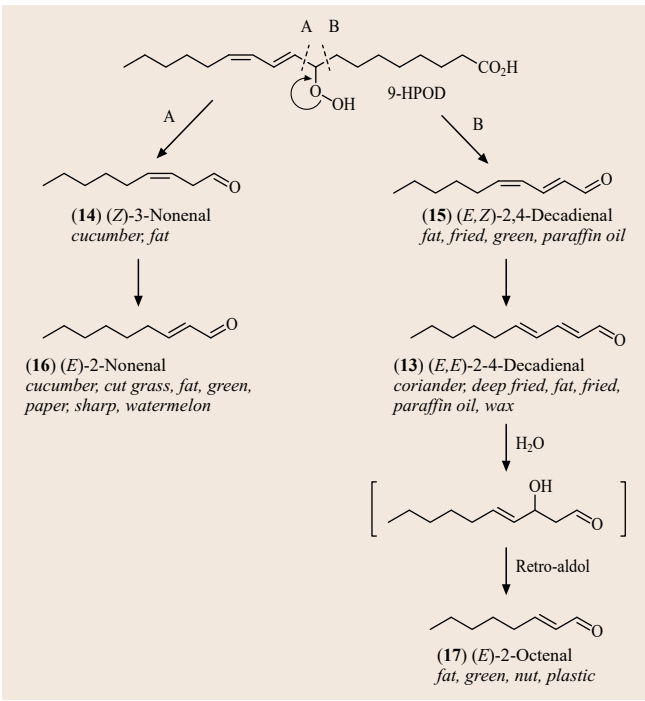


Fig. 11.13 Autoxidation of linoleic acid

oil, only C_6 aldehydes, such as (*Z*)-3-hexenal **(10)** and (*E*)-2-hexenal **(11)**, which are responsible for the *green* odor of olive oil, are formed [11.42]. Whereas 9-HPOT was more abundant in the reaction of linolenic acid with olive fruit tissue, the corresponding C_9 aldehydes, such as **(12)** were absent because the hydroperoxide lyase present in olive is highly specific toward 13-HPOT (Fig. 11.12) [11.42].

However, the enzymatic pathway to aldehydes is not the only one and also not the most common in foods. Autoxidation during cooking or storage forms a large variety of potent odorant aldehydes. Thereby, it is important to note that no enzyme is needed for the thermal cleavage of fatty acid hydroperoxides. Thus, (*E,E*)-2,4-decadienal **(13)** is formed from 9-HPOD, as is shown in Fig. 11.13 [11.43]. Unsaturated aldehydes may also react with water and degrade through a retro-aldol reaction to give (*E*)-2-alkenals with two fewer carbon units. The mechanistic aspects of flavor compound formation from lipids have been reviewed in detail by *Fitz* et al. [11.44]. Table 11.2 lists the most important aldehydes in fats and oils, most of which are used as flavor ingredients in the food industry. The possible precursors according to *Fitz* et al. [11.44] are also shown. A recent study of olive oil put the aldehydes and the fatty acids in perspective, thereby allowing the confirmation of the origin of certain aldehydes [11.45]. The occurrence of less common branched-chain fatty aldehydes in beef has been described in the literature [11.46, 47]. These aldehydes are believed to originate from microorganisms in the rumen of bovine animals [11.48]. They are then incorporated into the lipid phase of the muscular tissue in the form of plasmalogen, which is a special kind of phospholipid (Fig. 11.14). Aldehydes, including unusual branched-chain aldehydes, are bound to glycerol by an enol ether bond and are subsequently released by hydrolysis during cooking [11.48]. 12-Methyltridecanal **(30)** is the most important member of this family of aldehydes.

Note that the occurrence information indicated in the right column of Table 11.2 is not exhaustive. Only those foods are listed in which the compound has been discovered in the highest quantity, when this information is available.

Another class of aldehydes can be found in fat-containing food forms from Maillard reactions [11.50]

Table 11.2 Important odorous aldehydes and their occurrence in fat-containing or fat-based foods

Aldehyde	Precursors [11.44]	Odor [11.37]	Occurrence [11.37]
Hexanal **(18)**	13-LOOH	Apple, fat, grass, green, green fruit, oil	Common
(*Z*)-3-Hexenal **(10)**	13-LnOOH	Apple, cut grass, green, green leaf	Olive oil
(*E*)-2-Hexenal **(11)**	Isomerization of **(10)**	Almond, artichoke, fat, floral, green, green apple	Olive oil
(*Z*)-4-Heptenal **(19)**	Retro aldol of **(24)**	Biscuit, cream, fat, tallow	Chicken, milk
Octanal **(20)**	11-OOOH	Citrus, fat, green, oil, pungent, soap, sweet	Beef, chicken, cheese, olive oil
(*E*)-2-Octenal **(17)**	Retro aldol of **(13)**	Fat, green, nut, plastic	Butter oil
(*E*)-2-Nonenal **(21)**	9-LOOH, 12-AnOOH	Cucumber, fat, green, paper	Chicken
(*Z*)-3-Nonenal **(22)**	10-LOOH	Cucumber, fat	Chicken, olive, peanut
(*E,E*)-2,4-Nonadienal **(23)**	Unknown	Deep fried, fat, green, wax	Butter
(*Z,Z*)-3,6-Nonadienal **(12)**	9-LnOOH	Green, cucumber, avocado [11.49], fat, soap	Butter
(*E,Z*)-2,6-Nonadienal **(24)**	Isomerization of **(12)**	Fat, cucumber, chicken skin, fruity, pear [11.49]	Chicken
Decanal **(25)**	11-OOOH + retro aldol	Floral, green, orange peel, penetrating, tallow	Beef, chicken
(*E*)-2-Decenal **(26)**	9-OOOH	Fat, fish, paint, tallow	Chicken
(*E,E*)-2,4-Decadienal **(13)**	9-LOOH	Coriander, deep fried, fat, fried, paraffin oil, wax	Chicken, butter
(*E,E,Z*)-2,4,7-Decatrienal **(27)**	9-LnOOH	Fat, green, cucumber, sweet, mandarin [11.49]	Fish
(*E*)-2-Undecenal **(28)**	8-OOOH	Fat, green, soap	Chicken, butter
(*E,E*)-2,4-Undecadienal **(29)**	8-LOOH (hypothesis)	Fatty, animalic	Chicken
12-Methyltridecanal **(30)**	Plasmalogens (phospholipids)	Cooked meat, fat, meat broth, sweat, tallow	Beef

OOOH, LOOH, LnOOH, AnOOH: hydroperoxides of oleic, linoleic, linolenic, or arachidonic acid, respectively.

or by enzymatic degradation reactions. They originate from amino acids and form through Strecker degradation (Fig. 11.15). These aldehydes can be considered as minor compounds in fat-containing foods, as compared with lipid-derived aldehydes. However, they can impart a distinctive odor (Table 11.3).

11.2.4 Ketones

Ketones have less importance than aldehydes in the flavor of fat-containing foods (Table 11.4). They are usually found in lower quantities, and only a few ketones can be considered as key odor components of meat fat or vegetable oils. A notable exception is mold-ripened cheese, such as blue cheese. The formation of methylketones (2-alkanones) is the result of the β-oxidation of fatty acids [11.51]. Fatty acids appear to slow down the growth of *Penicillium roquefortii* and their oxidation in carefully controlled conditions in cheese making may be a detoxifying mechanism used by the mold [11.52]. By feeding *P. roquefortii* spores with uniformly labeled lauric acid, Dartey and Kinsella showed that a homologous series of C_3–C_{11} methyl ke-

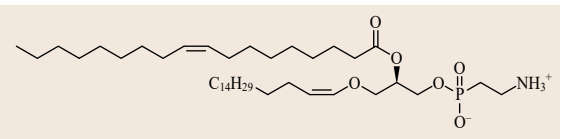


Fig. 11.14 Structure of a plasmalogen (CAS RN-144371-68-6). Note the enol ether linkage whose cleavage would give stearic aldehyde

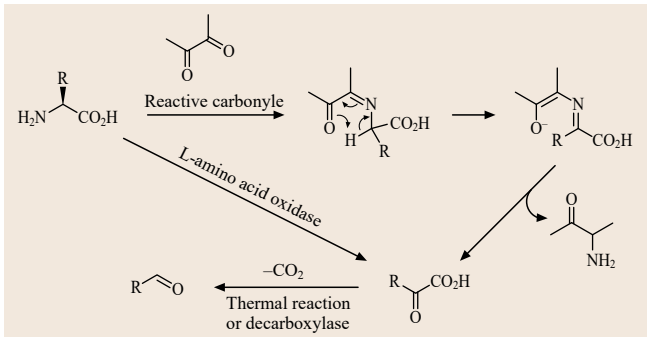


Fig. 11.15 The Strecker degradation pathway to aldehydes

Table 11.3 Strecker aldehydes

Aldehyde	Precursors	Odor [11.37]	Occurrence [11.37]
Phenylacetaldehyde **(31)**	Phenylalanine	Sweet, floral, honey, green	Chicken, beef, cheese
2-Methylpropanal **(32)**	Valine	Burnt, caramel, cocoa, cooked, green, malt, pungent	Fish, butter
2-Methylbutanal **(33)**	Isoleucine	Almond, cocoa, fruit, hazelnut, malt	Chicken
3-Methylbutanal **(34)**	Leucine	Almond, cocoa, fresh green, fruit, malt, ripe fruit, solvent, sweet	Beef, peanut
3-(Methylthio)propanal (methional) **(35)**	Methionine	Cooked potato	Beef, chicken, cheese

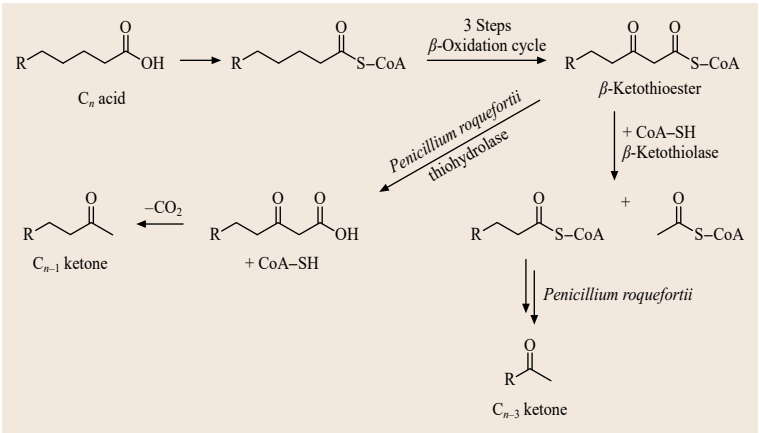


Fig. 11.16 Formation of methyl ketones in Roquefort cheese

tones was produced [11.53], which demonstrated the mechanism of Fig. 11.16.

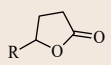
1-Octen-3-one **(40)** and (*Z*)-1,5-octadien-3-one **(42)** are found in only trace quantities in fat-containing foods. However, these compounds are such potent odorants that they may contribute to the overall flavor, as shown in butter oil by GC-O [11.55].

(*E*)-5-Methyl-2-hepten-4-one, filbertone **(44)**, is a characteristic and potent odorant of hazelnut [11.56]. It is likely to be formed during the roasting of the nuts in the course of the Maillard reaction, which involves sugars and amino acids, but not lipids. Thus, raw hazelnut was shown to contain only trace amounts of **(44)**, whereas the boiling of the defatted paste in water continuously generated this ketone. It also formed continuously during the roasting of hazelnuts and is found in the oil [11.54].

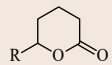
11.2.5 Lactones

Lactones are ubiquitous in foods. In 1976, Maga exhaustively summarized the occurrence of lactones in many foods, along with their levels and sensory properties [11.57]. The lactones are well-known key aroma compounds in fruits, such as peach, apricot, and coconut, but they are also important in fat-containing foods: vegetable oils (coconut, soybean, cottonseed) or meat fat (beef, chicken, pork, lamb/mutton). Lactones identified in foods possess either a five-membered ring (γ-lactones = 4-olides) or a six-membered ring (δ-lactones = 5-olides). Many γ- and δ-lactones are used by the flavor industry, as reviewed in the literature [11.58]. Interestingly, the annual 1987 world consumption of lactones was 125 t of their native form in foods and 20 t as a flavor additive. Figure 11.17 and Table 11.5 list the most abundant saturated lactones in fat-containing foods, which are also the most important lactones used by the flavor industry [11.58].

γ- and δ-lactones bear a stereogenic center at C(4) or C(5), respectively. Many studies have been performed to determine the enantiomers of lactones in natural products. *Palm* et al. used GC analysis with chiral columns [11.62] and *Lehmann* et al. used multidimensional GC [11.63] for the analysis of dairy lactones. There is not much difference in odor quality between the (*R*)- and (*S*)-enantiomers of the same lactone, but



γ-Lactones (alkyl-4-olides)
R = H **(45)**, CH_3 **(46)**, C_2H_5 **(47)**
C_4H_9 **(48)**, C_5H_{11} **(49)**, C_6H_{13} **(50)**
C_7H_{15} **(51)**, C_8H_{17} **(52)**



δ-Lactones (alkyl-5-olides)
R = CH_3 **(53)**, C_3H_7 **(54)**
C_4H_9 **(55)**, C_5H_{11} **(56)**, C_6H_{13} **(57)**
C_7H_{15} **(58)**, C_9H_{19} **(59)**

Fig. 11.17 Odorous saturated lactones

Table 11.4 Odorous ketones in fats and oils

Ketone	Precursors [11.44]	Odor [11.37]	Occurrence [11.37]
2-Pentanone **(36)**	Free fatty acids	Ether, fruit, sweet	Cheese
2-Heptanone **(37)**	Free fatty acids	Fruit, green, nut, soap	Cheese
2-Nonanone **(38)**	Free fatty acids	Fragrant, fruit, green, hot milk, soap	Cheese
3-Undecanone **(39)**	Free fatty acids	Fresh, green, orange, rose	Cheese
1-Octen-3-one **(40)**	10-LOOH + oxidation of 1-octen-3-ol **(41)**	Earth, metal, mushroom	Trace in beef, cheese, chicken, butter
(*Z*)-1,5-Octadien-3-one **(42)**	10-LnOOH + oxidation of 1,5-octen-3-ol **(43)**	Geranium, metal	Trace in beef and butter, fish oil
(*E*)-5-Methyl-2-hepten-4-one (filbertone) **(44)**	Maillard reaction [11.54]	Hazelnut, nut	Hazelnut

Table 11.5 Odorous lactones in fat-containing foods

Lactone	Precursors [11.44]	Odor [11.37]	Occurrence [11.37]
γ-Butyrolactone **(45)**	Ascorbic acid [11.57], ketoglutarate	Weak butter [11.58]	Beef [11.59]
Pentan-4-olide **(46)**	Saturated fatty acid – δ-oxidation pathway [11.60]	Herb, sweet	Beef, chicken, pork, cheese
Hexan-4-olide **(47)**	δ-Oxidation	Coumarin, sweet	Butter, beef, chicken
Octan-4-olide **(48)**	δ-Oxidation	Coconut, fruity, sweet [11.58]	Butter, beef, chicken
Nonan-4-olide **(49)**	δ-Oxidation	Coconut, fatty [11.58]	Butter, beef, chicken
Decan-4-olide **(50)**	δ-Oxidation	Fruity, peach, apricot [11.58]	Butter, beef, chicken, pork
Undecan-4-olide **(51)**	δ-Oxidation	Strong, fruity, peach, apricot [11.58]	Butter, beef, chicken, pork
Dodecan-4-olide **(52)**	Oleic acid [11.61]	Fat, peach, butter [11.58]	Cheese, butter, beef, chicken, pork
Hexan-5-olide **(53)**	δ-Oxidation	Dairy, milky, creamy [11.49]	Beef, chicken, pork, butter, cream
Octan-5-olide **(54)**	δ-Oxidation	Coconut, hay, goat [11.58]	Beef, chicken, pork, butter, cream, milk
Nonan-5-olide **(55)**	δ-Oxidation	Fat, cream, milk, butter, oily [11.58]	Beef, chicken, pork, butter, milk
Decan-5-olide **(56)**	δ-Oxidation	Peach, oily, coconut [11.58]	Beef, chicken, pork, lamb, milk, butter, cream
Undecan-5-olide **(57)**	δ-Oxidation	Cream, fat, peach [11.58]	Butter, cream, milk
Dodecan-5-olide **(58)**	δ-Oxidation	Strong, peach, butter, coconut [11.58]	Cheese, butter, beef, chicken, pork
Tetradecan-5-olide **(59)**	δ-Oxidation	Creamy	Beef, chicken, pork, butter
(*Z*)-6-Dodecen-4-olide **(60)**	Linoleic acid [11.61]	Coconut, fruity, peach [11.49]	Butter

the perception threshold may be different. The (*R*)-enantiomer predominates in milk, cream, butter, and cheese for most of the lactones ($R/S > 80/20$ for **(50)**, **(52)**, **(56)**, **(58)**, **(59)**), with the surprising exception of octan-5-olide **(52)**. The high enantiomeric excess is evidence for an enzymatic pathway. As early as 1969, TGs containing 4-hydroxyoctanoic and 4-hydroxynonanoic acid were isolated from butterfat and were shown to give the corresponding lactones **(48)** and **(49)** when heated in water [11.64]. Based on the findings obtained after radiolabeled [1-^{14}C] lauric acid (C_{12}) was infused into the lactating glands of goats, a δ-oxidation pathway was proposed to occur in saturated fatty acids. These experiments eventually yielded radiolabels only in γ–C_{12} and δ–C_{12} lactones, thus proving that C_{12} hydroxy acids had been produced [11.60]. This interesting work has been overlooked, in consideration of the low number of subsequent citations. Another pathway implies the hydration of unsaturated fatty acids combined with the β-oxidation degradation of fatty acids (Fig. 11.18) [11.61]. This mechanism explains why only even carbon number lactones are present in milk. It is in agreement with the observed enantiomeric excessof lactones and also the configuration of the double bond found in (*Z*)-6-dodecen-4-olide **(60)**, as shown in Fig. 11.18. An industrial fermentative process for the synthesis of lactone provides further evidence in favor of this mechanism [11.65].

Table 11.5 shows the major lactones usually found in fat-containing foods. Although they may not all be potent, they are important in many foods. They provide sweet, fruit, peach to fatty, coconut, and creamy notes. For saturated lactones, the fatty character increases with the chain length.

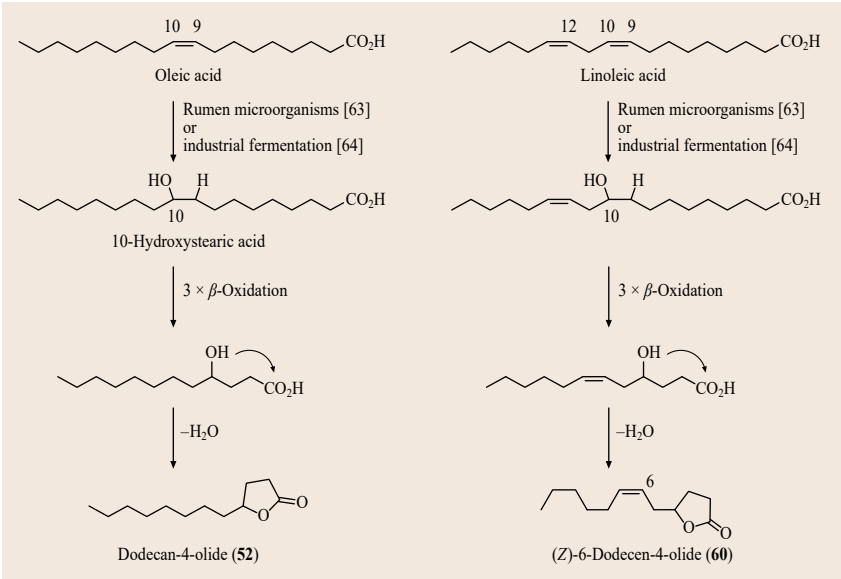


Fig. 11.18 Lactone formation in milk products (after [11.61])

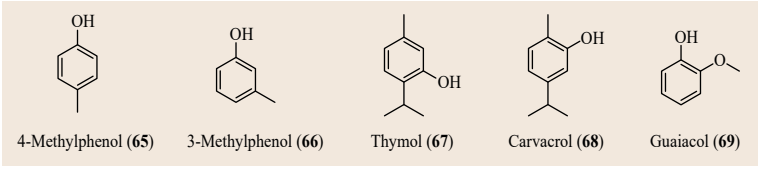


Fig. 11.19 Odorous phenols in fats and oils

11.2.6 Alcohols

Alcohols are not important contributors to the flavor of fat-containing foods (Table 11.6). The thresholdlevels of alcohols are one or two orders of magnitude higher than those of the corresponding aldehydes [11.44]. However, hexanol **(61)**, (*Z*)-3-hexenol **(62)**, and (*Z*)-2-pentan-1-ol **(63)** have been shown to contribute to the green odor of olive oil [11.40]. Alcohols in oils are formed through the same LOX pathway as the aldehydes shown in Fig. 11.12, but a reductase acts as the next step. Natural leaf alcohols, such as **(62)** or (*E*)-2-hexen-1-ol **(63)** are produced industrially in high yield from linolenic acid or linseed oil, using cloned lipoxygenase, HPL, and baker's yeast [11.66]. Phenylethanol **(64)** is not a lipid-derived compound. It is the reduction product of the Strecker aldehyde phenylacetaldehyde **(31)**, as shown in Fig. 11.15.

11.2.7 Phenols

Phenols constitute a class of powerful odorants in foods. They are rarely found in fats and oils, but may contribute to the overall aroma when present. Their odor ranges from woody, smoky to medicinal or animalic and fecal. Table 11.7 and Fig. 11.19 present a few phenols that were identified in fat-containing foods.

11.2.8 Furans

Furans are ubiquitous in fats and oils [11.37]. They are products of the autoxidation of polyunsaturated fatty acids. Thus, 2-pentylfuran **(70)** can form from linoleic acid. Thefirst steps are shown in Fig. 11.20. Aldehyde **(14)** can react with singlet oxygen to give a peroxide that eventually cyclizes upon loss of water [11.67]. 2-Pentylfuran **(70)**, 2-ethylfuran **(71)** of Fig. 11.20, and higher 2-alkylfurans were identified in beef, chicken and pork, or heated butter [11.37]. In many foods, furans such as furfural or 2-acetylfuran are the result of sugar degradation and are important flavor compounds [11.37]. Generally, however, furans that are formed from lipids are not important odor compounds because their concentrations are around or below their odor thresholds in lipophilic matrices.

11.2.9 Nitrogen-Containing Heterocycles

Most of the aroma-active nitrogen-containing compounds, such as pyrazines, are Maillard reaction products. This reaction of amino acids and sugars also

Table 11.6 Odorous alcohols in fats and oils

Alcohol	Precursors [11.44]	Odor [11.37]	Occurrence [11.37]
1-Hexanol **(61)**	Linoleic acid	Banana, flower, fruit, grass, green	Beef, cheese, butter
(*Z*)-3-Hexenol **(62)**	Linolenic acid	Banana, grass, green fruit, green leaf	Butter, olive
(*E*)-2-Hexen-1-ol **(63)**	Linolenic acid	Fat, grass, green, leaf, pungent, sweet, walnut	Olive
Phenylethanol **(64)**	Phenylalanine	Floral, herb, honey, lilac, pungent, rose, spice	Chicken, cheese

Table 11.7 Odorous phenols in fats and oils

Phenol	Precursors	Odor [11.37]	Occurrence [11.37]
4-Methylphenol **(65)**		Fecal, medicinal, phenol, smoke, spice, stable	Fish, cheese, chicken
3-Methylphenol **(66)**		Fecal, medicinal, phenol, plastic, sharp, smoke, spice	Cheese, pork
2-Isopropyl-5-methylphenol **(67)**	Terpene pathway [11.39]	Medicine, wood	Cheese, fish, olive
5-Isopropyl-2-methylphenol **(68)**	Terpene pathway [11.39]	Caraway, spice, thyme	Cheese
2-Methoxyphenol **(69)**	Lignans	Burnt, medicine, smoke, sweet, wood	Olive

produces low molecular-weight amines, such as ammonia, which may subsequently react with lipid oxidation products to yield the nitrogen-containing heterocycles found in heated fats. Thus, model thermal reactions between an amino acid and 2,4-decadienal **(13)** invariably give 2-pentylpyridine **(72)** as a major volatile compound (Fig. 11.21), together with an extensive list of other compounds [11.68]. A series of homologous 2-alkyl pyridines **(72)–(75)** was recently identified in chicken fat [11.69]. Table 11.8 gives their odor description when tasted at a concentration of 10 ppm in water. These compounds are potent odorants whose role in fats and oils has been overlooked.

Similarly, pyrroles, such as **(76)** of Fig. 11.22 can be found in the fat phase of cooked meat. Pyrroles are not considered to be potent flavor components, which is in contrast with pyrrolines such as 2-acetylpyrroline **(77)**. 2-Acetylpyrroline is formed from the amino acid proline, not from lipids. However, it is found in chicken and beef [11.37]. It is an extremely potent compound with an odor threshold of as low as 0.02 $\mu g/m^3$ [11.39].

Pyrazines (Fig. 11.23) are important aroma compounds in foods and are formed by the Maillard reaction. They are found in oils made from roasted seeds or from nuts. In this case, they play a major role in the respective flavors of these foods (Sect. 11.3.1).

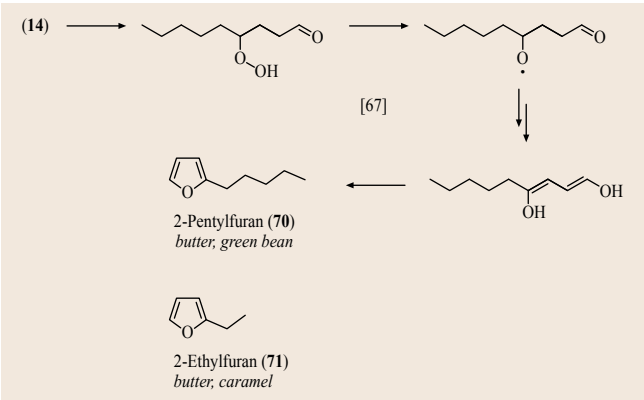


Fig. 11.20 Odorous furans in fats and oils

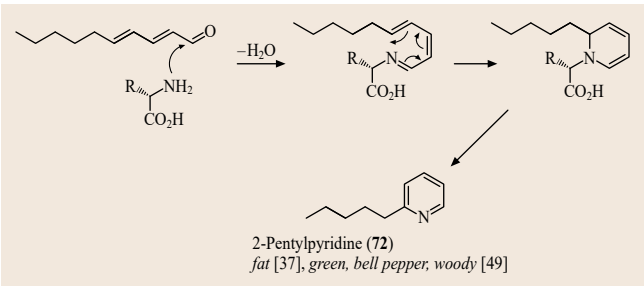


Fig. 11.21 Formation of 2-pentylpyridine (72)

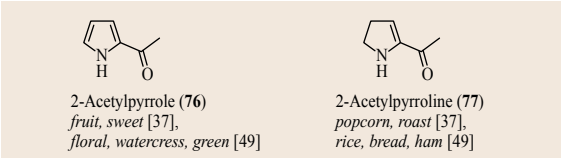


Fig. 11.22 Odorous pyrroles in fats and oils

11.2.10 Other Heterocycles

Sulfur heterocycles, such as thiophenes (Fig. 11.24) and thiapyrans may originate from lipids. Thus, the reaction of hydrogen sulfide with 2,4-decadienal forms either 2-hexylthiophene or 2-pentyl-2*H*-thiapyran [11.70]. Thiophenes are not regarded as important odor contributors to food flavors.

Thiazoles and thiazolines (Fig. 11.25) can also form in fatty and roasted foods through the reaction of cysteamine with a lipid-derived aldehyde or acid [11.70]. These heterocycles may greatly contribute to the flavor of oils such as sesame (Sect. 11.3).

Table 11.8 Odorous pyridines in fats and oils

Pyridine	Precursors	Odor [11.69]	Occurrence [11.37]
2-Pentylpyridine **(72)**	2,4-Decadienal	Fruity, green, woody, vegetable, licorice, aniseed	Beef, chicken, lamb, peanut
2-Butylpyridine **(73)**	2,4-Nonadienal	Fruity, green, fatty, chemical	Chicken, lamb
2-Hexylpyridine **(74)**	2,4-Undecadienal	Fruity, green, fatty, oily	Chicken [11.69], lamb
2-Heptylpyridine **(75)**	2,4-Dodecadienal	Fruity, green, woody, fatty	Chicken [11.69]

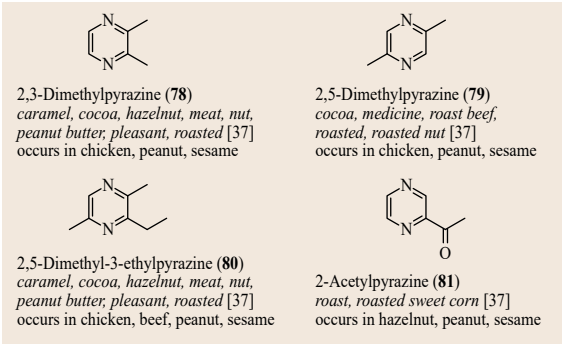


Fig. 11.23 Odorous pyrazines in fats and oils

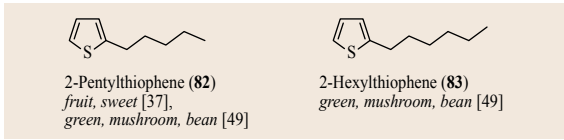


Fig. 11.24 Odorous thiophenes in fats and oils

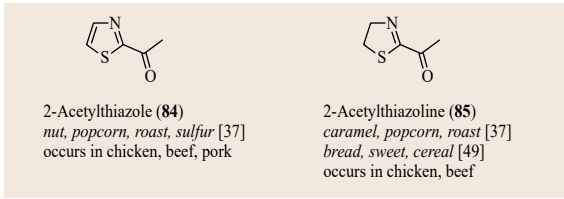


Fig. 11.25 Odorous thiazole and thiazoline in fats and oils

11.3 The Aroma of Fats and Oils

The aroma of foods can be studied for many purposes. For the flavor industry, the creation of a flavor that is the closest to a genuine cooking recipe is the main goal. For agronomists, understanding which compound is a key aroma compound or is detrimental to the overall flavor will help in selecting the right plant hybrids or modifying cultivation practices. The food industry also benefits from knowledge about aroma compounds. Understanding the stability of key aroma compounds during storage or cooking is important to maintain good quality even under cost constraints.

GC-MS analysis of a food extract does not provide sufficient information to fully understand a food's flavor. The key compounds, many of which are found in trace amounts, have to be sorted out from hundreds of components. The identification of key aroma compounds is often done by GC-O [11.71]. In the following section, a selection of the most important fats and oils of the human diet is reviewed. Their odor-contributing compounds, based on recent GC-O studies, are given in the form of tables wherever possible.

The odor activity value (OAV) is often additionally used to evaluate the contribution of individual compounds to the odor profile of food extracts. It represents the ratio between the quantity of a compound divided by its odor threshold in the same matrix. When the OAV is greater than 1, the compound is considered to have an important odor contribution. In aroma extract dilution assay (AEDA) analysis, the analytes are not quantified. The aroma extract is diluted stepwise and each diluted solution is then analyzed by GC-O. The flavor dilution (FD) factor corresponds to the last dilution step of the extract in which the analyte is still perceived. The higher the FD factor, the more the compound contributes to the overall flavor of the extract. The nasal impact frequency (NIF) method uses more panelists, whose task is to report the detection of odorants eluting from the GC and, if possible, provide a description of the odor. The compounds are then classified by the number of panelists that detected each compound. It is hoped that all of these GC-O data will be helpful in understanding the flavor of foods. However, such data are never sufficient to perfectly re-create a natural flavor (personal experiences of this author). Quantitation of the most contributive odorants may help greatly, but the flavorist has to *interpret* the data and use his/her own skills to create a flavor composition that is close to the real food product.

11.3.1 Vegetable Oils

The vegetable oils presented here can be used as such, for example, in salad dressing. They can also be used for cooking or frying, a process during which certain molecules will degrade and others will form. Such processes are not discussed further here.

Olive Oil

Table 11.9 summarizes recently obtained GC-MS and GC-O qualitative and quantitative data [11.17, 40, 72–76]. *Reiners* and *Grosch* [11.76] used distillation for the preparation of the extract and GC-MS and GC-O analysis. After having identified the main odorants, the oil was spiked with deuterated reference compounds for the accurate quantitation [11.76]. There are many differences in the results of these studies. However, it is clear that C_6 aldehydes constitute the key elements of the odor of fresh olive oil, whereas a few ketones and esters may give fruity undertones. Terpenoids and hydrocarbons are found in high quantities, but do not contribute much to the odor.

Peanut Oil

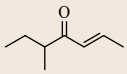
The contribution of flavor compounds to the aroma of peanut oil was analyzed by GC-O using AEDA [11.77]. Table 11.10 shows the compounds that possessed an FD of more than 1. Although numerous aldehydes were found amongst the most important compounds, other chemical classes, not only those that are lipid-derived, contributed to roasted or sweet notes. In particular, pyrazines in aromatic roasted peanut oil were shown to increase upon roasting [11.78]. Many of these pyrazines have a very low odor threshold level when tasted in oil: 2,5-dimethylpyrazine **(79)** is detected at 2 ppm, 2,3-dimethylpyrazine at 50 ppb, and 2,5-dimethyl-3-ethylpyrazine **(80)** at 24 ppb.

Hazelnut Oil

Table 11.10 (see the previous section on peanut oil) shows the result of AEDA analysis for hazelnut oil. Aldehydes were of less importance, whereas one ketone, filbertone **(44)** (Fig. 11.26), gave the specific odor to the oil. This ketone was accurately quantified using the deuterated analog **(44)** as an internal standard and was much higher in roasted hazelnut oil (316 ppb) than in unroasted hazelnut oil (6 ppb) [11.54].

Sesame Oil

Sesame oil has a very characteristic odor and is used for seasoning, particularly in East Asian dishes. In the manufacture of sesame seed oil, the seeds are roasted to develop the roasty odor. In 1996, *Shimoda* et al. identified and quantified 100 flavor compounds in sesame oils made from different roasting processes [11.14]. They used hydrodistillation (Sect. 11.1.1) followed by GC-MS analysis to show that the amount of all of the chemical classes of aroma compounds was increased by a factor of 4–6 upon deep roasting as compared with light roasting [11.82]. Table 11.11 shows the quantitative data expressed in ppb.



(*E*)-5-Methyl-2-hepten-4-one
= Filbertone (**44**)
hazelnut, nut [37]

Fig. 11.26 Filbertone, a typical aroma compound of hazelnut

2-Methylprop-1-ene-1-thiol (**86**)
sulfurous, meaty [81]

(*E*)-3-Methylbut-1-ene-1-thiol (**87**)
sulfurous, meaty [81]

(*Z*)-2-Methylbut-1-ene-1-thiol (**88**)
sulfurous, meaty [81]

(*Z*)-3-Methylpent-1-ene-1-thiol (**89**)
sulfurous, meaty [81]

Fig. 11.27 New thiols in roasted sesame seeds (after [11.79, 80])

2-Ethyl-4-methyl-3-thiozoline (**90**)
tropical, fruity, peach, sulfurous (250 ppb in water) [83]

2-Isoprppyl-4-methyl-3-thiazoline (**91**)
strong, chocolate, gassy, peach, roasted coffee (800 ppb) [83]

Fig. 11.28 Two new thiazolines in sesame oil (after [11.81])

In 2010, the roasting of sesame seeds was studied by *Tamura* et al. [11.79]. Dichloromethane extraction of the ground roasted seeds, followed by SAFE (Sect. 11.1.1), was used to isolate the volatile compounds. Remarkably, 11 thiols were firmly identified, including previously unknown 1-alkenyl-1-thiols **(86)–(89)** (Fig. 11.27). These thiols have not yet been shown to occur in sesame oil. It is likely that high-sensitivity GC-MS or GC-O methods will eventually show their odor contribution, especially when considering their very low odor thresholds [11.80].

Apart from that, flavor scientists recently identified two thiazolines **(90)** and **(91)** (Fig. 11.28) in sesame oil [11.80], which might also contribute to the odor.

Table 11.9 Aroma compounds in olive oil

Compound Acids	%SPME [11.18]	%SDE [11.18]	mg/kg SPME [11.40]	OAV [11.40]	GCO qual [11.72] (or [11.76] when indicated)	GCO Qual [11.73]	µg/kg oil [11.74]	Odor threshold [11.75] (µg/kg)	OAV [11.75]	FD [11.76]	µg/kg oil [11.76]
Acetic acid			Croatia	Croatia	Pungent	Vinegar				16	6830
Propanoic acid					Aromatic, Pungent						
Butyric acid						Sweaty					
3-/2-Methylbutanoic acid										16	81
Hexanoic acid						Sweaty					
Alcohols	**41.94**	**20.19**	**12.721**								
1-Penten-3-ol	0.65	0.28			Wet earth		1986				
Pentan-1-ol					Pungent		45				
2-Methylpropan-1-ol					Ethyl acetate-like						
2-Methylbutan-1-ol					Fish oil						
3-Methylbutan-1-ol	1.03	0.44			n.d.	Malty					
(*E*)-2-Pentenol	0.14	–					39				
(*Z*)-2-Pentenol	0.96	0.4	0.316	1.3	Banana		206				
1-Hexanol	11.7	4.94	3.824	9.6	Fruity, aromatic		1748	400	1.2		
(*E*)-3-Hexenol	0.35	0.12	0.116	0.1	Fruity, aromatic		17	1500	0.02		
(*Z*)-3-hexenol	10.44	4.47	6.870	6.3	Leaf-like [11.76]	Leaf-like	181	6000	0.1	< 8	684
(*E*)-2-Hexenol	15.92	7.41	1.595	0.3	Green, grassy	Leaf-like	2573	8000	0.1		
(*Z*)-2-Hexenol	0.17	0.06			Green fruit		12				
1-Octen-3-ol	0.09	0.24									
1-Heptanol	0.13	0.21									
2-Ethyl-1-hexanol	0.16	0.22									
(*Z*)-Hepten-2-ol	0.05	0.11									
1-Octanol	0.13	0.36									
1-Nonanol	0.08	0.53									
(*Z*)-6-or 4-nonenol	0.02	0.11									
Benzenemethanol	0.21	0.12									
2-Phenylethanol	0.36	0.45				Sweet, winey					

Table 11.9 (continued)

Compound	%SPME [11.18]	%SDE [11.18]	mg/kg SPME [11.40]	OAV [11.40]	GCO qual [11.72] (or [11.76] when indicated)	GCO Qual [11.73]	µg/kg oil [11.74]	Odor threshold [11.75] (µg/kg)	OAV [11.75]	FD [11.76]	µg/kg oil [11.76]
Phenols	**0.09**	**0.12**									
Phenol	0.09										
Ethylphenol		0.12									
Guaiacol						Phenolic, burnt				64	28
Aldehydes	**17.04**	**35.92**	**9.752**								
Pentanal					Sweet, fruity		6974				
3-Methylbutanal			0.007	1.3	Malty [11.76]					< 8	62
2-Methyl-2-butenal					Solvent-like						
Hexanal	0.47	1.63	0.433	5.8	Green, apple	Green	769	75	3.9	8	1770
(*E*)-3-Hexenal					Artichoke, green, flowers			450	1		
(*Z*)-3-Hexenal	0.07	0.12			Green leaves, grassy	Grassy		2.8	155	16	36
(*Z*)-2-pentenal					Green, pleasant						
(*E*)-2-Pentenal	–	0.15	0.083	0.3	Green, apple		20				
Heptanal	0.06	0.45									
(*E*)-2-Hexenal	15.63	7.68	9.203	21.7	Green, apple-like [11.76]	Green, apple-like	3479	1125	12.6	32	6770
Octanal	0.08	0.27	0.026	0.5	Citrus-like [11.76]					32	382
(*E*)-2-Heptenal	0.16	1.92									
2-Octenal					Fruity, soap						
Nonanal	0.34	5.03				Citrus-like				< 8	
(*E,Z*)- or (*E,E*)-2,4-Hexadienal	–	0.45									
(*E,Z*)-2,4-Heptadienal	0.05	1.5									
(*E,E*)-2,4-Heptadienal	–	0.7									
Decanal	–	–									
Benzaldehyde	0.12	0.05									
(*Z*)-2-Nonenal					Green-fatty [11.76]					32	28
(*E*)-2-Nonenal	–	0.23			Paper-like, fatty [11.76]	Paper-like, fatty				16	91

Table 11.9 (continued)

Compound	%SPME [11.18]	%SDE [11.18]	mg/kg SPME [11.40]	OAV [11.40]	GCO qual [11.72] (or [11.76] when indicated)	GCO Qual [11.73]	µg/kg oil [11.74]	Odor threshold [11.75] (µg/kg)	OAV [11.75]	FD [11.76]	µg/kg oil [11.76]
Aldehydes	**17.04**	**35.92**	**9.752**								
(*Z*)-3-Nonenal					Green, harsh [11.76]					8	
(*E*)-2-Decenal	0.04	4.09									
Undecenal	–	0.46									
(*E*,*E*)-2,4-Nonadienal					Deep-fried [11.76]					8	49
(*E*,*Z*)-2,4-Decadienal	–	4								< 8	255
(*E*,*E*)-2,4-Decadienal	–	7.19				Deep-fried				16	422
trans-4,5-Epoxy-(*E*)-2-decenal					Metallic [11.76]					16	32
Vinylbenzaldehyde	0.02	–									
Ketones	**5.17**	**1.52**	**0.458**								
Butan-2-one					Fragrant, pleasant						
3-Pentanone	4.82	1.26	0.234	0	Sweet		944				
1-Penten-3-one			0.224	320	Sweet, strawberry		96			8	26
4-Methylpentan-2-one					Sweet						
Heptan-2-one					Fruity						
2-Octanone	0.1	0.09			Fruity, mushroom-like						
4-Octanone	–	–									
6-Methyl-5-hepten-2-one	0.2	0.17			Fruity						
1-Octen-3-one					Mushroom-like [11.76]					32	1.4
(*Z*)-1,5-Octadien-3-one					Geranium-like [11.76]					32	0.05
2-Nonanone					Fruity						
Phenylethanone	0.05	–									

Table 11.9 (continued)

Compound	%SPME [11.18]	%SDE [11.18]	mg/kg SPME [11.40]	OAV [11.40]	GCO qual [11.72] (or [11.76] when indicated)	GCO Qual [11.73]	µg/kg oil [11.74]	Odor threshold [11.75] (µg/kg)	OAV [11.75]	FD [11.76]	µg/kg oil [11.76]
Esters	**8.65**	**7.20**	**0.063**		**Sweet, aromatic**						
Ethyl acetate					Sweet, strawberry, apple						
Ethyl propanoate											
Ethyl-2-methylpropanoate					Fruity [11.76]	Fruity				16	1.4
Ethyl-2-methylbutyrate			0.002	2.9	Green, pungent, sweet	Fruity				32	2.1
Ethyl-3-methylbutyrate						Fruity					
Butyl acetate					Banana						
3-Methylbutyl acetate			0.011		Putty-like, unpleasant	Fruity					
3-Methyl-2-butenyl acetate											
Methyl pentanoate	0.29	0.11			Aromatic, ketone						
2-Methylbutyl pentanoate											
Methyl hexanoate	0.09	–									
Hexylacetate	1.67	1.17	0.050	0.1	Sweet, fruity		20	1040	0.1		
(*Z*)-3-Hexenylacetate	5.56	4.01			Green-banana, fruity	Banana-like	11	750	0.1	16	2250
(*E*)-2-Hexenylacetate	0.52	1.13					8				
Methyl nonanoate					Fruity						
Methyl decanoate					Fresh						
Methylbenzoate											
Methylsalicylate	0.52	0.78				Medicinal					
Furans											
2-Ethylfuran (**71**)					Sweet						
3-(4-Methyl-3-pentenyl)furan					Moldy						
Terpenic sesquiterpenic compounds	**4.39**	**22.65**									
Hydrocarbons	22.06	12.14									

Table 11.10 Aroma compounds in peanut and hazelnut oil

Compound	Odor	FD-factor for peanut oil [11.77]	FD-factor for hazelnut oil [11.77]
Acids			
Hexanoic acid	Sweaty	10	> 1000
3-Methylbutanoic acid	Sweaty	1	1000
Aldehydes			
(*Z*)-2-Nonenal	Fatty, green	1000	–
(*E,E*)-2,4-Decadienal	Fatty, deep fried	1000	100
(*E,Z*)-2,4-Nonadienal	Green	100	10
(*E,Z*)-2,4-Decadienal	Fatty, green	100	10
2-Phenyl-2-butenal	Green, phenolic	100	–
3-Methylbutanal	Malty	10	–
Octanal	Fatty	10	–
(*E*)-2-Nonenal	Fatty, green	10	100
Phenylacetaldehyde	Sweet, honey-like	10	100
(*E,E*)-2,4-Nonadienal	Fatty, deep-fried	10	100
(*E*)-2-Undecenal	Fatty	10	1
Hexanal	Green	1	100
(*E*)-2-Heptenal	Fatty, green	1	–
(*E,Z*)-2,6-Nonadienal	Cucumber-like	1	–
trans-4,5-Epoxy-(*E*)-2-decenal	Metallic, green		10
(*Z*)-2-Octenal	Fatty		1
Furfural			
(*E*)-2-Decenal	Fatty, green		1
Esters			
Ethyl-2-methylbutanoate	Fruity	1000	1
Ethyl isobutyrate	Fruity	100	1
Ketones			
(*E*)-5-Methyl-2-hepten-4-one	Sweet, hazelnut-like		1000
Lactones			
δ-Dodecalactone	Sweet	10	–
γ-Octalactone	Fruity, coconut-like		> 1000
N-heterocycles			
2-Ethyl-3,5-dimethylpyrazine	Roasty	1000	> 1000
2,3-Diethyl-5-methylpyrazine	Roasty	1000	> 1000
2-Acetyl-1-pyrroline	Roasty, sweet	100	–
2-Propionyl-1-pyrroline	Roasty	100	–
3-Ethy1-2,5-dimethylpyrazine	Roasty	100	1
2-Ethenyl-3,5-dimethylpyrazine	Roasty	100	–
2,5-(or 2,6-) Diethylpyrazine	Sweet	10	100
2-Methyl-5-((*E*)-1-propenyl)pyrazine	Sweet, earthy	10	–
2-Ethyl-5-methylpyrazine	Sweet	1	–
2-Ethyl-3-methylpyrazine	Roasty	1	1
Phenols			
2-Methoxyphenol (guaiacol)	Burnt	100	–
Vanillin	Vanilla-like	100	> 1000
2-Methoxy-4-vinylphenol	Spicy, phenolic	10	1000
Sulfur compounds			
3-Mercapto-2-butanone	Cooked meat-like	100	–
Dimethyltrisulfide	Sulfurous	100	–
2-Furfurylthiol	Sweet, smoky	100	–
Terpenoids			
(*E*)-β-Damascenone	Boiled apple-like	1000	100
D-Limonene	Lemon-like	10	> 1000

Table 11.11 Aroma compounds in sesame oil

Compound	Deep-roasted (ppb) [11.82]	Light-roasted (ppb) [11.82]	Impact odorant [11.14]
Pyrazines	**48.8%**	**49.2%**	
2-Methylpyrazine	779	195	
2,5-Dimethylpyrazine	735	158	
2,6-Dimethylpyrazine	383	82	
2-Ethylpyrazine	153	35	
2,3-Dimethylpyrazine	101	23	
2-Ethyl-6-methylpyrazine	237	47	X
2-Ethyl-5-methylpyrazine	212	40	X
2,3,4-Trimethylpyrazine	346	65	
2,6-Diethylpyrazine			X
2-Ethenylpyrazine	46	12	
3-Ethyl-2,5-dimethylpyrazine	542	104	
2-Ethyl-3,5-dimethylpyrazine	154	45	X
2-(1-Methylpropyl)pyrazine	23	5	
2-Ethenyl-6-methylpyrazine	55	9	
2,3-Diethyl-5-methylpyrazine	22	4	
3,5-Diethyl-2-methylpyrazine	92	21	
2-Methyl-6-(1-propenyl)pyrazine	47	8	
2-Isopropenylpyrazine	48	8	
2-Acetylpyrazine	178	35	
(*E*)-2-Methyl-6-(1-propenyl)pyrazine	51	11	
2,3-Dimethyl-5-isopentylpyrazine	28	tr	
Pyrroles	**5.0%**	**10.4%**	
2-Ethyl-1*H*-pyrrole	19	3	
1*H*-pyrrole	70	16	
3-Methyl-1*H*-pyrrole	26	tr	
1-Methyl-1*H*-pyrrole-2-acetonitrile	40	7	
2-Ethyl-4-methyl-1*H*-pyrrole			X
1-Ethyl-1*H*-pyrrole-2-carboxyaldehyde	29	5	
1-(1-Methyl-1*H*-pyrrol-2-yl)ethanone	22	tr	X
1-(1*H*-Pyrrol-2-yl)ethanone	71	17	
1*H*-Pyrrole-2-carboxyaldehyde	127	143	
Methylpyrrole-2-carboxylate	33	tr	
Pyridines	**2.3%**	**1.7%**	
1-(2-Pyridinyl)ethanone	16	tr	X
4-Pyridinylacetate	67	13	
Methyl4-pyridinecarboxylate	55	10	
2-Pyridinemethanol	58	9	
Thiazoles	**6.6%**	**5.9%**	
4-Ethylthiazole	35	7	
2,4-Dimethylthiazole	58	12	
2,5-Dimethylthiazole	115	20	
4,5-Dimethylisothiazole	39	8	X
4,5-Dimethylthiazole	110	22	X
4-Methyl-5-ethylthiazole	23	5	
2-Ethyl-5-methylthiazole	28	8	
2-Propyl-4-methylthiazole	123	21	X
2-Butyl-5-methylthiazole	45	5	
Furans	**9.3%**	**7.9%**	
2-Pentylfuran	50	8	
2-Furfural	51	10	
2-Furanmethylacetate	75	13	
5-Methyl-2-furfural	220	47	

Table 11.11 (continued)

Compound	Deep-roasted (ppb) [11.82]	Light-roasted (ppb) [11.82]	Impact odorant [11.14]
Furans	**9.3%**	**7.9%**	
Furfurylalcohol	316	49	
1-(5-Methyl-2-furanyl)-1-propanone	70	10	X
α-Methyl-α-vinyl-2-furanacetaldehyde	25	8	
Aldehydes	**11.8%**	**12.6%**	
Pentanal	36	15	
Hexanal	263	59	
2-Methyl-2-butenal	31	15	
Heptanal	30	7	
Octanal	26	6	
(*E*)-2-Heptenal	82	13	
(*E*)-2-Octenal	48	16	
(*E*,*E*)-2,4-Nonadienal	32	12	
(*E*,*Z*)-2,4-Decadienal	35	tr	
(*E*,*E*)-2,4-Decadienal	154	36	
Benzaldehyde	240	53	
α-Ethylidenbenzeneacetaldehyde	47	tr	
Ketones	**4.8%**	**4.2%**	
2-Heptanone	31	6	
2-Nonanone	62	11	
1-Phenylethanone	142	24	
1-(3-Methoxyphenyl)ethanone	177	37	
Alcohols	**3.3%**	**3.2%**	
Hexanol	79	12	
Octanol	26	9	
Dodecanol	69	12	
Benzenemethanol	69	16	
Benzeneethanol	47	10	
Acids	**1.7%**	**0.7%**	
Hexanoicacid	32	8	
Heptanoicacid	32	5	
Octanoicacid	86	tr	
Phenols	**4.1%**	**2.1%**	
Guaiacol	321	32	
2-Methoxy-5-(1-propenyl)phenol	33	7	
Esters	**0.5%**	**0.7%**	
Ethylacetate	45	12	
Sulfur	**0.9%**	**0.7%**	
2-Furanmethanethiol	40	6	
3-Formylthiophene	38	6	X
Others	**0.7%**	**0.8%**	
D-Limonene	27	7	
2,3-Dihydro-1*H*-indole	37	7	

11.3.2 Animal Fats

Milk and Butter

A thorough analysis of the flavor of milk was published by *Bendall* in 2001 [11.83]. The extraction of milk was performed by using SAFE (Sect. 11.1.1) and more than 60 aroma compounds were identified by GC-MS and GC-O using the NIF method (Table 11.12 and Fig. 11.29). The nitrogen-containing heterocycles indole **(92)** and skatole **(93)**, as well as phenol **(94)** and 4-propylphenol **(95)**, added the animalic notes to milk. γ-Lactones **(50)**, **(52)**, **(60)**, **(96)**, and **(97)** and δ-lactone **(56)** had high NIF factors.

Butter aroma has been the subject of numerous GC-O studies [11.55, 87]. These were reviewed in 2008 by *Mallia* et al. [11.85]. The list of aroma-

Table 11.12 Aroma compounds in milk products

Compound	Odor GC-O of milk [11.83]	Odor threshold ppb in water [11.83]	Odor GC-O of butter oil [11.85]	Concentration (ppb) in whole butter [11.86]	Concentration (ppb) in butter oil [11.55]
Acids					
Acetic acid	Vinegar	22 000			
Butyric acid	Vomit; feta cheese	1000	Butter, sweat, cheese, rancid	192	Present, not quantified
3-Methylbutyric acid	Slight grassy; cooked vegetables	250			
Pentanoic acid	Acidic; bready; cheese, dog	2100			
Hexanoic acid			Pungent, must, cheese, acrid	732	Present, not quantified
Dec-9-enoic acid	Wet paper; burnt dust				
Phenylacetic acid	Sweet peachy; old cotton; oats	1000			
Aldehydes					
2-Methylbutanal	Musty	0.9	Chocolate, fruity	4.9	–
3-Methylbutanal	Musty; wet dog	0.25	Chocolate	11.9	–
Pyruvaldehyde	Sweet; earthy	5000			
Hexanal	Cooked	10.5	Green, fatty	29	Present, not quantified
Hex-*cis*-3-enal	Grassy; fresh milk; warm milk	0.03			
Heptanal	Cheesy; caramel	3			
(*E*)-2-Hexenal	Wet grass; rancid fat; sour cream	27			
(*Z*)-4-Heptenal	Fresh milk; biscuity; apple shortcake	0.06	Green, fatty, cream, biscuit	Present, not quantified	0.3
Nonanal			Waxy, fatty, floral	43	–
(*E*)-2-Nonenal			Green, fatty, tallow	10	6.75
(*Z*)-2-Nonenal			Green, fatty	Present, not quantified	0.2
Nona-*trans*-2,*cis*-6-dienal	Green; fresh milk; green apple/tomato	0.01			
Nona-*trans*-2,*trans*-4-dienal	Fatty; old cardboard	0.06			
Esters					
Ethyl acetate	Sweet ester; solvent	5			
Ethyl 2-methylbutyrate	Juicy-fruit chewing-gum; berry-like	0.006			
Ethyl 3-methylbutyrate	Ester; green ester	0.03			
Ethyl oleate	Ester				
Ethyl linolenate	Fruity; sweet lactone				

Table 11.12 (continued)

Compound	Odor GC-O of milk [11.83]	Odor threshold ppb in water [11.83]	Odor GC-O of butter oil [11.85]	Concentration (ppb) in whole butter [11.86]	Concentration (ppb) in butter oil [11.55]
Ketones					
2,3-Butanedione	Butter; pastry	5	Butter	6.6	Present, not quantified
Pentane-2,3-dione	Milky	5			
1-Hexen-3-one			Vegetable, metallic	0.004	Present, not quantified
1-Octen-3-one	Mushroom; vegetable; dry hay	0.01	Mushroom	0.58	1.1
Acetoin	Caramel; butterscotch	800			
1-Nonen-3-one	Paint	0.000008			
(*Z*)-1,5-Octadien-3-one	Herbaceous, strong; warm milk; green grassy	0.0004			
Lactones					
δ-Hexalactone	Fruity; rolled oats		Cream, chocolate, sweet, aromatic	47.9	–
δ-Octalactone	Lactone fruity; sweet	400	Coconut, peach	72.8	Present, not quantified
cis-3-Methyl-γ-nonalactone	Sweet lactone; buttery; toast				
γ-Decalactone	Lollies; milky	11			
δ-Decalactone	Coconut; hot milk; fruity	100	Coconut, peach	1193	Present, not quantified
γ-Dodecalactone	Lactone; fruity; sweet floral	7	Peach	441	–
(*Z*)-6-Dodecen-4-olide	Fruit; dishcloth; burnt sugar	0.1 (alc. soln.)	Peach	–	Present, not quantified
δ-Dodecalactone	Sweet fruity	1000			
γ-Dodec-*cis*-6,*cis*-9-dienolactone	Muesli with honey; baby powder				
γ-Tetradecalactone	Hot milk				
δ-Tetradecalactone	Dry hay				
δ-Hexadecalactone	Lactone; hot; smoky				
2-Acetyl-1-pyrroline	Mouse-dirt; crackers; burnt chocolate pudding	0.0073 (starch)			
Indole	Hot rubber; jonquils; earthy	90			
Skatole	Melted/baked butter; garden dirt; lamb	3			

Table 11.12 (continued)

Compound	Odor GC-O of milk [11.83]	Odor threshold ppb in water [11.83]	Odor GC-O of butter oil [11.85]	Concentration (ppb) in whole butter [11.86]	Concentration (ppb) in butter oil [11.55]
Phenols					
Phenol	Leather; paint; shortbread	250			
p-Cresol	Soap; old milk	2			
4-Ethylphenol	Barny; burnt paper; dirty faecal	100			
4-Propylphenol	Wet hair; bitter				
Nitrogen					
2-*iso* Butyl-3-methoxypyrazine	Vegetable capsicum; woody/green; grassy	0.005			
3-Methyl-1*H*-indole			Mothball, feacal	12.6	Present, not quantified
Sulfur compounds					
Dimethyl disulfide	Rubber	12	Corn, fresh pumpkin	20	–
Dimethyl trisulfide			Garlic, sulfury	17.4	–
2-Methylthiophene	Milky; cooked vegetables				
S-Methyl thio-3-methylbutyrate	Cooked milk; lactone, cherry; shortbread	0.04			
Dimethyl trisulfide	Cheesy; rolled oats	0.008			
Benzothiazole	Dead match-head; popcorn, strong; musty	50			
Terpenoids					
α-Pinene	Solvent	120			
α-Thujene	Cooked; nutty				
α-Pinene	Soapy, fragrant; green				
2-Carene	Rolled oats; ethereal				
Limonene	Orange	200			
Linalool	Floral; fruity	1.5			
β-Caryophyllene	Berry-fruit; orange				
Phyt-2-ene	Grass; hay				
Phyt-1-ene	Grassy; cardboard				
Phyta-*trans*-3,*trans*-5-diene	Wood polish; paint				
Neophytadiene	Wood polish; burnt				
Phyta-*trans*-2,*cis*-4-diene	Fecal; burnt				
Phytol	Bitter; oats				

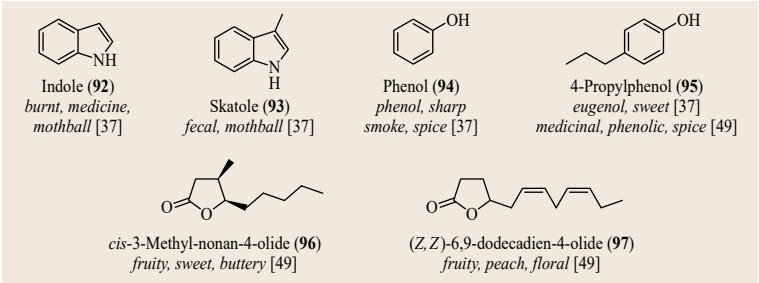


Fig. 11.29 Selected key aroma compounds in milk (after [11.83])

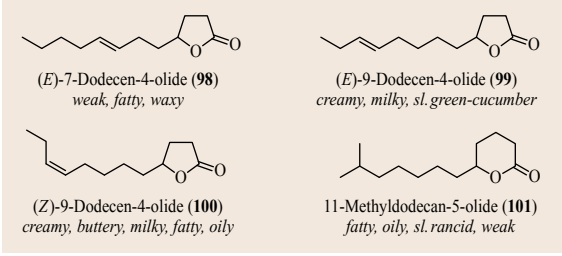


Fig. 11.30 New lactones in butter oil (after [11.84])

active compounds shown in Table 11.12 is adapted from this review. In sweet cream butter, the quantities indicated in Table 11.12 were obtained by static headspace analysis (Sect. 11.1.2) carried out in a large glass vessel [11.86]. Less quantitative data are available on the lone butter oil [11.55]. Recently, *Sarrazin* et al. carried out a study focused on the lactones in butter oil [11.84]. They distilled 1 kg of butter oil (Sect. 11.1.1) and discovered a series of new γ-lactones after systematic chemical synthesis [11.88]. Reconstitutions based on the lactone fraction and diluted in sunflower oil were carried out. The first reconstitution was only composed of the major lactones, **(50)**, **(54)**, **(56)**, **(58–60)** in the ratio actually found in butter oil. In addition to the major lactones, a second reconstitution also contained the minor lactones **(98–101)** of Fig. 11.30. The odor difference of the two reconstituted solutions were clearly perceived and the lactones **(98–101)**, although found in lower quantities, brought a more creamy and coconut character to the overall odor [11.84].

Cheese

The aroma of cheese has been exhaustively reviewed by *Curioni* and *Bosset* [11.41], who compiled the GC-O data from about 100 references in nine tables. They provided a comprehensive overview of the odor qualities and thresholds of many compounds sorted according to chemical class. In this review, thirteen different cheese types were taken into consideration and the main odorants were summarized based on the GC-O studies carried out in the cited references. The main odorants in Gongonzola, a typical blue cheese made from cow's milk in Italy, were found to be 2-heptanone **(37)**, 2-nonanone **(38)**, 2-heptanol (green, earthy), 1-octen-3-ol (mushroom-like) and ethylhexanoate (fruity, apple). In Camembert, a typical mold-ripened cow's milk cheese from France, a larger variety of compounds were identified as the main odorants, namely 1-octen-3-ol, 3-methylbutanal **(34)**, 2-undecanone **(39)**, 1-octen-3-one **(40)**, 2,3-butanedione (buttery), phenylethyl acetate (floral, rose-like), decan-5-olide **(56)**, methional **(35)**, methanethiol (sulfurous), dimethylsulfide (sulfurous), butanoic acid **(1)**, and 3-methylbutanoic acid **(6)**. In Gruyère, a typical hard cheese made from cow's milk in Switzerland, the main odorants were 2-methylbutanal **(33)**, (*Z*)-2-nonenal (fatty, tallowy), phenylacetaldehyde **(31)**, methional **(35)**, methanethiol, dimethyltrisulfide (cabbage-like, sulfurous), propanoic acid (fruity, pungent), butanoic acid **(1)**, 2-methylbutanoic acid **(7)**, and 3-methylbutanoic acid **(6)**. 2-Ethyl-3,5-dimethylpyrazine (earthy) and 2,3-diethyl-5-methylpyrazine (earthy) are also impact odorants formed during the cooking steps (34−54 °C) involved in the preparation of Gruyère cheese. In goat cheese, acids are the main odorants: nonanoic acid (goat-like), 4-ethylnonanoic (goat-like), and 3-methylbutanoic **(6)**.

Beef Fat

Most of the publications on beef flavor describe the analysis of roast beef with the meat being minced and roasted before extraction of the volatile compounds [11.89]. *Um* et al. analyzed heated beef fat flavor compounds isolated by supercritical CO_2 extraction (Sect. 11.1.3) [11.91]. Besides hydrocarbons, they identified 15 aldehydes, three ketones, four phenols, 10 carboxylic acids, six esters, and seven lactones, which are summarized in Table 11.13. *Umano* and *Shibamoto* used the headspace technique (Sect. 11.1.2), resulting in the identification of a greater number of more volatile compounds on average [11.92]. However, the absence of esters and lactones shows the inability of the technique to extract compounds of lower volatilities. An interesting setup was used by *Rochat*

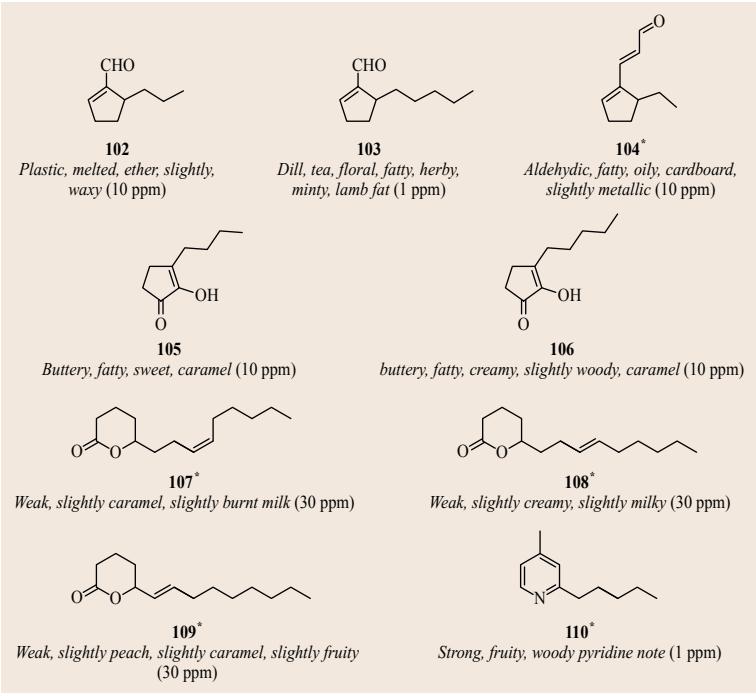


Fig. 11.31 Aroma compounds newly found in chicken (after [11.90]). Asterisks denote compounds not previously known in nature. Odor qualities given from tasting the compounds are shown at the indicated dosage in salted water

and *Chaintreau* for the analysis of roast beef [11.93]. They placed a Tenax cartridge to extract the headspace of the oven during cooking. It can thus be considered that the volatile compounds came from the fat being exuded during roasting. The study focused on carbonyl compounds; more than 50 of them were identified, among which were straight and branched-chain aldehydes, 2-alkenals and 2,4-alkadienals, and 2-alkanones. Their flavor impact was assessed by GC-O with the NIF method (see Sect. 11.3 above); these substances are also presented together with the obtained data in Table 11.13. Another GC-O study of beef fat headspace found the same key compounds, with 4-heptenal, 2,6-nonadienal, indole, and decan-5-olide also being considered as important aroma chemicals [11.94].

Pork

The volatile compounds identified in pork fat do not differ much from those of beef fat. The reader is referred to the review of *Werkhoff* et al. [11.47] for a comparison of beef, pork, and chicken meat, with an emphasis on sulfur compounds and long-chain aldehydes. A careful qualitative and quantitative analysis of roasted pork by GC-MS and GC-O provided detailed results (amounts, odor quality, NIF factors) [11.95].

Chicken

Considering the topic of the present chapter, that is, the flavor of fats, chicken perhaps constitutes the most interesting example because the fat resides in a precise location near the meat and skin. Therefore the chicken can be roasted and a different part, in particular the fat, can be analyzed separately. *Noleau* and *Toulemonde* published a thorough piece of work in which they identified and quantified nearly 200 compounds in the skin and meat of roasted chicken, 80 of which were new [11.96]. For skin and meat, they used the Likens–Nickerson apparatus (Sect. 11.1.1). The dripping fat was also analyzed with short-path distillation (Sect. 11.1.1) and more than 200 compounds were identified by GC-MS and quantified by GC-FID [11.97]. Again, the volatile part was composed of aldehydes (51 chemicals, accounting for 120 mg/kg of fat), ketones (34, 10 mg/kg), acids (19, 40 mg/kg), and lactones (24, 8 mg/kg) as the major components. *Delort* et al. subsequently followed the same approach to analyze distinct parts of cooked chicken, including its fat [11.90]. Short-path distillation was used to produce the fat aroma extract, which was fractionated further by chromatography over silica gel, and many new compounds could be identified (Fig. 11.31). The role of these compounds in the overall aroma remains to be assessed.

Table 11.13 Aroma compounds in beef

Compound	Concentration in heated beef fat ppm [11.91]	Amount in heated beef fat (GC FID %) GC-FID peak area % [11.92]	GC-O NIF, detection frequency % [11.93]	GC-O Odor quality [11.93]	GC-O Odor quality [11.94]
Acids					
Acetic acid	3.65				
Butyric acid	0.56				Fecal
3-Methylbutyric acid	0.31				
Pentanoic acid	0.15				
Hexanoic acid	2.09				
Heptanoic	2.18				
2-Ethylheptanoic	1.43				
Octanoic	3.85				
Nonanoic	8.92				Fat, cheese
Decanoic	3.22				
Aldehydes					
Acetaldehyde			21	Grilled (weak), acetaldehyde-like	
Propanal		tr.	74	Caramel, sweet, alcoholic, *cooked*, broth, spicy, earthy	
Acrolein		5.18			
Butanal	0.23	28	Smoky, fish, amylic, aldehyde-enal or dienal		
3-Methylbutanal			41	Bad, meaty, fish, rotten, aldehyde, valeric acid, fatty	
Pentanal	1.71	tr.			
(*E*)-2-Pentenal		0.32			
Hexanal	2.94	2.53	42	Green, fresh grass, aldehyde	Cut grass
(*E*)-2-Hexenal		0.55			Cut grass
Heptanal	0.76	3.06	53	Aldehyde, green	Fat yuck, sweaty, feet
(*Z*)-3-Heptenal		tr.			

Table 11.13 (continued)

Compound	Concentration in heated beef fat ppm [11.91]	Amount in heated beef fat (GC FID %) GC-FID peak area % [11.92]	GC-O NIF, detection frequency % [11.93]	GC-O Odor quality [11.93]	GC-O Odor quality [11.94]
Aldehydes					
(*Z*)-4-Heptenal					Fat yuck, sweaty, feet
(*E*)-2-Heptenal		2.07			
(*E*,*E*)-2,4-Heptadienal		tr.	37	Aldehyde, green, broth, spicy	
Benzaldehyde	0.07	0.51			
Octanal	1.66	2.57	32	Green, lemon, citrus, aldehyde	Citrus leaf
(*Z*)-3-Octenal		0.26			
(*E*)-2-Octenal		1.28	70	Aldehyde, green, floral, dienal, fatty, cardboard	
(*E*,*E*)-2,4-Octadienal			53	Mild, alcoholic, floral, green, lemon, aldehyde, sea	
Phenylacetaldehyde	tr.				
Nonanal	1.84	7.01	85	Sea, aldehyde, citrus, green, citronella grass	Fatty
(*E*)-2-Nonenal	0.94	1.32	80	Paper, fatty, iris, nauseating, aldehyde, dienal, wood, nut	
(*Z*)-2-Nonenal		0.42			
(*E*,*E*)-2,4-Nonadienal			74	Dienal, aldehyde, pan, swatted bug, insect, fatty, rancid	
(*E*,*Z*)-2,6-Nonadienal					Cucumber
Decanal		0.34	64	Fatty, rancid, meaty, burnt, tobacco, aldehyde, green, overcooked	
(*E*)-2-Decenal	3.77	1.45	48	Green, fat	Fat, cheese
Undecanal	0.73		53	Mild, alcoholic, floral, green, lemon, aldehyde, sea	
(*E*)-2-Undecenal		tr.			
(*E*,*E*)-2,4-Decadienal	0.75		51	Plastic, tailing odor	
2-Undecenal	2.62				
Dodecanal	0.65				
Tridecanal	0.48				

Table 11.13 (continued)

Compound	Concentration in heated beef fat ppm [11.91]	Amount in heated beef fat (GC FID %) GC-FID peak area % [11.92]	GC-O NIF, detection frequency % [11.93]	GC-O Odor quality [11.93]	GC-O Odor quality [11.94]
Esters					
Methyl octanoate	0.14				
Methyl nonanoate	0.22				
4-Methylheptanedioic acid methyl ester	1.30				
Methyl tetradecanoate	2.24				
Methyl hexadecanoate	1.98				
Methyl octadecanoate	1.65				
Ketones					
2-Butanone		tr.			Butterscotch, caramel
2,3-Butanedione			71	Butter	Butterscotch, caramel
Cyclohexanone		0.35			
2-Heptanone		0.38	32	Citrus, grapefruit, limonene, floral, cheese	
4-Octanone		tr.			
2-Octanone		0.31			
2-Methyl-3-octanone		0.23			
2-Nonanone		tr.			
2-Decanone		tr.			
3-Hydroxy-4*H*-pyran-4-one	0.10				
2-Tridecanone	4.98				
2-Hexadecanone	1.66				
2-Heptadecanone	5.27				
1-Octen-3-one			74	Old water, mushroom, cellar	
1-Nonen-3-one			85	Mushroom	
Alcohols					
Heptanol	tr.	0.39			
Octanol		tr.			
1-Octen-3-ol		0.51			Mushroom
Tridecanol	0.25				

Table 11.13 (continued)

Compound	Concentration in heated beef fat ppm [11.91]	Amount in heated beef fat (GC FID %) GC-FID peak area % [11.92]	GC-O NIF, detection frequency % [11.93]	GC-O Odor quality [11.93]	GC-O Odor quality [11.94]
Lactones					
γ-Nonalactone	0.47				
δ-Decalactone	0.79				Coconut
δ-Undecalactone	0.30				
δ-Dodecalactone	1.43				
δ-Tetradecalactone	23.30				
δ-Pentadecalactone	1.70				
δ-Hexadecalactone	17.60				
Furans					
2,5-Dimethyltetrahydrofuran	0.18				
2-Pentylfuran	1.10	0.79			
2-Hexylfuran		0.45			
2-Heptylfuran		tr.			
Phenols					
Phenol	2.09				
4-Methylphenol	0.33				
2,5-Dimethylphenol	2.25				
3,5-Dimethylphenol	3.33				
Nitrogen compounds					
Indole					Fecal, soil
Hydrocarbons					
Total	13.61	54.51			
Terpenoids					
Total	29.57	10.45			

11.4 Conclusion

The analysis of aroma compounds in fats and oils is a difficult task. Understanding the aroma of fat-containing foods requires many analytical skills, from the preparation of a good extract to the GC-MS identification of hundreds of chemical compounds. GC-O and/or quantitative studies are then needed to assess the role of each component. Many extraction methods are available and may be adapted to the needs of the analysis. Further fractionation can provide the identity of even more products that can play a role in the flavor. Aldehydes, acids, lactones, and ketones are common key contributors to the aroma of fats and oils. However, the sensory importance of sulfur compounds and nitrogen heterocycles should not be overlooked. Finally, it should be noted that the many components can be desirable or undesirable, such as in frying oil, which imparts good taste to foods but may also be responsible for deterioration in quality if not properly used [11.98]. A fundamental understanding of the diverse mechanisms of formation of these chemicals is thus the key prerequisite to maximizing or preventing the formation of certain compounds.

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12. Aroma Encapsulation and Controlled Delivery

Gary Reineccius

The area of protecting and delivering aroma compounds in a food application is most challenging. At this time, well over 8000 aroma compounds have been found in nature and they vary widely in physical and chemical properties which makes their protection and delivery very problematic (evaporation and chemical reactivity). Thus, one attempts to design encapsulation systems that protect the key aroma compounds but yet deliver them when needed using cost-effective and legally approved materials and methodologies. This chapter provides strategies used to accomplish these goals. Overviews of materials and methods used in encapsulation and controlled delivery are presented. The final section offers insights into unmet needs in this area.

There is no question that volatile compounds that contribute to sensory perception (aroma compounds) are extremely important to characterizing the flavor of a food. Until fairly recently, it was felt that they were the only chemicals contributing significantly to flavor perception. Food manufacturers treated taste as if was an afterthought, that is, if a product needed a sweet taste, one could use any sweet compound (most likely the cheapest) and flavor character would be satisfactory. We have since learned that taste and aroma are intimately linked and the human brain has certain expectations of congruency to elicit a true flavor (Chap. 47).

The long held belief that aroma was the only consideration in creating a flavor resulted in a great deal of research on identifying, determining the importance of, stabilizing and delivering aroma compounds on eating. This chapter will focus on stabilizing and controlling the release of flavor compounds in foods. Stabilizing is very important since aroma compounds are often chemically reactive and are, by definition, volatile. They need to be protected from loss from manufacture to consumption; this is often done by employing some process of encapsulation. To deliver these protected aroma compounds they must be released from the encapsulant. This brings us to the topic of controlled release, that is, when do we want them released. Ideally, they should be released from the encapsulant just as the food goes in the mouth so they may be protected until the very last moment and then be released into the food to create the desired perception when masticated or simply swallowed. This chapter will discuss the concepts of encapsulation to provide the protection of aroma compounds and then their controlled release at some step in the food preparation process.

12.1 Diversity in Aroma Compounds

In order to address the topic of aroma encapsulation, it is worthwhile to set the stage by discussing what compounds one wants to protect, what we want to protect them from, and what the issues are in terms of controlling their delivery. This book is focused on odor compounds: those compounds found in foods that stimulate the sense of smell. Essentially, this must consider all volatile compounds present in a food that are transferred to the breath during eating at adequate concentrations to influence perception.

At this time, more than 8000 volatile chemicals, and thus potential contributors to odor, have been identified in food products. These compounds differ tremendously in terms of volatility, solubility, and reactivity. Volatility, expressed as boiling point, can range from temperatures of ca. −60 °C (e.g., hydrogen sulfide) to ca. 285 °C (e.g., vanillin). An aroma compound can be totally immiscible in water or totally insoluble; others can easily be dissolved in water. In terms of reactivity, some aroma compounds are extremely stable to normal temperatures, acidities, ambient light or the presence of oxygen. Others are very labile. If a food flavor was composed of only one known compound and we had to protect it from one known reaction, our task would be simple. Unfortunately, nature has chosen to make our world much more complicated and also much richer. While one aroma compound may reproduce a specific food flavor, the perceived flavor is poor and not true to nature. Nature uses many compounds to provide depth and character. The result is we must protect many, often very different, aroma compounds to deliver a quality flavor. The breadth of physical and chemical properties as well as reactivity displayed by aroma chemicals makes designing encapsulation processes extremely challenging.

12.2 Chemical Reactions that Lead to Flavor Loss

Aroma chemicals, by their nature, are subject to loss during storage due to chemical reactions or evaporation. As noted earlier, encapsulation is a method used to protect aroma compounds from these losses. While numerous different chemical reactions can occur within a flavoring or between the flavoring and the environment, we will focus only on the major chemical reactions. These are oxidation, the Maillard reaction, and some miscellaneous reactions. Note, we are not attempting to deal with the development of off flavors in foods (see Chaps. 5, 10), but only a narrow discussion of how flavors added to a food might be lost during storage or preparation. Each of these reactions will be briefly discussed.

12.2.1 Oxidation

While essential oils will undergo numerous degradation reactions (e.g., polymerization, isomerization, dehydration, and thermal rearrangements), the primary degradation reaction is oxidation (Fig. 12.1, [12.1]). Mono terpenes tend to be the most liable compounds in essential oils (limonene in citrus oils). The pathway shown in Fig. 12.1 illustrates that limonene oxide and carvone tend to be major oxidation-end products. These two compounds are primarily responsible for the off flavor associated with limonene-free radical oxidation.

Other essential oils, such as phenolic oils (clove, savory, thyme, and origanum), mint, and aldehyde-based oils (e.g., oil of bitter almond) will also deteriorate due to oxidation. Oil of bitter almond has benzaldehyde as a characterizing component which will oxidize to benzoic acid that has a harsh, biting effect in the back of the throat. The sensitivity of essential oils to oxidation is often the primary reason for the encapsulation of a flavoring, limiting the exposure of the flavoring to oxygen.

A different cause of deterioration in essential oils is light exposure. For example, anise oil will readily degrade on light exposure to give off notes. The first step in degradation is the isomerization of *trans*-anethole to *cis*-anethole followed by subsequent oxidation to anisaldehyde (vanilla-like odor; Fig. 12.2a). An additional reaction, the reaction of anethole with anisaldehyde will readily occur to form 4,4-dimethoxystilbene (Fig. 12.2b; no odor reported).

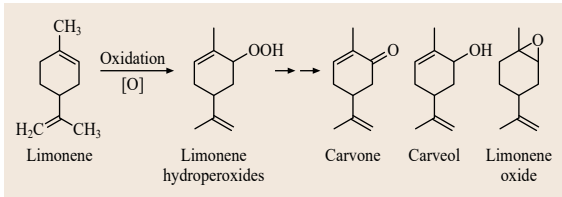

Fig. 12.1 Pathway for the oxidation of limonene (after [12.1])

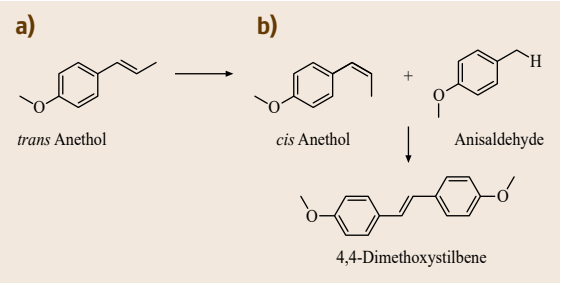

Fig. 12.2 **(a)** Oxidation of *t*-anethol to anisaldehyde. **(b)** Reaction product of *t*-anethol and anisaldehyde to form 4,4-dimethoxystilbene (after [12.1])

12.2.2 Maillard Reaction

While in food chemistry we tend to think of the Maillard reaction as a reaction between amino acids and reducing sugars, in flavor chemistry, this definition is broadened to the reaction of any amine with any carbonyl compound. With this broadened definition, we become very concerned about the unsaturated carbonyls and amines used in flavorings and their opportunity for reaction. This reaction does not necessarily result in the formation of off flavors, but the loss of desirable flavoring components.

Unsaturated carbonyls (most often aldehydes)
+ amines (may be from several sources)
→ Schiffs base (no flavor)

Unsaturated carbonyls are fairly common while few amines are used as flavoring components. The most common amines used in flavoring are methyl or ethyl anthranilate (grape flavoring and some essential oils). Due to differences in amount and frequency of use, we are most commonly concerned about the loss of the aldehydes. Figure 12.3 illustrates the sources of reactants that lead to the loss of carbonyls.

A statement was made earlier that the primary result of the Maillard reaction in this context is a loss of flavor (as opposed to the formation of an off flavor), in the case of reaction with the high potency sweeteners, not only the flavoring agents (the unsaturated aldehydes) are lost but also the sweetener. This is a primary reason that aspartame is not used in some chewing gums (lemon [citral] or cinnamon [cinnamic aldehyde] flavored gums; [12.2]).

If we decide to encapsulate our flavoring to offer some protection against such reactions, we must choose our flavor encapsulation system carefully. The primary consideration here is to not use a protein in the encapsulation wall system. If so, the carbonyl components of the flavoring will react with the free amino groups in the protein encapsulant and will lead to complete losses of the flavoring in a short time [12.3].

12.2.3 Miscellaneous Reactions–Internal Reactions

By internal reactions, we are considering reactions that can occur within a given flavor formulation. When one prepares a complex mixture of organic chemicals (creates a flavoring) many reactions may occur (dimerization, aldol condensations, ester formation, sulfide formation, etc.). A reasonable question is why these reactions do not occur in nature resulting in flavor degradation and the answer is they do. However, in many cases flavor is formed enzymatically, very rapidly on chewing (vegetables), so little time is available for the chemical reaction to occur prior to eating. In another instance such as during the roasting of coffee beans, the flavor is created during heating but gets locked into a dry matrix where diffusion to other reactants is not allowed (within the bean). A final example is the natural flavor is very dilute and thus reactions take place very slowly; however, this is not the case in a concentrated commercial flavoring.

To meet this particular challenge, a unique form of encapsulation is used: molecular encapsulation. Molecular encapsulation is often called inclusion complexing and involves the interaction of individual flavor molecules with an encapsulating material such as a cyclodextrin or amylose. These encapsulating materials possess unique structures that *tuck* individual flavoring compounds into a safe place in them where they are protected from the environment and each other. This method will be discussed in some detail later in this chapter.

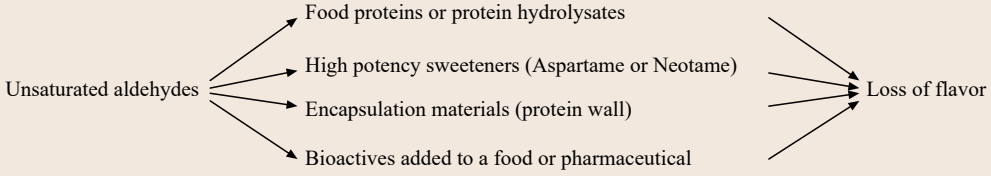

Fig. 12.3 Components that contain amines that may react with carbonyl-containing flavoring materials

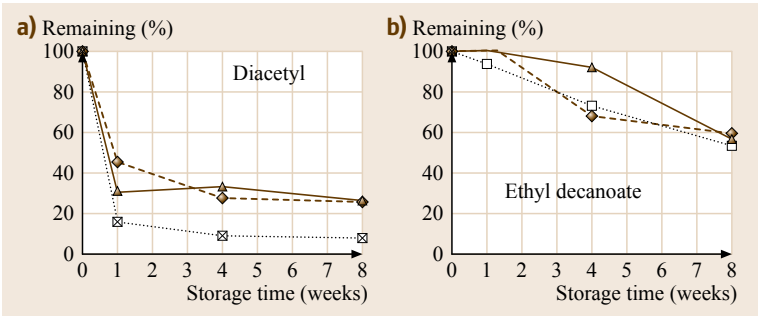

Fig. 12.4a,b Retention of two model aroma compounds when put in microwave popcorn package prepared as a liquid flavoring (*dotted lines*), spray dried in N-Lok (*dashed lines*) and spray dried in gum acacia (*solid lines*) **(a)** diacetyl **(b)** ethyl decanoate (after [12.4])

12.2.4 Reaction with the Food Matrix

This mechanism of flavor loss has already been partially presented above when discussing Maillard reactions (carbonyl flavor compounds with free amino groups on proteins). As noted, this reaction can be a very significant means of flavor loss. Beyond Maillard-type reactions, proteins also offer other reactive sites for flavor reaction. An example is the formation of sulfides from any thiol-based flavor compounds reacting with the free sulfhydryl groups on cysteine. One could envision acetal formation again involving protein side chains and aldehydes. Anyone working in the high protein bar business appreciates how difficult it is to form and keep a desirable flavor in high protein food products.

12.3 Evaporation as a Mechanism of Flavor Loss

Thinking back to the earlier description of the physical properties of aroma compounds, some aroma compounds have extremely low boiling points (e.g. hydrogen sulfide has a boiling point of −60 °C, methanethiol boils at 5.95 °C, and acetaldehyde at 20.2 °C) and thus, would be readily lost due to evaporation if not adequately sealed into some encapsulated structure. There are numerous aroma chemicals that boil close to or just above room temperature. The high vapor pressure and small size of some aroma chemicals make it nearly impossible to keep them in any system – even our best encapsulation systems: This is illustrated in Fig. 12.4 where model aroma compounds were encapsulated by spray drying, put in a model microwave popcorn package (oil, popcorn, and salt) and then stored. In a matter of one week, the diacetyl concentration had dropped by 85% when not encapsulated but still lost 55–70% when encapsulated (Fig. 12.4a). Despite diacetyl being a reasonably large molecule with a boiling point of 88 °C and it was encapsulated, it was readily lost from the capsules. This is likely due to the high polarity of the molecule. For comparison purposes, data are also shown for a much less volatile and larger compound, ethyl decanoate. Losses for this compound differed little whether encapsulated or added as a liquid (Fig. 12.4b).

12.4 Techniques for Preserving Flavor

Understanding that all aroma compounds are volatile and many are reactive, the industry searches for ways to make them more stable to extend shelf-life. This typically involves protection from oxygen, light, and reactions within the flavor system or with the encapsulation or food matrix. Various encapsulation processes have been developed for these purposes. The major methods will be touched upon (detailed reviews are available on the methods – see reviews [12.5–8]).

12.4.1 Plating

Plating of flavorings involves adding a flavoring to a dry carrier of some type and simply mixing the system to spread the flavoring across the surface and into any pore structure of the carrier. This is often done simply to better distribute a potent flavoring (perhaps a spice oil) in a final food product (e.g., sausage). Some early work by *Bolton* and *Reineccius* [12.9] demonstrated that one could offer substantial protection to flavor compounds

if they were plated on a silica substrate. The very porous silica was able to adsorb the flavoring into very small pores and thereby offer significant protection against evaporation and oxidation. This approach has found some application in the industry most recently for the delivery of savory flavorings.

More recent work on silica has focused on sol–gel approaches [12.10–12]. The sol–gel techniques involve allowing the polymerization of silica monomers to form a structure around pockets (or droplets) of flavor. Sol–gel approaches have found some application in the fragrance industry but not the flavor industry [12.13].

12.4.2 Encapsulation

Encapsulation is a very general term for any process that puts some type of protective *shell* around an active material (flavoring). When one goes through the literature, one notes that many different materials and processes are proposed for flavor encapsulation. With that said, only four processes find broad application in the food/flavor industry. They are spray drying, extrusion, inclusion complexing and coacervation: the largest volume of dry, encapsulated flavorings is done by spray drying. Each of these processes offers unique benefits and weaknesses. Since all of these methods are discussed in the review articles cited above, only brief statements will be made for each.

Spray Drying

Spray drying (Fig. 12.5) starts by preparing an aqueous emulsion consisting of the flavoring material (generally water insoluble) and the encapsulating material (water soluble; generally a gum acacia, modified starch, or maltodextrin-based dispersion) in a mix tank. The material is solubilized and then homogenized to make a very fine dispersion of the flavoring in the solution. This emulsion is atomized into hot air (e.g., 200 °C) where the particles dry rapidly. The powder is separated from the exhaust air stream in a *cyclone*, collected, screened and bagged. The flavoring material is in the form of a dry emulsion called matrix encapsulation – droplets of flavoring dispersed in the dry encapsulating material (Fig. 12.6). This process is very popular for several reasons including low cost, equipment readily available, high flavor load (ca. 20%), and the process/product performs well (retention of flavoring and barrier to oxygen). The down side includes secondary processes are required to modify physical properties (instantize or afford controlled release) and not well suited to very low boiling compounds.

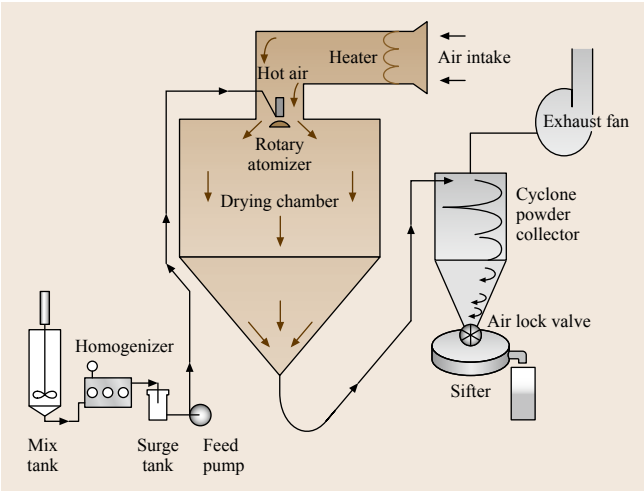

Fig. 12.5 Spray dryer used to encapsulate flavoring materials

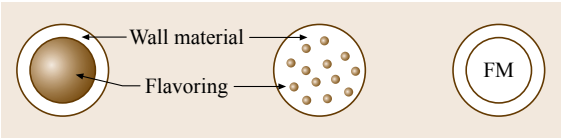

Fig. 12.6 Particle structures occurring in encapsulation systems. (*left* – shell and core structure; *middle* – matrix structure; *right* – molecular inclusion (one flavor molecule in a cyclic glucose structure))

Extrusion

The extrusion process for flavor encapsulation originated from a very simple process of making a flavored hard candy (sucrose and water). The flavored hard candy was ground to provide a powdered flavoring. The traditional commercial process (Fig. 12.7a) involves dispersing a flavoring into a hot, extremely viscous carbohydrate melt. The melt is forced through a small orifice (die) into a vat of cold isopropanol where it rapidly forms a glassy matrix on cooling. Mixing in the vat breaks the extruded strands into small pieces and allows some dehydration. Further air drying results in stable, dry particles. The final particles have a matrix structure and are much larger than those produced in spray drying (ca. 500 μm vs. 10–50 μm). The most recent innovations in this process are to use a twin-screw extruder to form the carbohydrate melt and flavor emulsion (Fig. 12.7b). Substantial work has been devoted to finding alternatives to the cold isopropanol bath. The use of an extruder has provided opportunities to reduce moisture content, reduce the time the flavor is held at high temperatures, and develop a continuous process. Lowering moisture content is particularly valuable in that the less moisture put into the product, the less effort

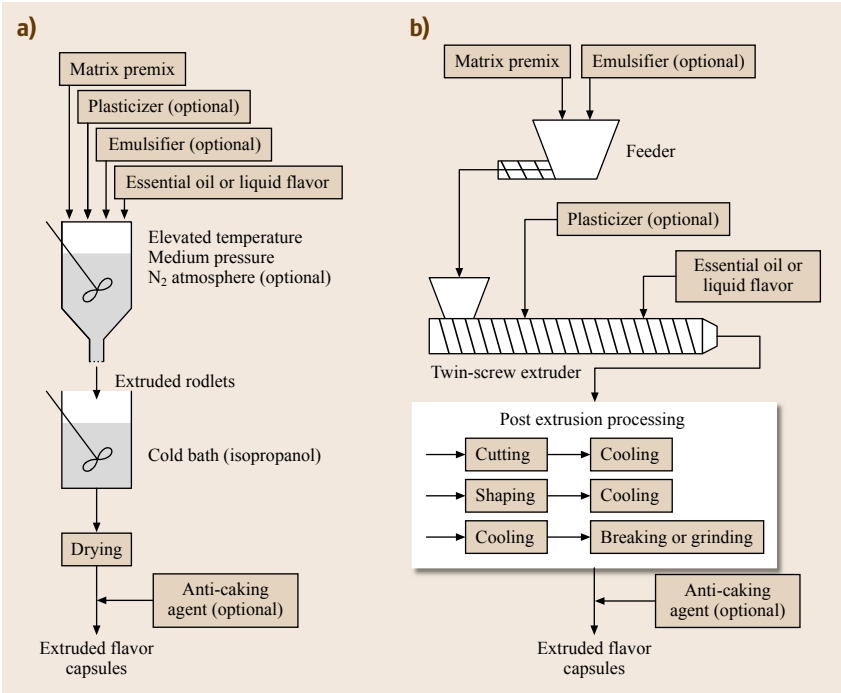

Fig. 12.7a,b Systems for producing extruded flavoring materials: **(a)** traditional system; **(b)** modern extruder system (after [12.7])

is needed to remove it to produce a dry, stable, glassy particle. Advantages of this process (in all configurations) tend to be a long shelf-life (excellent oxygen barrier properties) and large particles. The negatives may be low load (typically ca. 10%), large particles (can also be a positive), slowly soluble in cold water, high temperature process, less than ideal emulsion quality and cost. Despite the list of negatives being longer than positives, it is an important process in the industry.

Molecular Inclusion

Molecular inclusion involves the use of very specific host molecules (cyclodextrins and/or amylose) that offer sites that protect any molecules included in them [12.14]). Figure 12.8 shows the structure of the most commonly used cyclodextrin, the beta form. It is interesting that a cyclic structure composed of glucose forms a very hydrophobic core when placed in water. Flavor molecules tend to be hydrophobic and thus move into the center of the cyclodextrin where they are well protected from oxidation or other chemical reactions. The amount of flavoring that can be loaded into cyclodextrins depends upon the molecular weight of the flavoring material: One molecule of cyclodextrin includes one molecule of flavoring. Thus the loading typically ranges from ca. 9% to 15% by weight.

Amylose, and amylopectin in certain cases, also offers opportunities for forming inclusion complexes [12.15]. Amylose will form a single helix in the presence of aroma compounds that offers a hydrophobic central cavity (hydrophilic exterior) for including aroma compounds. There is evidence that aroma compounds may also be bound in between the helices. There is potential for initiating flavor release using enzymes (amylase), temperature, or moisture. While there are numerous patents on using amylose for this purpose, it has found less use for this purpose than cyclodextrins due to several factors including the physical properties of the amylose (solubility, clarity in solution, etc.)

Coacervation

The process of (complex) coacervation, as applied in flavor encapsulation, originated with carbonless papers in mid-1950s [12.15]. Complex coacervation involves making a coarse emulsion of a flavoring in an aqueous solution of edible food polymer (must have emulsifying properties) and then the addition of a second oppositely charged edible polymer (typically use a combination of a protein, such as gelatin and a hydrocolloid, such as gum acacia). The pH is controlled such that the polymers form a coating around the droplet. The coating typically has to be hardened by crosslinking. This can be done in various ways, for example, chemically using glutaraldehyde or enzymatically using *trans*-glutaminase.

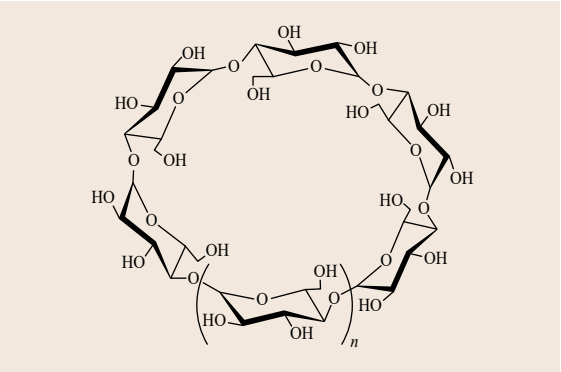

Fig. 12.8 Structure of β-cyclodextrin (after [12.14])

Coacervation is the only encapsulation process that makes a true core and shell structure (other processes result in matrix or molecular encapsulation structures, Fig. 12.6). Coacervation offers some unique advantages not imparted by other encapsulation processes including a wide range of particle size, high loadings, good oxidation stability, having a unique release mechanism (diffusion), and high recovery of flavoring. There are numerous weaknesses to the use of coacervation for flavor encapsulation which include: 1) Water soluble flavor components are lost to the process water; 2) aggregation of the coacervate particles makes control of particle size problematic; 3) process and materials costs are high relative to competing processes; and 4) the protein wall component raises issues of reactivity, allergenicity (labeling) and vegetarian status. As will be discussed later, this process has a unique controlled release mechanism.

Microbial Cells

The potential for using yeast cells for the encapsulation of sensitive materials has been recognized for nearly 40 years [12.16]. The preferred material is empty yeast cells, that is, those that have been treated to remove most of their cytoplasmic cell interior and then spray dried [12.17, 18]. The cells are loaded with flavoring materials by diffusion.

Substantial work has been published on defining the encapsulation and release mechanisms of flavorings from microbial cells [12.19–21]. As one would expect, the movement of flavoring compounds into the cells is dependent upon compound hydrophobicity. *Dardelle* et al. [12.18] reported that compounds with a $\log P > 2.0$ will achieve an encapsulation efficiency of more than 50%. Encapsulation efficiency decreases as $\log P$ decreases.

This method is particularly attractive for imparting thermal stability to flavoring for it has been reported that flavors are not released from dry yeast cells until temperatures approaching 250 °C are attained [12.20, 21]. As with most food polymers, the cell permeability is dependent upon water activity. However, while most encapsulation materials become permeable at water activities around 0.3, the yeast cells were impermeable below a water of ca. 0.7 [12.21].

Other Methods

There are numerous other encapsulation methods listed in the patent and scientific literature. However, they see very limited to no commercial usage and thus they are not going to be discussed in this book chapter.

12.5 Controlled Delivery of Food Aroma

As was mentioned in the introduction to this chapter, one purpose for encapsulating a flavoring material is to impart controlled release properties to it. Looking at the processes used in flavor encapsulation, one can see that the most commonly used processes for flavor encapsulation (spray drying, extrusion and molecular encapsulation) release the flavoring compounds on dissolution. This follows since all of the wall materials used in these encapsulation processes are water soluble. There is a substantial need in the industry to develop processes that afford the controlled release of flavorings, that is, protect the flavoring as long as possible from undesirable reactions and then release for sensing during eating.

Capsule dissolution (flavor release) can occur during manufacturing, home preparation, or during eating depending upon the product and processing. For example, water may be added to the flavoring when making a batter, dough, sauce, broth, or some other item for consumption. Adding water to a dry encapsulated flavor immediately releases the flavoring: the flavor was protected during storage until use but not during further processing or preparation.

The goal may be to delay the release of flavor (protect it) during the baking or frying of a food product. Developing a process that is based on temperature (thermal release) could protect the flavoring from chemical reactions with the food, or losses due to evaporation resulting in a food with better flavor, or more realistic flavor. An issue to keep in mind when approaching this goal is that flavorings are largely lipophilic. Thus, if one puts flavor into a lipophilic shell so that it is not released on water contact, the flavor will be permeable to the wall and over time the flavor will be lost from

the particle to the product environment. The alternative is to put the lipophilic flavor into a hydrophilic shell and coat the final capsules with a water barrier to slow release. The first process provides a wall that is impermeable to the flavoring and most often, oxygen. Then a secondary coating is applied to make the initial particle slowly soluble. These processes are discussed in the following sections.

12.5.1 Diffusion-Controlled Release

It is often assumed that to achieve controlled release (delayed release), one must have particles that are perfectly impermeable to the flavor in any environment – humidity, or contact with water or oil. That is not the case for this is an impossible goal. The only encapsulation process that one might apply to flavorings that would meet that requirement would be to put the flavor into glass or metal shells. One cannot make a particle out of any food ingredient that would not leak flavor, given enough time, or when put into water. The problems are the flavoring materials (high vapor pressures and small molecular size), and the approved wall materials cannot provide a barrier to water over significant time periods. Thus, we do not seek (the impossible) to develop capsules that are totally impermeable but those that minimally will delay flavor release on contact with water or on heating.

One can delay flavor release in various ways. The simplest is to entrap the flavoring in an insoluble matrix: One that will not dissolve on contact with water, for example, corn protein (diffusion controlled release). That limits applications to those products where insoluble particles are acceptable (texture issue on eating). The release rate would be dependent upon the diffusion rate through the matrix and the particle size (smaller particles yield a faster release). This approach has found application in chewing gum applications [12.22, 23]. The fact that the particle is not soluble limits its applications in many other products.

An alternate means of imparting diffusion-controlled release is to use coacervated particles. If one crosslinks the capsule wall matrix, the capsules become insoluble and flavor can be released only by diffusion through the capsule wall. One has some control over the flavor-release properties of these particles. Since this is a diffusion controlled release, flavor release starts slowly and becomes constant for some time. The rate of flavor release is dependent upon several factors including the capsule size, flavor-loading level and both the flavoring composition and the capsule wall material. Particle size influences the flavor release rate since the smaller the particle, the more surface area it has per unit weight. Surface area determines the opportunity for the flavoring to reach the food matrix. The flavor loading level (amount of flavor in the capsules) determines the proportion of the particle that is wall material – the barrier against diffusion. Capsules with low flavor loadings have a large proportion of wall material (thick walls with small central cores of flavoring) and thus, the flavoring only slowly is released. The type of flavoring also determines the release rate. A very light, volatile flavoring (such as a butter or a fruit flavoring) tends to be made up of low molecular weight, highly volatile components. These types of compounds readily diffuse through food polymers resulting in high release rates. Finally, the wall material used in flavor encapsulation may be more or less dense (or cross linked) thereby providing a stronger barrier to flavor release (diffusion).

12.5.2 Controlled Release Using Coatings

It is fairly common to encapsulate a flavoring using one of the traditional methods (spray drying, or extrusion) and then apply a secondary process to afford controlled release properties to it. The primary encapsulation may be used to impart oxygen-barrier properties and decrease the volatility of the flavoring so that a secondary process can be applied. The secondary process may be to slow the particle dissolution or add thermal release properties. As one would expect, there are various materials to use in coating and ways to apply the desired coating.

Coating Materials

The materials used depend upon the application purpose. Coatings can be based on nearly any food material and can be either water soluble or fat soluble. The most common lipophilic coatings are lipids (hydrocarbons, beeswax or solid fats), a shellac or a modified cellulose (e.g., ethyl cellulose). If lipids are used, they´ must have a high enough melting point so that they do not melt in storage so the product cakes, but yet melt in the final application (e.g., baking or frying) to allow hydration of the core flavor capsule that results in flavor release.

The coating may function to provide protection to the flavoring and/or controlled release properties. An example of the coating providing both functions is the patent of *Patel* [12.24]. *Patel* [12.24] encapsulated high potency sweeteners (e.g., aspartame) in a shellac coating using fluidized bed or roller bed coating techniques. The shellac limited water uptake by the sweetener and protected it from other undesirable reactions (e.g., aldehydic flavor components) both providing a longer shelf-life to the product and controlled release properties. The patent of *Castro* and *Witke* [12.25] used coating techniques to offer a chew-

ing gum that changed flavor during chewing. A core flavoring particle was prepared with one flavor type and then a second flavoring was coated around that core. The two encapsulating materials, the core and the coating placed around it differed in polarity (solubility). Thus, the outside layer might be very water soluble and release its flavor quickly and then the inner layer which would be a less soluble material would slowly release its flavor to give a different flavor character to the gum.

Shellacs are often suggested in the patent literature as coating materials. A shellac is a resinous material obtained primarily from Thailand and India that is secreted by the female lac bug (Wikipedia). It is soluble in ethanol and forms a very good water barrier when dry. *Song* et al. [12.26] used shellac to impart controlled release properties to a chewing gum flavoring that was initially loaded into porous beads. The porous beads carried the flavoring and the shellac provided controlled release. The largest deterrent to using a shellac is the need to dissolve it in ethanol for application. Ethanol is a controlled substance, expensive and flammable.

Coating Methods

Fluidized bed – one of the most common techniques used to apply coatings in the industry (Fig. 12.9). As the name suggests, a bed of material (e.g., spray dried flavor) to be coated is suspended in an air flow and a coating material (e.g., lipid) is sprayed onto the powdered flavoring. (There are numerous possible configurations for such a process.)

The coated material is either cooled (if it is a molten lipid coating) or dried if the coating is applied in a solvent (water or alcohol) to form a durable shell. While this sounds fairly simple, it is not. The primary problem is that the preferred starting material is a spray-dried flavoring (due primarily to cost). Unfortunately, the spray-dried flavoring generally is a fine powder ranging in size from 10 to 50 μm (depending upon the spray dryer used). It is difficult to coat a very small particle since the coating material is also delivered as a spray and thus has similar dimensions: one is attempting to coat a particle with another particle of the same size. One tends to get agglomerates that eventually offer an effective barrier but one delivers a lot of coating and not much flavor. It is very difficult to coat particles less than 100 μm in diameter.

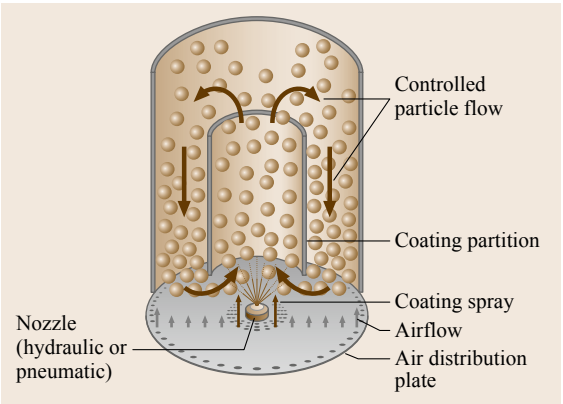

Fig. 12.9 Fluidized bed coating system

Granulation

Granulation is a term that tends to be used very broadly in the industry. In effect, it may be any process that results in a granular product. In the pharmaceutical industry, it is more limited describing a process in which powder particles are forced to agglomerate to form larger, multi-particle structures called granules (Wikipedia). Granulation can be done by wet or dry processes. The dry process involves compaction followed by grinding to the desired size. This is most often used to increase the particle size. The wet process involves spraying a liquid into an initial spray-dried material while tumbling or fluidizing the initial powder. If we wish to coat the initial powder, the liquid would contain a coating material which when dry serves to impart controlled release properties.

A different but somewhat similar process also shows up in the patent literature: one where a flavor-containing emulsion is sprayed into a chamber containing a dry (unflavored) powder. The emulsion *adsorbs* the dry neutral powder onto each droplet thereby providing a *coating*. An example of this process is given by *Johnson* [12.27]. He made an initial emulsion of modified starch, water and a perfume formulation. Corn starch was then placed in a small agglomerator and the starch was fluidized by hot air. The emulsion was sprayed into the chamber using a two-fluid atomizer. The resultant product was a relatively large particle (> 700 μm).

12.6 Unmet Needs

The materials and processes available for imparting controlled release properties to food flavorings are very limited. The primary limitations are imposed by both cost constraints and legal usage. One cannot help but look with envy at the pharmaceutical industry. The fundamental processes and materials available to the food flavor industry have changed very little over the last 50+ years. Having said that, innovations are occur-

ring which offer incremental improvements. The three largest flavor companies have been granted ca. 100 patents since 2000 on encapsulation (SciFinder search on key words *flavor* and *encapsulation*). We would expect this innovation to continue.

Three innovations that would be valuable in this author's view are:

1. The development of a positively charged food polymer
2. The ability to produce large particles by spray drying (ca. 100 μm)
3. The ability to apply uniform, hydrophilic and/or hydrophobic, nano-thickness coatings.

The need for a positively charged food polymer relates to needs in coacervation: a protein usually serves as the positively charged food polymer for complex coacervation. Proteins tend to be relatively expensive, must be labeled (allergens), and are reactive. Chitin has been used in place of protein but this polymer also has functional drawbacks. We need an inexpensive, positively charged food polymer to make coacervation more cost-effective and less problematic.

The desire for producing large particles in spray drying stems from our need for an inexpensive method to produce a primary flavored particle that is large enough to easily coat for imparting controlled release properties. Furthermore, it would be desirable that the particles have a core and shell structure rather than the typical matrix structure. Core and shell structures permit higher flavor loadings to partially offset the dilution due to using coating material.

The last need this author suggests is a better way to coat particles: A method that can uniformly apply a very thin coating to the small particles we can produce economically today. That would likely be a vapor phase coating process. One could certainly expand upon this short list of needs but innovations in these three areas would be very helpful.

12.7 Conclusions

The volatility, permeability, and reactivity of aroma compounds make them problematic to maintain in a food product during storage, distribution, and preparation. These needs have resulted in several methods of encapsulation being practiced in the food/flavor industry including spray drying, extrusion, coacervation, and inclusion complexing. These processes are fairly old and despite innovation, cannot provide the stability often needed to satisfy market demands. A similar statement can be made for our ability to obtain the desired controlled release properties to these encapsulated materials. We simply lack the materials and processes required to adequately accomplish controlled delivery. That is not to say that we have made not progress but that there is still a great deal of unmet need in the field.

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13. Physico-Chemical Interactions in the Flavor-Release Process

Anne-Marie Seuvre, Andrée Voilley

The perception of flavor is induced by the release of aroma compounds in the vapor phase. The olfactory perception is not only related to the nature of aroma compounds initially present in the food, but also to their distribution between the different phases. After a description of the interactions established between the aroma compounds and different constituents of food, this chapter looks at physico-chemical characteristics of aroma compounds and at the composition and properties of food matrices. Then, in order to understand the behavior of aroma compounds in the matrices, study methods of interactions are described. The assessment of the release is done by determining the partition coefficients and mass transfer between phases. The conclusion opens the way on the preservation of aroma compounds.

Foods are complex products containing volatile and nonvolatile compounds. Among the volatile compounds, flavor compounds may be naturally present in foods or added to compensate, for example, loss or damage arising either during the manufacturing process or during storage.

Aroma results from the balance between the different aroma compounds and will depend, in particular, on the concentration and composition of the food matrix. The chemical nature of aroma compounds, the composition and the structure of a food are the main characteristics influencing both the transfer of aroma compounds in the food and their release. Food matrices are often multiphase, containing liquid (aqueous or lipid) solid and vapor phases. The distribution of an aroma compound in a food depends on its affinity for the different phases, and its ability to be released into the vapor phase. This distribution, responsible for the availability of aroma compounds, will modify sensory perception when smelling a food or during consumption. This distribution is characterized by the partition coefficient of vapor versus matrix which determines the maximum amount of each odorant available in the vapor phase for perception and indicates the presence or absence of the type and extent of interactions between aroma compounds and the respective matrix.

The nature of the different nonvolatile constituents such as proteins, lipids, carbohydrates and salts has a great impact on the retention of aroma compounds in food matrices. Indeed, physico-chemical interactions can be established between flavor compounds and food constituents, and can modify the distribution of

aroma compounds in the food. So they can increase or decrease the release of aroma compounds and thus modulate their availability, thus influencing their sensory perception.

In recent years, interest in low-sugar or low-fat products has increased, and to satisfy the wishes of consumers, the industry has had to change the formulation of a food in several cases. However, the change in composition of the food may, consequently, cause a change in its structure and its sensory properties, particularly in terms of the overall aroma intensity and the individual aromatic notes perceived in the nose and in the mouth.

To predict the release of aroma compounds from a food matrix, instrumental and sensory studies are needed to develop mathematical models to describe and predict this complex process. These mathematical models are based on physico-chemical properties such as diffusion, transfer rate and volatility of each odorous molecule. Some models are dedicated to the partitioning of flavor compounds between the different phases of the food and the vapor phase, others are directed to the release of aroma compounds in the mouth by taking into account the physiological parameters.

The understanding of the phenomena of release/retention of aroma compounds from the physico-chemical point of view, in particular through the study of vapor-liquid equilibria between the food matrix and the gas phase using thermodynamic and kinetic approaches, is thereby an essential step. These equilibria are governed by the particular interactions that take place between the components of the matrix and the volatile compounds. The amounts of released aroma substances will depend not only on the concentration of aroma compounds in the matrix, but also on the nature of both odorants and matrix. The chemical functionalities, the conformational aspects and the structural features of the food matrix have to be considered. The interactions between the food matrices and the odorous molecules thus appear as a determinant of the balance between retention and release.

In the food sector, the aroma compounds are distributed between the different phases (vapor, liquid and/or solid phases, aqueous or lipid phases) according to their affinity and taking into account the possibility of the mixing of several phases. The objective of the thermodynamic equilibrium between the phases is to quantitatively describe the distribution of each compound present in the different phases (vapor, liquid or solid) of the food by the determination of the respective partition coefficients. The two coefficients most commonly used are the partition coefficient of vapor-medium, where the medium can be a liquid, a solid or a food matrix, and the coefficient of liquid-liquid partition between two immiscible liquids. The kinetic approaches study the rate of distribution of compounds within the food, their distribution in each phase at different times and their transfer from one phase to another.

13.1 Nature of the Interactions Aroma Compounds–Matrices

One of the essential variables in the study of the thermodynamic equilibria is the chemical potential μ_i of a component i. Indeed, a single or multiphase system is in equilibrium when μ_i is the same at any point in each phase of the system for a given temperature and a given pressure. The chemical potential μ_i is by definition

$$\mu_i = \left(\frac{\delta G_i}{\delta N}\right)_{\mathrm{P,T}} , \tag{13.1}$$

where G_i is the free enthalpy of the phase in question and N the total number of moles in this phase.

In a mixture of perfect gases and in isothermal and isobaric conditions, the chemical potential μ_i of a constituent i is a function of its partial pressure in the system

$$\mu_i = \mu_i^{0\,\mathrm{vap}} + RT \ln \frac{p_i}{p^0} , \tag{13.2}$$

where $\mu_i^{0\,\mathrm{vap}}$ is the reference chemical potential of the component i in the gaseous state under standard conditions of temperature and pressure, R is the gas constant, T the temperature (K), P the pressure (Pa), p_0 the reference pressure (101 325 Pa), p_i the partial pressure of component i in the gas mixture.

In general, in food systems, the vapor phase above a product is considered as an ideal gas mixture consisting of water vapor and volatile compounds.

In solution, the chemical potential of a component i is a function of its activity a_i (dimensionless number)

$$\mu_i = \mu_i^{0\,\mathrm{liq}} + RT \ln a_i , \tag{13.3}$$

where $\mu_i^{0\,\mathrm{liq}}$ is the chemical potential of the pure component i in the liquid state in standard conditions of temperature and pressure.

In an ideal solution, $a_i = x_i$, so the chemical potential μ_i of component i is a function of its molar fraction

Table 13.1 Activity coefficients of aroma compounds in water at 25 °C

Aroma compound	[13.1]	[13.2]	[13.3]	[13.4]	[13.5]	[13.6]
Isoamyl acetate	5016	4650	4400			
Acetaldehyde	3.3	3.0	3.4	4.2		
Benzaldehyde	1436		1040		1456	
D-Linalool	8631		14 000	25 000		
cis-3-Hexenol	381					
D-Limonene	86 631	33 400	47 000	77 500		
Acetophenone	1689		1200			
2-Heptanone	5629		4700			
2-Octanone	9452		11 300	13 200		
2-Nonanone	58 036		43 000			16 300

in the liquid phase, x_i

$$\mu_i = \mu_i^{0\,\mathrm{liq}} + RT \ln x_i \,. \tag{13.4}$$

In a real solution (not ideal), $a_i = x_i \gamma_i$, and the equation is

$$\mu_i = \mu_i^{0\,\mathrm{liq}} + RT \ln x_i + RT \ln \gamma_i \,, \tag{13.5}$$

with γ_i the activity coefficient of component i.

The application of the equations given above requires the definition of a reference state for all the constituents of the mixture. The pure liquid solute i is the reference state: for $x_i = 1$, $\gamma_i = 1$ and $a_i = 1$. The chemical potential of the reference is that of the pure compound at a given temperature and pressure.

The activity coefficient γ_i depends on the temperature, pressure and composition of the system. However, in the pressure range of interest, the activity coefficient varies little with pressure.

For any component i in an ideal solution, γ_i is constant and equal to 1 (Raoult's law). This solution corresponds to the mixture of molecules having a very similar structure or interactions having the same nature.

For any component i of a real solution, γ_i is not equal to 1 (Henry's law) and represents the deviation from ideality. Molecules have very different structures. In addition, the nature and the number of solute-solvent interactions differ by comparison to solute-solute interactions and solvent-solvent interactions.

Two factors are involved in the deviation from ideality:

- Taking two components, A_1 and A_2, the interactions between these components in a solution are of a much lower order of magnitude compared to the interactions between A_1 and A_1 and between A_2 and A_2: then the partial pressure of component i in the vapor phase is greater than the ideal case, therefore the activity coefficient is greater than 1 (positive deviation).
- The interactions between components A_1 and A_2 in solution are of an order of magnitude higher than the interactions between A_1 and A_1 and between A_2 and A_2: then the partial pressure of component i in the vapor phase is lower than in the ideal case, therefore the activity coefficient is less than 1 (negative deviation).

In the case of an infinitely diluted solution, interactions between solute molecules can be neglected and the activity coefficient of component i noticed γ_i^{∞} is constant.

Table 13.1 presents some value examples of activity coefficient of aroma compounds in water at 25 °C.

Interactions are due to attractive or repulsive forces between groups. Their range and intensity vary according to their nature (Table 13.2).

13.1.1 Attractive Forces

These forces include several types of interactions: Coulomb forces, van der Waals forces, hydrogen bonds and hydrophobic interactions. They depend, in particular, on the presence of electric charges and on the polarity of the different bonds of the interacting molecules. A bond is called apolar if the electrons participating are divided equally between the two polar nuclei and polar if one of the two atoms, more electronegative than the other, attracts the electron cloud more strongly.

Coulomb Forces

The Coulomb forces apply to a charged molecule, that is, an ion and another ion or a dipole. Their intensity depends on the nature of the second dipole involved. The forces between ions are intense and long-range, and their energy is an inverse function of the distance between charges. Interactions between ion and dipole are also long-range but decrease as the square of the distance between the ion and the center of the dipole. It should be noted that the intensity depends strongly on the orientation of the dipole. The interactions play

Table 13.2 Intensity and range of the physico-chemical interactions (after [13.7])

	Origin	Intensity	Range	Energy (kJ/mol)
Attractive forces	**Coulomb forces**			
	Ion-ion	Very intense	Very long $\frac{1}{r}$	≈ 750
	Ion-fixe dipole	Intense	Long $\frac{1}{r^2}$	≈ 250
	Ion-mobile dipole	Medium	Medium $\frac{1}{r^4}$	$\approx 2,5$
	Van der Waals			
	Dipole-dipole	Weak	Short $\frac{1}{r^6}$	$< 2,5$
	Dipole-apolar molecule	Weak	Short $\frac{1}{r^6}$	$< 2,5$
	Apolar molecule-apolar molecule	Medium	Short $\frac{1}{r^6}$	$\approx 2,5$
	Hydrogen bond	Intense	Medium	< 40
	Hydrophobic interaction	Intense	Long	< 25
Repulsive forces		Very intense	Very short $\frac{1}{r^n}$ with $n < 9$	

r: intermolecular distance

an essential role in the orientation of the dipoles especially for their solvation. Finally, interactions between ions and mobile dipoles are less intense and decrease with the distance at exponent four between the ions and the dipole center. Moreover, these interactions are independent of the orientation of the dipole which remains in a state of thermal rotation.

Van der Waals Forces

Under this designation are grouped orientation, polarization and induction forces. They all involve a dipole, either permanent or instantaneous. Orientation forces, also called dipole-dipole interactions or Keesom forces, result from the interaction of two permanent dipoles.

Polarization forces arise from the interaction between a permanent dipole and the dipole that it induces on a neighboring molecule and correspond to the term called Debye.

Dispersion forces, also called London forces, occur between nonpolar molecules and are created by the interaction of instantaneous dipoles. They come from the movement of electrons whose momentary configurations may correspond to a nonuniform charge distribution, that is, a dipole. In turn, this dipole induces a dipole in a neighboring molecule resulting in a force of attraction. In vacuum, the energy of all these interactions decreases with the distance between the centers of the dipoles at exponent six.

Hydrogen Bonds

These are the bonds between electronegative atoms such as oxygen or nitrogen and hydrogen atoms involved in a covalent bond with other electronegative atoms. They are of a very special nature because they only, and specifically, involve hydrogen atoms whose small size and tendency to positively polarize allow interaction with the neighboring electronegative atoms. This type of bonding only occurs in limited cases, but is of great practical importance. In fact, it participates in the stabilization of macromolecules such as proteins, nucleic acids or polysaccharides such as amylose, and gives them their spatial structure (ternary). Their energy is between 10 and 40 kJ/mole.

Hydrophobic Interactions

In biochemical and pharmacological fields, hydrophobic interactions are often described as a type of interaction in itself, although they result from the combination of several elementary interactions (polar, electrostatic, dispersive and repulsive) in addition to entropic effects of hydrophobic hydration [13.7]. They concern molecules or portions of apolar molecules which therefore have only very low affinity for water. The hydrophobic effect comes from the nonaffinity of these solutes for water and results in a collection of apolar molecules and a reorganization of solvent molecules.

13.1.2 Repulsive Forces

Repulsive forces result from the electronic orbital overlap when the molecules get closer to a very short distance. Then the positively charged nuclei repel each other. These forces have a very short range and an extremely important growth when molecules get closer.

13.2 Physico-Chemical Characteristics of Aroma Compounds

The aroma compounds are organic molecules of low molecular weight (< 400 Da) whose vapor pressure at atmospheric pressure and at room temperature is sufficiently high so that they are partially in the vapor state in the gas atmosphere and can cause an olfactory stimulation when they come into contact with the olfactory mucosa [13.8].

Aroma compounds are present in very small amounts in food; their concentrations vary from a few milligrams per ton (ppb) to a few milligrams per kilogram (ppm). On the other hand, they are generally present in very large numbers [13.9], especially for food produced by thermal processes (coffee) or in combination with fermentation (bread, beer, cocoa, tea) that contain more than 500 flavor compounds. Among these aroma compounds, only a limited number are of importance for the overall food aroma; these are the substances whose concentrations in the food exceed the thresholds of their sensory perception. The threshold of perception of an aroma compound depends on its vapor pressure, which changes depending on the temperature and the environment.

Aroma compounds in foods belong to all chemical classes [13.8–10] with several examples being presented in the following):

- Terpene compounds: hydrocarbons (limonene, myrcene), alcohols (linalool, menthol), aldehydes (geranial), ketones (menthone, carvone)
- C_{13}-norisoprenoids compounds: damascenone, 3-hydroxy-β-ionone
- Esters: isoamyl acetate (3-methylbut-1-yl ethanoate), ethyl butanoate, etc. (fruity flavor)
- Lactones: γ-decalactone (peach), δ-decalactone (coconut), etc.
- Carbonyl compounds: benzaldehyde, C_6 aldehydes (green notes: hexanal, trans-2-hexenal, ...), methyl ketones (2-heptanone and 2-nonanone as characteristics of blue cheese), butanoic acid (rancid butter), 2-methyl-butanoic acid (Roquefort)
- Phenols: raspberry ketone (fruit), eugenol (clove), thymol (thyme) and vanillin (vanilla), etc.
- Sulfur compounds: which represent about 10% of the compounds identified in the volatile fraction of food (sulfides, disulfides or trisulfides, thiols, ...)
- Heterocyclic compounds: pyrazines (coffee, cocoa, chips and meat), furanones (smell of caramel, bread, beef broth, ...), thiazoles and benzothiazoles, especially isobutylthiazole (fresh note of tomato).

Commonly, food flavors are mixtures of volatile compounds with different physico-chemical properties; consequently, this phenomenon leads to the preferential retention of some of them in a specific food matrix and thus to a change in sensory properties of a specific food product [13.11, 12]. Several physico-chemical characteristics of the aroma compound are involved in their retention by a food matrix. They include molecular dimensions, chemical functions, polarity of the odorous compound, hydrophobicity, volatility and diffusivity.

13.2.1 Molecular Dimensions

This parameter has a direct impact on the diffusivity of aroma compounds but also plays a role in the establishment of interactions with matrices. For example, starch, which is a mixture of amylose and amylopectin, interacts in aqueous solutions with aroma compounds. This retention is mainly attributed to the inclusion of the odorous molecules in the amylose helix and, to a lesser extent, in those helix structures being formed by amylopectin [13.13–16]. The interactions are of low energy and the dissociation constant varies according to the length of the carbon chain of the solute [13.14].

The effect of the molecular size of the volatile compound has been studied in the case of interactions with polysaccharides and especially with glucose polymers. These polymers seem to interfere in the number and nature of interactions, essentially determining the importance of contact with the hydrophobic regions of these polysaccharides, and thus the importance of hydrophobic interactions and/or van der Waals forces.

In the case of the interaction between the volatile compound and β-cyclodextrins, the molecular dimensions of the ligand are also a parameter determining the stability of the complexes. The β-cyclodextrins have the shape of a torus with a diameter of 0.78 nm and an internal volume of 262 $Å^3$ [13.17–19]. Molecules with a size of the same order of magnitude can therefore be included. However, it was observed that smaller molecules were less well retained [13.11]. The affinity constant of alcohols evolves in the direction methanol < ethanol < 2-propanol < 1-butanol [13.20]. Similar results were obtained for acids or ketones [13.21, 22].

According to *Kano* [13.23], the nature of the predominant interactions between a volatile compound and β-cyclodextrin depends on the size of the compound included. If its size is close to the size of the cavity, van der Waals interactions would predominate, whereas if the compound has a size substantially smaller than that of the cavity, hydrophobic interactions dominate.

The shape of the molecule determines the importance of contact with the cavity of cyclodextrins and thus the strength of the bonds [13.24]. The nature and strength of the interactions are also determined by the nature of the chemical functions carried by the aroma compound.

The conformation of polysaccharides also affects the ability to interact with the flavor compounds [13.25]. For example, amylose or starch hydrolysates retain aroma compounds in solution unlike dextrans [13.26]. These polysaccharides are glucose polymers but in the case of the first two, the glucose units are linked by connections $\alpha 1 \rightarrow 4$ whereas they are connected by links $\alpha 1 \rightarrow 6$ in the case of dextrans. The presence of ramifications can also affect the ability of aroma fixation. The volatile compounds have a greater affinity for the amylose constituted of linear chains as amylopectin, having ramifications. However, it was shown that interactions may exist between the polymer and the volatile compounds if the length of the linear portions is sufficient [13.14, 15].

However, the most outstanding effect of the conformation on the polysaccharides ability to interact with volatile compounds arises from the cyclization of the chain dextrins to lead to the formation of cyclodextrins.

13.2.2 Chemical Functions

The chemical functions of the aroma compounds affect their retention by the matrices. For example, it seems that the polysaccharides retention follows the order: alcohols > ketones, esters > acids. These functions influence the distribution of the electric charges of the compound and therefore its polarity, whose characteristic is also affected in the nature of the interactions established by the aroma compound with the polysaccharides.

Alcohols are generally better retained than the other components. It has thus been observed that 1-hexanol and 2-propanol are better sorbed by β-cyclodextrins and starch hydrolysates with dextrose equivalent (DE) 20, 31 and 61.5 than diacetyl (diketone) or ethyl acetate [13.27]. Their proportion among the sorbed compounds represents 17 and 7.5% of the total moles of sorbed compounds against 6.9 and 4.8% for diacetyl and ethyl acetate, respectively. Another study on freeze-dried starch hydrolysates reported a retention rate of benzyl alcohol higher than those of fifteen other volatile compounds [13.28]. The retention rate of benzyl alcohol varies between 45 and 83% depending on the nature of the carrier (freeze-dried starch hydrolysates) while that of the esters does not exceed 49.5% and that of the acid is less than 7%.

Regarding ketones, they seem to be less retained than alcohols and as much as esters. *Maier* [13.29] observed a lower sorption of ketones by starch compared to that of alcohols with the same chain length.

When the retention of ketones is compared to that of esters, few differences were noted. However, there are too few studies comparing the effect of these functions to draw conclusions [13.25].

Esters also appear to be less well retained by polysaccharides than alcohols but are better retained than acids. Thus the study of the retention of a mixture of 16 aroma compounds by lyophilized starch hydrolysates showed a lower retention of esters compared to alcohols, and very low retention of acids (Fig 13.1) [13.28]. Less than 7% of the acids were retained, regardless of the considered support, whereas the esters and alcohols were retained at more than 10% and in certain media up to 83%.

However, there are exceptions to the preferential retention of alcohols by the polysaccharides. For example, only 21% of initially added amounts of oct-1-en-3-ol and 33% of 1-octanol were retained during the spraying of starch hydrolysates DE 36.5 while 41% of octanal was retained [13.30]. This example shows that the chemical functions are not sufficient to explain the differences in retention depending on the nature of the volatile compound.

13.2.3 Polarity

The polarity of the volatile compound further plays a role in establishing interactions with the matrix. For example, during the dehydration treatment of polysaccharides, the polarity of the volatile compounds affects their retention: the most polar aroma compounds are generally less retained. The polarity of the volatile compound plays a role in establishing interactions with polysaccharides and in the stability of complexes between aroma compounds and the matrix, as is the case for complexes between aroma compounds and β-cyclodextrin. But the nature of the support is also to be considered: β-cyclodextrins have a hydrophobic cavity in which the flavor molecules are included. The strength of the bonds increases with increasing contact of the hydrophobic part of the compound included within the wall of the cavity of β-cyclodextrin [13.23].

The polarity also affects the interactions with mono- or disaccharides: *Roberts* et al. [13.31] suggested that the interaction of the volatile compounds with sucrose or glucose depends on the polarity of the volatile compound. The polar compounds are excluded from aqueous solutions of sucrose or glucose because of the structuring effect of these sugars on the water. The apolar compounds are retained through interaction with the

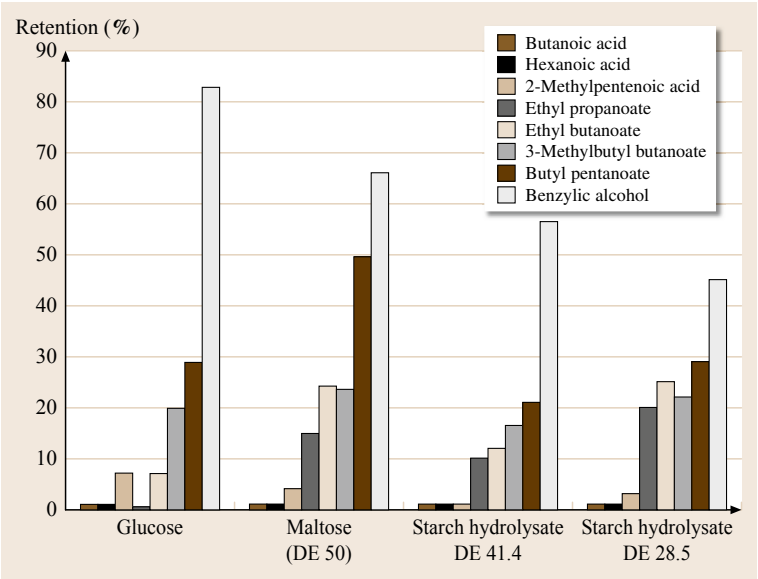

Fig. 13.1 Influence of chemical functions of aroma compounds on their retention in mixtures containing freeze-dried starch hydrolysates and glucose (after [13.28])

hydrophobic regions of the sugars. However, there are some exceptions to this trend.

Aroma compounds have generally a hydrophobic nature and have rather different physico-chemical characteristics. The water solubility and hydrophobicity allow the evaluation of their affinity for the organic and aqueous phases. Another physico-chemical characteristic is the saturated vapor pressure which represents the volatility of the molecule in its pure state. It can be measured or estimated. A static method, suitable for measuring low saturated vapor pressure, is to measure the pressure above the pure product when the thermodynamic equilibrium is reached. Different methods of estimation of the saturated vapor pressure are described in the literature [13.32–40].

13.2.4 Hydrophobicity

The hydrophobicity is represented by the log P value which corresponds to the logarithm of the partition coefficient of one compound between water and n-octanol. This value can be calculated with the help of the gas chromatography (GC) experimental determination of the concentrations of the chosen aroma compound in the two phases, or estimated [13.41]. The value of log P may be estimated by a group contribution method [13.42] but it does not take into account the position of the chemical groups in the molecule and the intramolecular interactions. Aroma compounds having a log P of less than 1 are considered polar and are relatively soluble in water (Fig. 13.2) [13.1]. This characteristic is important to consider: a hydrophilic compound readily diffuses through a matrix of the same nature, unlike a hydrophobic compound [13.43, 44]. A polar carrier is better for apolar compounds, as are most of the aroma compounds and vice versa for polar compounds.

Hydrophobicity and volatility are characteristics that predict the ability of a flavor to reach the gas phase, but the release of volatile compounds also depends on kinetic phenomena that additionally must be taken into account when regarding their transfer from one phase to another.

13.2.5 Volatility

A physico-chemical characteristic to consider is saturated vapor pressure which represents the volatility of the molecule in its pure state. It can be measured or estimated [13.32]. Saturated vapor pressure can partly explain the differences of the aroma compound ability to transfer to the vapor phase. But even more than this characteristic, the value of the volatility in solution needs to be taken into account [13.30]. This value characterizes the behavior of a diluted compound: the higher it is, the more easily it evaporates. It depends on the boiling temperature, on the molecular weight and on the solubility in the dilution medium. It can be modified by the existence of interactions with the components of the matrix [13.45–48] since the aroma compounds establish low energy bonds with nonvolatile molecules. For example, the addition of a 15% (m/m) glucose solution to a 1-propanol solution increases its volatility whereas the addition of starch hydrolysate (DE 5) reduces the volatility of this compound [13.49, 50].

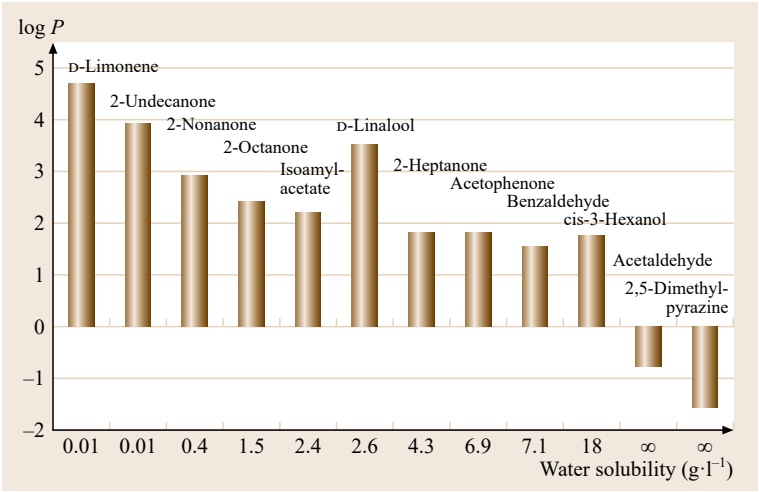

Fig. 13.2 Comparison between hydrophobicity and water solubility of aroma compounds at 25 °C (after [13.1])

13.2.6 Diffusivity

The ability of the volatile compounds to be retained by a matrix depends on their solubility and on their diffusion coefficient in this matrix [13.28, 43, 51]. The more soluble a compound may be, then the higher the diffusivity in the matrix may be and, accordingly, the more facilitated the transfer is. This is why the molecular volume, which is indirectly related to the molar mass, helps to predict differences between diffusivity of aroma compounds. The greater the volume is, the lower the release rate is [13.52, 53].

13.3 Composition and Properties of the Matrices

The release of aroma compounds of a food depends on several factors. Studies from model environments have shown that the aromatic quality of volatile compounds in the vapor phase depends on their physico-chemical properties, their initial concentration and their interactions with the media components. Several authors have studied the behavior of aroma compounds in relatively simple systems such as water, in the presence of carbohydrates, proteins and lipids. The authors have demonstrated the effect of the medium composition on the retention of aroma compounds. In foods, lipids play a dominant role in the behavior of aroma compounds. Thus, when a product is reduced in fat content, strong changes in the aromatic quality of the product are caused. This problem can be solved by a new and specifically adapted formulation of the aroma. However, the use of ingredients such as polysaccharides and proteins capable of binding aroma molecules is desirable.

Most foods are relatively structured media (emulsions, gels, foams). Conformational changes of the various ingredients (especially proteins) caused by treatments during the manufacturing of the product play a role in the physico-chemical behavior of aroma compounds.

13.3.1 Carbohydrates, Proteins, Lipids, Salts

Carbohydrates

In general, the presence of carbohydrates in an aqueous solution induces a decrease in the relative volatility of compounds compared to that in water. However, carbohydrates such as mono-and diosides cause an effect of *salting-out* that is to say, an increase in volatility compared to that in water [13.16].

Interactions between carbohydrates and aroma compounds are generally low energy and depend on many factors such as the nature and concentration of volatile compounds and carbohydrates [13.54–57]. These interactions can be of different types: adsorption, formation of inclusion complexes and modification of the solute diffusivity in the system [13.58], they result in changes in the volatility and diffusion coefficient of the solute. Thermodynamic and kinetic approaches have been conducted on the retention of various aroma compounds by gels and solutions of polysaccharides [13.59–61].

Carbohydrates have a particularly important role in the formulation of new products. Indeed, in the food industry, carbohydrates are used as substitutes for conventional ingredients. For example, carboxymethyl

cellulose and starch preparations are used as substitutes for fat [13.16].

Carbohydrates are most commonly used by the food industry [13.53, 62]. They consist mainly of sucrose, starch and starch derivatives, gums.

Oses. Sucrose, one of the most common ingredients in the food industry, is also used as a textural sensory agent and flavor carrier, either alone or in mixture. Some studies have been conducted on the sweetness-texture interactions [13.63, 64]. Alone, it is a medium on which volatile aromas are sorbed or encapsuled. The very low cost of this method of retention is its main advantage. However, the compounds are not encapsulated and then evaporate or oxidize easily. But, when the encapsulation is performed by co-crystallization, the aroma compounds are retained in the pores formed between the microcrystals of sucrose pellets and only those located on the surface are exposed to evaporation or degradation [13.65].

Finally, sucrose mixed with starch hydrolysates is very commonly used as a matrix for encapsulation by extrusion [13.66–68]. In all cases this support is inexpensive and readily dissolves in water. However, it has a sweet taste that may limit its utilization. Carbohydrates are the most common encapsulation materials in the food industry but also some proteins are interesting flavor carriers. The behavior in sucrose solutions depends on their concentrations. *Roberts* and *Acree* [13.69] showed that the presence of sucrose in aqueous solution at high concentration induces a decrease of the release of aroma compounds by an increase in the viscosity of the solution and a decrease of the water activity in the solution. *Lubbers* and *Guichard* [13.70] also showed a decrease in the release of aroma compounds in sugar syrups containing 60% dry matter. *Mei* [13.71] observed that the addition of sucrose and fructose to yogurt has a suppressive effect on the release of certain aroma compounds. But in sucrose solutions at lower concentrations, other authors [13.72, 73] showed an increase in the release of aroma compounds in the presence of sucrose, described as *salting-out* [13.73, 74].

Starch and Starch Hydrolysates. Many studies have focused on the interactions between aroma compounds and starches [13.75–78]. The retention is influenced by numerous factors such as the botanical origin of starches, the applied treatment as well as the nature of aroma compounds [13.79]. Different mechanisms are involved, such as hydrogen bonds and hydrophobic interactions, and also the formation of inclusion complexes [13.55, 75, 76]. The higher polarity and solubility of aroma compounds, as well as their shape and size, influence the structure and stability of such complexes. It may even be a competition between the compounds for a binding site [13.80].

Starch hydrolysates are obtained from the partial hydrolysis of starch by enzymatical or acidic treatment, or by using a combination of these two ways. The resulting products are mixtures of oligo-and polymers of glucose, characterized by their DE. Those having a DE between 3 and 20 are called maltodextrins, while those having a DE greater than or equal to 20 are called glucose syrups.

Their hydrophilic nature does not allow them to form stable emulsions in the early stages of encapsulation and requires the addition of an emulsifier to ensure the retention of flavors [13.53, 62]. An alternative is to use them in combination with other compounds having emulsifying properties (arabic gum or proteins) [13.81, 82].

For a similar solid content, their viscosity and their ability to retain aroma compounds decrease with the increase of their DE [13.53, 83]. These retention differences are explained by the highest diffusivity of aroma compounds in starch hydrolysates solutions with high dextrose equivalent [13.53, 84] and also by the greater mobility of the insoluble fraction of flavors in such environments [13.84]. However, the assumption of the existence of interactions between volatile compounds and starch hydrolysates was also issued [13.15]. It is based on the fact that these materials cause a color reaction with iodine, thus revealing the existence of a complex, and is further reinforced by the discovery of complexes between dextrins over nine glucose units and sulfate dodecyl and sodium [13.85]. This study also demonstrated a greater ability to form complexation with increasing degree of polymerization and consequently lowering the DE. Interactions between aroma compounds and starch can arise from two kinds of interactions. First, by the involvement of hydrogen bonds between the hydroxyl groups on the outside of the amylose helices and aroma compounds, and secondly, the inclusion complexes where the aroma compounds are trapped inside the amylose helices through hydrophobic interactions [13.86–88]. Some authors explain the retention and the release rate of aroma compounds in part by the crystal structure (Type-V) of amylose [13.89]. Increasing the concentration of starch in dairy gel models generally results in a decrease in the release of aroma compounds [13.90].

In contrast, the protection against oxidation increases with dextrose equivalent as the high dextrose equivalent products have a high content of reducing sugars. This property is a major advantage of these media, as a protection against oxidation is carried out without the addition of antioxidant, with starch hydrolysates

being not considered as food additives [13.91]. Associated with their low prices and their neutral taste, these properties make it one of the most common flavoring materials in the food industry.

Modified Starches. Modified starches have been developed to combine both the emulsifying properties of arabic gums and the low cost of starch hydrolysates. To improve its functional properties, starch can be crosslinked or grafted, but only grafted starch is used as a flavor carrier. Modified starches, although they are more expensive than starch hydrolysates, have the advantage of being able to form stable emulsions with aroma compounds, often with hydrophobic compounds [13.81, 92, 93]. They are obtained by grafting lipophilic groups such as octenyl succinate (0.3%) on starch, which is a hydrophilic substance. These molecules are therefore amphiphilic and have excellent emulsifying properties, which gives them a very high retention capacity. In addition, after rehydration, the reconstituted emulsions are stable and their droplet diameter is in the micrometer-range, giving the product an attractive appearance [13.92]. These materials are considered as food additives, designated by the code E1450, unlike starch hydrolysates [13.94].

β-Cyclodextrins. Among the derivatives of starch used as aroma carriers, there are also β-cyclodextrins (Fig. 13.3).

Although known since the beginning of the 20-th century, they have been used in the food industry only since the 1970s. They are now allowed as aroma carriers in USA, Japan and in the European Union [13.95]. The European regulation of 11 November 2011 allows their presence (additive E459) at a concentration of 1000 mg/kg in the final food. They are homopolymers of seven D-glucose units linked by $\alpha 1 \rightarrow 4$ (Fig. 13.3). They have the shape of a truncated cone having two outer rings of hydroxyl groups. Primary hydroxyl groups are located at the narrow end of the torus formed by these polysaccharides, while the wider end is lined with secondary hydroxyl functions. These OH functions provide the β-cyclodextrins solubility of 18.8 g/l in water at 25 °C [13.19]. Their ability to retain aroma compounds is related to the hydrophobicity of their cavity whose dimensions are compatible with the inclusion of numerous aroma compounds [13.19, 62, 96]. Upon the considered compounds, one or two molecules or a portion of the flavor molecules may be included in the cavity. *Sanemasa* [13.97] studied the molar ratio of aroma compounds and β-cyclodextrins after encapsulation for twenty-two volatile compounds, and found that it varies between 0.8 and 2. *Szejtli* [13.95] noted in a journal devoted to these molecules that the stoichiometry of the complex aroma/β-cyclodextrin compound ranges between 0.5 and 2.

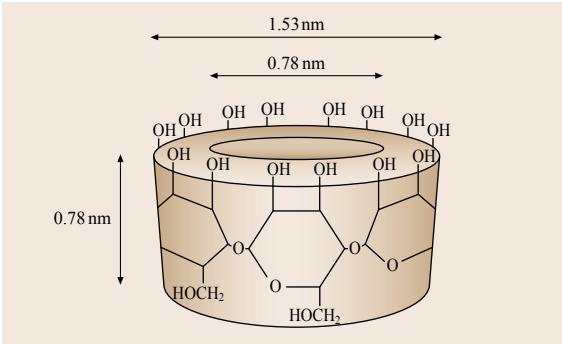

Fig. 13.3 Representation of a β-cyclodextrin molecule

The ability of these materials to reduce the volatility of aroma [13.25], their chemical or thermal degradations and their desired application are factors which can compensate for their higher prices than starch hydrolysates [13.17, 98, 99].

Gums. Gums are also widely used as flavor carriers and among them, arabic gum is the most common [13.100]. It is obtained from the exudate of Acacia Senegal and some other species of acacia. Its supply and its price are subject to the vagaries of the harvest. However, its large storage capacity makes it an interesting medium. This ability is due to its composition. Arabic gum consists of polymers of glucuronic acid, rhamnose, galactose and glucose. It also includes 5% of proteins that give it its emulsifying properties and thus higher retention capacity than starch hydrolysates at which it is sometimes added during atomization.

Xanthan gum is also widely used in food industries because it is both soluble in hot or cold water. Its structure consists of a main chain of β-D-glucose residues. One molecule of glucose out of two carries a side chain of trisaccharides (α-D-mannose, β-D-glucuronic acid and β-D-mannose terminal). Xanthan is stable under acidic medium (an advantage compared to starch and its derivatives), it supports cycles of freezing and thawing without notable syneresis and it also has an excellent compatibility with many compounds and salts. Finally, Xanthan has good emulsifying properties (a vinaigrette to 60% of oil is stable for over a year by the addition of 0.3% xanthan). *Bylaite* et al. [13.101] aimed at better understanding the release of aroma compounds from xanthan-thickened food model systems. The viscous food model was developed by increasing xanthan concentration to vary the *macroscopic viscosity* of the food model. The aroma release was evaluated in the xanthan-thickened solutions by assessing the partitioning and mass transfer behavior of 20

selected aroma compounds (5 aldehydes, 4 esters, 5 ketones, 3 alcohols, and 3 terpenes) having a wide range of physico-chemical properties. Interactions between flavor compounds and xanthan were assessed by measuring air-liquid partition coefficients K of aroma compounds in pure water and in the xanthan solutions by static headspace gas chromatography. The mass transfer of aroma compounds was estimated by dynamic headspace gas chromatography. To specifically understand the influence of viscosity on molecular diffusion of aroma compounds and thus abolish the effects of eddy diffusion, the dynamic headspace analyses were carried out with solutions under stagnant conditions. Their results had shown that limonene and some of the esters and aldehydes exhibited decreased K values in the presence of xanthan, indicating that the release of these volatile aroma compounds was reduced due to interaction with the xanthan matrix. They observed that the degree of interaction depended on the physico-chemical characteristics of the aroma compounds. A similar tendency was observed at nonequilibrium with the decreases in release rates being most pronounced for limonene, followed by the esters and aldehydes, with no effect for ketones and an apparent *salting-out* effect for alcohols. They concluded that the reduction in flavor release by xanthan was thus dependent on the physico-chemical properties of the aroma compounds and was apparently a result of the aroma-xanthan interactions and not influenced by the viscosity of the system itself.

Other gums, such as guar gum, are also largely used in the food industry. Guar gum consists of a polysaccharide composed of galactose and mannose. The backbone is a linear chain of β 1,4-linked mannose residues to which galactose residues are 1,6-linked at every second mannose, forming short side-branches. *Jouquand* et al. [13.57] have studied two guar samples differing mainly in their mannose/galactose ratio but with a similar molecular weight. The impact of these variations on aroma retention was determined by measuring the partition coefficient of ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate in polysaccharide solutions prepared at 0.1% (w/w). They observed that the ethyl hexanoate retention depended on the mannose/galactose ratio. A high density of galactose residues induced a release of this nonpolar compound. A competition between galactose residues and ethyl hexanoate to bind water molecules could explain this *salting-out* effect. This result pointed out the impact of H-bonds on the retention of this compound. On the contrary, the high retention of ethyl decanoate was clearly linked to the molecular weight of guar samples involving hydrophobic interactions with the macromolecules.

Proteins

The knowledge of aroma-protein interactions is essential to the food industry. Indeed, several proteins used for their functional properties can bring unwanted flavors (off-flavors) and cause problems of acceptability of the product [13.102]. Furthermore, proteins can selectively retain the components in a mixture of flavorings and cause an imbalance in the perceived flavor. The binding capacity of aroma compounds by proteins may also be useful in the formulation of new products, especially for the flavor of low-fat foods. For this reason, the retention of flavors, and the factors that affect flavor release in media containing proteins are widely studied [13.88, 102, 103].

The fixation of flavors to food proteins (and likewise of off-flavors) must be considered by the food industry in the use of functional ingredients such as proteins. In formulating new products, several parameters must be evaluated and determined such as the binding affinity and the degree of release of flavor, and the partitioning of each aroma compound between the various components of the food. Although the functional properties of the protein are those requested, in some cases the protein preparations have undesirable aromas and the release of these compounds limits the use of these preparations [13.104].

The protein materials are mainly represented by gelatin, a water soluble protein derived from collagen [13.62]. Together with water, it forms a semi-solid colloid gel. Gelatin thereby firstly forms a solution of high viscosity in water which sets to a gel on cooling. Gelatin is also soluble in most polar solvents. It can be associated with starch hydrolysates to improve their emulsifying properties and thus their retention capacity. Mixed with arabic gum, it is the material used for encapsulation by coacervation. Other proteins such as gluten, casein, albumin or soy protein can also be used as an encapsulation support, but are less common [13.105].

Various bonding mechanisms are possible between the flavor compounds and proteins such as reversible bonds whose nature is probably hydrophobic and/or electrostatic as suggested by the influence of the temperature, the length of the carbon chain and the force ion [13.102, 106]. Many authors have highlighted the existence of hydrophobic binding sites [13.58]. A chemical reaction between the substrate and the volatile compound is also possible, giving rise to a covalent bond, for example, the formation of a Schiff base between the lysine or cysteine moieties and an aldehyde [13.107].

Proteins are capable of binding with aroma compounds by specific interactions. In most cases, they cause a decrease in their volatilities [13.108]. *Weel* et al. [13.109] specified that the perception of flavor

from flavored whey protein is determined by texture rather than by release. The retention of apolar flavor compounds by proteins depends on the existence of hydrophobic regions in the macromolecule which must be accessible to the ligand. The protein denaturing agents cause the unfolding of the tertiary structure of the protein and alter the structure of the hydrophobic regions. These agents are useful to highlight the role of the conformation of the hydrophobic interactions between small molecules and proteins.

In most cases, interactions between proteins and flavor compounds are reversible, they are of hydrophobic type or employ hydrogen bonds [13.110, 111]. However, there are also irreversible covalent interactions such as between aldehydes and available NH residues. A nonreversible binding has been demonstrated with the fixation of (E) hex-2-enal on milk proteins [13.112]. Numerous model media have been studied to reveal the importance of proteins (notably dairy proteins such as caseins, lactoglobulin and bovine serum albumin) in the retention of aroma [13.113–115]. *Hansen* [13.116] showed early on that milk proteins cause a decrease in perceived intensity for vanillin, benzaldehyde and D-limonene.

The heating of proteins usually causes unfolding. Indeed, when increasing the temperature, the bonds, in particular hydrogen bonds which stabilize the protein structure, become lower thus causing the unwinding of the polypeptide chain. This unfolding induces the exposure of hydrophobic side chains that become available for the formation of molecular associations [13.117, 118]. *Burova* et al. [13.119] observed that the bovine serum albumin (BSA) denaturation by acid or thermal treatment acted negatively on the covalent binding between vanillin and native bovine serum albumin. More recently, *Tavel* et al. [13.120] confirmed the strong affinity of β-lactoglobulin to β-ionone and also an affinity for guaiacol. Both aroma compounds showed greater retention by partially denatured (heated) β-lactoglobulin than by native β-lactoglobulin. The less tightly packed structure of the heated protein promotes a binding with aroma compounds, especially with the central cavity; this cavity becomes more accessible by heating that induces the favorable conformational change which in turn results in the retention of aromas.

The effects of heating on the protein and the related impact on the release or retention of aroma compounds directly impact the formulation of foods. Accordingly, the heat treatment of products containing proteins sensitive to heat can change the suitability of the product [13.104]. However, this phenomenon depends on the composition of the food process conditions and on the nature of aroma compounds.

The nature and strength of interactions between aroma compounds and proteins are influenced by physical or chemical treatments which alter the conformational status of the protein and cause significant changes in ligand binding properties. This results in increased or decreased binding of a given compound by the proteins according to the intensity and duration of treatment.

The final result of the retention of volatile compounds by proteins may be a change in the sensory perception of flavor. For example, *Hansen* and *Heinis* [13.107] observed a decrease in the intensity of the flavor by using benzaldehyde and limonene in the presence of milk protein, but in the case of citral no difference in perception was noted. Other dairy products were studied such as yogurt; data on the release of aroma compounds in such systems are available in the literature [13.121–124].

The β-lactoglobulin was used as a model for the study of protein-ligand interactions because its conformation and physical properties have been relatively well studied [13.103]. Interactions between aroma compounds and β-lactoglobulin depend on the conformational state of the protein, temperature, pH and the presence of other ingredients such as salt, lactose and residual lipids [13.125].

Espinosa-Diaz [13.1] and *Seuvre* et al. [13.126–128] also used β-lactoglobulin as a model to study the interactions between the protein and the aroma compounds. Their results show that physico-chemical interactions between aroma compounds and β-lactoglobulin depend on the nature and on the initial concentration of the flavor compound and on the pH of the protein solution. The influence of protein conformation on flavor retention has also been shown, with monomers having a greater affinity than dimers. Moreover, water has been shown to be necessary to the formation of aroma-protein hydrophobic interactions. The volatility of the aroma compounds can be altered by the structure of the food matrix; in dispersed systems (water-lipid), β-lactoglobulin is less available to interact with flavor molecules because of its partial adsorption at the lipid-water interface. The physical barrier formed by β-lactoglobulin at this interface also modifies the aroma transfer. This phenomenon further depends on the respective pH. Flavor release from food matrix likewise depends on the nature of the aroma compounds. Their behavior as a function of the food matrix structure and composition shows that flavor release is influenced basically by the presence or absence of interactions with the nonvolatile constituents of the medium.

Proteins and carbohydrates do not exert any barrier properties to water. When such properties are desired, lipids are preferably used.

Lipids

This substance class includes mono-and diglycerides, and hydrogenated oils and phospholipids which are also called lecithins [13.62, 82]. As emulsions, they are used to flavor many beverages. As coatings, they are used to control the release by increasing the temperature or to obtain water barrier properties. Their melting temperature is determined both by the length of their carbon chains and their degree of unsaturation (Table 13.3). The shorter and the more unsaturated fatty acid chains are, the lower the melting point is. The judicious choice of the type of used lipid allows controlling the release of aromas. In addition, their hydrophobicity makes them little permeable to hydrophilic compounds, which gives them a large retention capacity for these substances in comparison to protein and carbohydrate matrices.

The fat content of a food strongly affects the perception of flavor [13.130]. *Ebeler* et al. [13.131] studied the influence of lipids on the release and on the sensory perception of menthone and isoamyl acetate. Their results show a decrease of the concentration in the vapor phase and of the perceived intensity of these two compounds in the presence of soybean oil compared to the values obtained in water. In general, the perception thresholds of aroma compounds are higher with a dissolution in oil than with a dissolution in water. For example, the detection limits of octanoic acid and γ-decalactone are 60 times higher in oil compared to those in water. For 2,4-decadienal this factor is 600 [13.58]. A decrease in the concentration of volatile compounds in the vapor phase was observed when the amount of lipid increases in the medium: water, skim milk, whole milk and vegetable oil [13.132]. The addition of lipids in an aqueous medium causes a redistribution of aroma between the water, the lipid molecules and the vapor phase. Aroma compounds are generally hydrophobic, their concentration is often high in the lipid phase of the food and reduced in aqueous and vapor phases [13.133]. These factors should be considered when formulating new products with reduced fat. *Brauss* et al. [13.134] observed that altering the fat content affected flavor release in a model yogurt system and *van Ruth* et al. [13.135] pointed out the influence of the lipid fraction on the release of 20 aroma compounds from oil-in water emulsions.

Several authors consider that the decrease of the volatility of aroma compounds in lipids is mainly due to a phenomenon of solubilization [13.58]. Regarding the nature of interactions between aroma compounds and lipids, *Plug* and *Haring* [13.136] revealed the existence of hydrophobic interactions and van der Waals forces. *Le Thanh* [13.137] showed the presence of hydrogen bonds between fatty acids and volatile compounds.

Even in a small proportion, lipids strongly influence volatile aroma compounds: 1% of vegetable oil added to the aqueous phase substantially reduces the concentration of aliphatic C4 to C8 aldehydes in the vapor phase [13.138]. This trend is even more important for higher chain length or generally a higher number of carbon atoms constituting the aroma molecules: *Jouenne* [13.3] observed that the activity coefficient of 2-octanone decreased by a factor of 4 in the presence of 1% miglyol while for 2-nonanone this factor was 10. When the compound is hydrophilic, the opposite effect is observed: *Land* and *Reynolds* [13.139] showed that the concentration of diacetyl in the vapor phase is higher above oil than water. The effect of fat on the retention of hydrophobic aroma compounds is higher than that of proteins [13.126] or that of carbohydrates [13.140].

The nature of the fat moiety, especially the length of the carbon chain and the degree of saturation of the fatty acids constituting the lipid, also influences the coefficient of vapor-liquid partitioning of aroma compounds [13.137, 141]. The melting point depends on the composition of fat; accordingly, small changes in temperature around the melting point of the lipid affect the solid/liquid ratio. This solid/liquid fraction can affect the coefficient of vapor-liquid partition. Sorption of volatile compounds in liquid food lipids is greater than that of solid [13.29]. The amount of sorbed substance depends on the length of the carbon chain of the triglyceride fatty acids: moreover retention increases with the degree of unsaturation of the fatty acids [13.29]. The behavior of solutes in liquid lipids obeys Henry's Law which is not the case for solid lipids [13.142].

The influence of different chemical groups of solutes on their partition coefficients between an organic solvent and an aqueous phase is highlighted from the coefficients of liquid-liquid or vapor-liquid partitioning. In the homologous series of aliphatic alcohols, the coefficients of liquid-liquid partitioning (cyclohexane/water and hexadecane/water) increases with the number of carbon atoms [13.143]. The same trend was observed by analyzing the vapor of solutions of homologous series of aldehydes and ketones in oil: the

Table 13.3 Melting temperature of lipids (after [13.129])

Lipid		Melting temperature (K)
Fatty acids	Stearic acid C_{18}	344
	Oleic acid $C_{8:1}$ Δ^9	286
	Linoleic acid $C_{18:2}$ $\Delta^{9,12}$	368
	Linolenic acid $C_{18:3}$ $\Delta^{9,12,15}$	262
Triglycerides	Trimyristin (C_{14})	330
	Tripalmitin (C_{16})	339
	Triolein (C_{18})	346

vapor pressure decreases with the length of the aliphatic chain [13.144]. The comparison between liquid-liquid partition coefficients of two aroma compounds with the same chain length but with different chemical function shows significant differences. For example, the partition coefficient in cyclohexane/water of 2-hexanol was 1.68 [13.145] while that of 2-hexanone has been reported to be 11.10 [13.146]. This difference is explained by the difference of polarity of the chemical groups. The hydroxyl group is more polar than that of the carbonyl group.

The flavor-lipid interactions must be considered especially in the use of fat substitutes. Indeed, in addition to the consequences related to texture, the removal of fat in most foods causes an imbalance in the aroma and the appearance of off-flavors, which causes a decrease in the acceptability of the product by the consumer [13.147].

Salts

The effect of salts on the volatility of aroma compounds must also be taken into account. *Voilley* et al. [13.148] showed a significant release phenomenon of octanol and acetone in the presence of calcium chloride (20%). The same trend was observed by several authors with sodium chloride [13.137, 149]. This effect of *salting-out* of odorants in the presence of salts can be explained by a change in the structure of water causing an increase in the effective concentration of aroma compounds in the solution thus increasing their volatility [13.150]. Based on the fact that consumption of salt is too high (9–10 g/day in France for example) compared to the values that are recommended for daily intake (6–8 g/day), there is an increasing interest in the production of low sodium-content foods. However, salt perception interacts with aroma perception. *Lauverjat* et al. [13.151] studied the physico-chemical properties involved in the mobility and release of salt and aroma compounds in model cheeses. They determined the values of NaCl water/product partition coefficients to highlight interactions between proteins and NaCl. They observed that

> *these interactions were not modified by the product composition or structure. On the contrary, the composition and the structure of the studied model cheese influenced diffusion of NaCl and both the partition and the diffusion of aroma compounds.*

These authors also concluded that *both physiochemical and cognitive mechanisms contributed to perception*.

Moreover, salt (sodium chloride) is also used as a carrier for flavors. Sodium chloride may retain flavors by impregnation. This process, though economical, is not a real flavor encapsulation and then aromas evaporate or oxidize easily.

13.3.2 Macro- and Microstructure

An emulsion is a heterogeneous system containing a liquid dispersed in another in the form of droplets of microscopic size or colloidal [13.152]. Two main types of emulsion are found in food:

- *Lipid-in-water* emulsion which is a dispersion of fat in the form of lipid droplets (diameter of 0.1 to a few tens of micrometers in average) in water (continuous phase).
- *Water-in-lipid* emulsion, in which case the fat (in high concentration) is the continuous phase and water is the dispersed phase. Butter and margarine belong to this type of emulsion [13.153].

The aroma is determined by the presence of volatile molecules in the vapor above the emulsion [13.154]. The perception of aroma depends on the precise location of aroma inside the emulsion components. Most odorants are perceived more intensely when they are present in the aqueous phase rather than in the oil phase [13.155, 156]. An emulsion may be divided into four phases. The flavor molecules will be distributed within each of them: inside the droplets, in the continuous phase, in the oil-water interfacial area and the vapor above the emulsion [13.157]. The relative concentration of aroma compounds in each of these phases depends on their molecular structure, the affinity of the aroma compound for each phase and the respective phase properties [13.141].

Two factors influence the release of odorants from the emulsion: their partition coefficients at equilibrium (liquid-liquid, vapor-liquid) that determine the degree of the odorant concentration gradients at the interface, and their mass transfer coefficients that determine the rate at which the molecules move from one phase to another.

Influence of the Nature of the Continuous Phase of an Emulsion

The physico-chemical characteristics of aroma compounds largely determine the partition coefficient between the vapor phase and different solvents in which they are present (water or oil). If an apolar flavor compound is added to an apolar solvent, then the volatility decreases as the molecular weight increases (the value of the partition coefficient vapor-apolar solvent decreases). Indeed, the number of favorable attractive interactions increases between the flavor compounds and the nonpolar solvent with the increase in the size of the

compound that is to say with the length of the carbon chain in the case of homologous series [13.138]. However, if a nonpolar compound is added to a polar solvent, the volatility increases as the molecular weight increases [13.158]. Nonpolar compounds are less volatile in apolar solvents than in polar solvents [13.138]. Indeed, when a nonpolar compound is introduced into a polar solvent, a relatively large number of hydrogen bonds between water molecules must be replaced by relatively weak interactions of van der Waals-type, which is energetically unfavorable due to the hydrophobic effect. Polar compounds are less volatile in polar solvents than in apolar solvents, because the hydrogen bonds formed in the polar solvent are stronger than the van der Waals interactions formed in a nonpolar solvent [13.157].

Thus, the nature of the continuous phase of the emulsion, oil or water, can have an influence on the release of volatile compounds in the vapor phase. *Salvador* et al. [13.159] showed that for diacetyl the transfer rate towards the vapor phase is greater for an oil-in-water emulsion than for a water-in-oil emulsion with the same oil content (50% v/v) and emulsified with the same type and amount of emulsifier (0.5%, v/v sucrose ester). This variation of the transfer rate of the flavor compounds from the dispersed phase towards the continuous phase would be due to structural differences of the oil-water interface.

Influence of Droplet Size and Interface of an Emulsion

In the case of an oil-in-water emulsion, the release rate of aroma compounds from the emulsion increases as the droplet size decreases. The rate is highest for droplets smaller than 10 micrometers [13.157]. If the droplets are coated with an interfacial membrane, the release rate can be significantly reduced and will depend on the type of emulsifier [13.160].

However, no difference was observed by *Druaux* et al. [13.161] with the variation of the size of the lipid droplets of an oil-in-water emulsion for the volatility of diacetyl and 2-nonanone if the size of the fat globules is multiplied by 12 (0.5–6.1 μm). *Charles* et al. [13.162] showed an increase of the release of hydrophobic compounds and a decrease of the hydrophilic compounds when the size of fat globules in a salad dressing decreases. The higher viscosity of the salad dressing with a fine granularity could explain the lower release of hydrophilic compounds (transfer through the aqueous phase is a limiting factor). Hydrophobic compounds are more easily released through increased contact between the small globules and air (direct transfer from the fatty phase towards the vapor phase) and by reducing the thickness of emulsifiers at the globule surface (transfer towards the aqueous phase). *Van Ruth* et al. [13.135] also showed that the particle size has a very important effect on the vapor-emulsion partition coefficient, since the partition coefficient of 18 out of 20 compounds was affected. Generally, a decrease of the vapor phase concentration is observed when the particle size increases. The droplet diameter change modifies the concentration of the emulsifier on its surface, and the ratio *interface volume/emulsion volume*. The diameter also affects the kinetics of the aroma compounds release, which is in relation with the viscosity change. *Miettinen* et al. [13.163] studied the effects of the composition and structure of a rapeseed oil-in-water emulsion on the release of a nonpolar compound, linalool and a polar compound, diacetyl by static headspace and sensory evaluation by smelling. The lipid content strongly affected the release of linalool but not diacetyl. They also found a small effect of the type of emulsifier both by means of headspace and sensory analyses. For diacetyl, the release is more pronounced from emulsions at 5 and 50% rapeseed oil containing modified potato starch as emulsifier compared to those containing sucrose stearate. For linalool, the same trend was found for the 5% rapeseed oil emulsion, but the opposite trend was observed for the 50% emulsion. In addition, they observed that the decrease in the size of the oil droplets, obtained by high-pressure homogenization, increased the release of linalool but had no effect on diacetyl liberation.

Emulsifiers

To obtain a stable emulsion, a surfactant substance must be present to protect newly formed droplets against immediate recoalescence. This substance is the emulsifying agent [13.152].

In food, there are two major classes of emulsifying substances: emulsifiers with low molecular weight and macromolecular emulsifiers. Among the low molecular weight emulsifiers ($\leq 1000\,g/mol$), monoglycerides are the most commonly used emulsifying additives besides lecithins and other synthetic molecules such as polysorbates. Macromolecular emulsifiers are proteins, mainly from egg or milk [13.164].

The amphiphilic emulsifiers are often classified according to what is called hydrophilic-lipophilic balance (HLB). It is a semi-empirical concept which depends on the ratio between the hydrophilic and hydrophobic groups. If the HLB value is high, the emulsifier has a predominant hydrophilic character and therefore a preferential solubility in water. For a low HLB value, solubility in oil is higher [13.152]. The amphiphilic character of these molecules allows them to stabilize a naturally unstable system such as two immiscible phases such as oil and water.

To be effective, an emulsifier must, first, facilitate the creation of a new interface by lowering the interfacial free energy and, secondly, provide some stability to the droplet, that is, by forming a protective adsorbed layer on its surface [13.152]. The emulsifier is adsorbed at the oil-water interface, which has the effect of reducing the interfacial tension [13.165]. At the oil-water interfaces, surfactants tend to orient themselves so that their hydrophilic polar head (for example, carboxyl or hydroxyl group) is in the aqueous phase and the hydrophobic chain (lipophilic hydrocarbon chain in general) in the lipid phase [13.152]. An emulsion can be characterized by the size and distribution of its droplets, its density, the ratio of volume fractions of the two phases and viscosity.

Milk proteins are used to stabilize *lipid-in-water* emulsions [13.166]. The proteins form an interfacial membrane and thus stabilize the system against flocculation, coalescence and creaming, through electrostatic repulsions and/or steric hindrance [13.167, 168]. However, during adsorption at the oil-water interface, the conformation of the protein can be modified. Thereby β-lactoglobulin can partially unfold and the sulfhydryl groups which are commonly buried in the protein monomer become available to react with other sulfhydryl groups and form disulfide bridges [13.169]. This change of conformation can, in turn, induce a modification in the retention of aroma compounds.

It was also observed that salts can also change the emulsifying properties of β-lactoglobulin. In fact, these properties decrease with increasing NaCl concentration in the presence of calcium ions. The effect of calcium is dominant and can be reduced by the additional presence of NaCl in the solution [13.168].

13.4 Methods to Study Interactions and Their Role on Transfers

By studies on the macroscopic scale, it is possible to highlight these interactions to determine the number of binding sites and the affinity of aroma compounds for these sites. The first approach to the study of the interactions is therefore to characterize them at the macroscopic scale.

13.4.1 Experimental Highlight and Characterization of the Interactions

The detection of physico-chemical interactions between flavors and other components is based on the measurement of the equilibrium vapor-liquid or liquid-liquid, with or without the involvement of changes of state in the aroma compound [13.170]. The used methods are static or dynamic, depending on how the thermodynamic equilibrium is reached at the time of measurement (Table 13.4). But whatever the method (static or dynamic), the interactions are quantified by determining a retention percentage or a fixed aroma amount on the carrier.

For Low Water Contents

The sorption of aroma compounds in a chamber at controlled water activity allows to detect the retention of aroma compounds in relation to the hydration of the carrier and, therefore, synergies or competitions between aroma and water. Water as a plasticizer may actually promote the fixation of volatile compounds but by interacting with the polar sites of the macromolecules, water also competes with the ligand. In low water content media, few interactions between nonvolatile components and aroma compounds can occur.

In Aqueous Solution

The vapor analysis or headspace analysis consists in analyzing the vapor phase above the liquid phase (or solid) when the equilibrium between the two phases is reached. The sampling step of the vapor phase is im-

Table 13.4 Methods to study the physico-chemical interactions at the macroscopic scale

State of hydration of the support	Method	Equilibria	
		Static	Dynamic
Low water content	With change of state of the aroma compound	Sorption [13.1, 27, 171]	
Solution	With change of state of the aroma compound	• Headspace [13.60, 150, 158, 172, 173]	• Exponential dilution [13.174–177]
	Without change of state of the aroma compound	• Liquid-liquid partition [13.178] • Dialysis at equilibrium [13.179–182]	• Liquid chromatography [13.183] • Dynamic liquid chromatography with coupled columns [13.3, 176]

portant: indeed, the vapor-liquid equilibrium should not be modified by sampling. After a variable time to reach equilibrium, sampling can be done with a gas-tight syringe and injected into the gas chromatograph [13.131, 150, 184] by overpressure in the vial connected to the chromatograph [13.133, 158] or by driving the vapor with an inert gas [13.27, 148]. The advantages and disadvantages of the different types of sampling are known [13.185]. To underline the retention of the aroma compounds by the medium, the comparison of the vapor-liquid partition coefficients obtained in water and the considered medium is achieved.

The method is called dynamic when one of the two phases (liquid or vapor) circulates (equilibrium is displaced). The exponential dilution consists in following over time the depletion of aroma compounds in the vapor phase. An inert gas is bubbled through the liquid phase and causes the depletion of the vapor phase. The kinetic analysis is realized on the vapor phase [13.3, 49, 174, 175].

It is possible to combine the static headspace with a trapping system that does not require calibration [13.185]: the liquid and vapor phases reach equilibrium inside a syringe by moving the piston. The gas mixture is trapped on porous polymer (such as Tenax) and then desorbed and analyzed by gas chromatography (purge and trap method). Reverse gas chromatography is another technique to study the interactions between aroma compounds and macromolecules such as starch or β-cyclodextrin [13.87, 186, 187]. The column is filled with the macromolecule to be studied, which constitutes the stationary phase, and is placed in the oven of the gas chromatograph. At t_0, a known amount of flavor is injected onto the column, the carrier gas then further drives the aroma compounds. The interaction between the ligand and the macromolecule increases the retention time of the ligand. The free aroma compounds are analyzed by a flame ionization detector. This technique permits the determination of the partition coefficient, but also other thermodynamic parameters such as the coefficient of solubility, the adsorption enthalpy and the free energy of adsorption, and also permits quickly the determination of sorption isotherms.

These above methods require the volatilization of aroma compounds. Other methods to study the interactions do not require a change of state of the compound. The static techniques are based on the equilibrium between the two immiscible or partially miscible liquid phases. The partition coefficient depends on the interactions between the compound and the components of each phase. The methods applied to highlight the interactions do not allow determining the nature of the bonds. To validate the assumptions made on the basis of observations at the macroscopic scale, it is necessary to use spectroscopic methods and molecular modeling. Spectroscopic methods such as infrared spectroscopy [13.137, 188] or Raman [13.188], nuclear magnetic resonance (NMR) [13.188, 189] or electron paramagnetic resonance [13.188, 190] allow achieving this objective. These methods are based on the absorption of energy by molecules during their irradiation but differ in the energy level of the electromagnetic radiation. They provide information on the mobility and the molecular conformation of the respective molecules.

13.4.2 Modeling and Prediction of the Release of Aroma Compounds

Modeling can help in predicting the release (or retention) of aroma compounds and can be generally considered in two ways. Firstly, mathematical models based on the physico-chemical properties, and secondly, molecular models giving the conformational aspects of molecules based on the interactions of the atom groups.

Mathematical Models

Physiological factors influencing the release and aroma perception during the consumption of solid food can be quantified both instrumentally and sensorically. Such investigations are useful to predict the release of aroma compounds in a food matrix. To achieve this aim, mathematical models based on physico-chemical properties such as diffusion, transfer rate and volatility have been developed. The models can be classified in two groups. Some authors [13.138, 172, 191] focused on the partitioning of flavor between the different phases of the food and the vapor phase compounds. Other authors [13.31, 154, 192–200] oriented their research towards the investigation of the release of aroma compounds in the mouth, taking into account the respective physiological parameters.

The first model was proposed by *Buttery* et al. [13.138]. This equation was used to estimate the volatility of aroma compounds in three-phase systems, oil/water/air, taking into account the partition coefficients of oil/air and water/air, and the volume fractions of oil and water in the mixture. Using this model, *Guyot* et al. [13.201] found a good correlation between experimental and theoretical values of the vapor-liquid partition coefficients when the oil content of the emulsion increases. *Carey* et al. [13.191] measured the vapor-emulsion partition coefficients for 39 aroma compounds. The experimental values were correlated to 72 physico-chemical parameters determined by the software CAChe (Computer-Aided Chemistry and Biochemistry) to define the parameters playing an important role in the release, such as water

solubility, log P or dipole moment. As a result, an empirical model has been developed predicting the effect of lipids based on these three physico-chemical parameters and on lipid concentration.

Marin et al. [13.172] modeled the liberation of aroma compounds from a nonstirred aqueous solution. The system is initially at thermodynamic equilibrium. At time t_0, an air flow passes through the vapor phase to simulate the opening of a package containing food. Thereby, the aroma compounds present in the vapor phase above the food are diluted by the air flow. In the course of these studies, the model was also validated by experimental measurements. Overall, the authors showed that the release of aroma compounds depends on the vapor-liquid partition coefficient, on the mass transfer coefficients in the two phases (liquid and vapor) and also on the gas flow.

For the release of aroma compounds in the mouth, other parameters, such as chewing, temperature, dilution with saliva, should be considered. *McNulty* and *Karel* [13.192] considered odorant transfer from the lipid phase into the aqueous phase when the equilibrium between the two phases is modified by dilution with saliva. They hypothesized that only the aroma compounds in the aqueous phase contribute to the perception and that the volatile lipophilic compounds are transferred from oil to water upon dilution by saliva. Their model predicted that the release of volatile compounds increases with the oil/water partition coefficient, with the volume fraction of the lipid phase and with the dilution of the emulsion.

Overbosch et al. [13.154] underlined that partition between phases is not the only factor to take into account and highlighted the importance of the degree of flavor release, defined as the flow of matter from one phase to another per unit of time and surface area. *Roberts* and *Acree* [13.69] developed a model based on the parameters temperature, viscosity, fat content and liquid-liquid partition coefficient to take into account the composition and the texture of the product. *De Roos* and *Wolswinkel* [13.193] defined a model of multiple extractions to simulate the effects of mastication. This model takes into account both the distribution of aroma compounds in different phases and the resistance to mass transfer at interfaces. *Brossard* et al. [13.202] suggested that in viscous systems, the aromatic perception of lipophilic compounds would depend on a combination of factors such as the maximum concentration of flavor in the lipid phase, the rate of transfer from the lipid phase towards the aqueous phase and the possibility of release of aroma compounds of the lipid phase to the vapor phase.

The team of *Harrison* and *Hills* studied these factors from a theoretical point of view and developed predictive models of release [13.196, 200, 203]. These models include both the resistance to mass transfer at interfaces and the volatility, as well as the effects of the salivary flow and the breath [13.194, 195, 198, 199]. *Hills* and *Harrison* [13.203] basically developed a theoretical model to calculate the release of aroma compounds from a homogeneous solid food, using the theory of the double layer (*two-layer theory*) to simulate the mass transfer of the food compound to the saliva in the mouth. Their model proposed that the rate-limiting step in flavor release (from a boiled sweet) was the presence of two liquid films. One was adjacent to the boiled sweet, where solubilization of the sugar matrix occurred and one related to the bulk liquid layer. The model covered the release of flavor from the solid phase (the boiled sweet) to an aqueous phase and some experimental evidence was obtained by monitoring the release of a dye from a sweet sample into the liquid phase. *Harrison* and *Hills* [13.196] further developed a mathematical model describing the release of aroma compounds from gels of gelatin and sucrose. They showed that the release of aroma compounds from these gels depends on the melting point of the gel which is related to the concentration of sucrose and gelatin in the respective gel. For a low concentration of sucrose, the limiting step is the heat transfer allowing melting of the gel, but for high concentrations of sucrose, sucrose must diffuse out of the gel to reduce the melting point. *Harrison* et al. [13.199] also developed models to take into account release of aroma compounds from emulsions into the gas phase in the mouth. They considered that the limiting step of the aroma compounds transfer from food towards the vapor phase is the resistance at the food-air interface. They incorporated the partition coefficients into their calculation model, and the influence of viscosity on the transfer coefficient, as well as the lipid fraction and the size of fat globules. The model predicted that the initial release of the odorants is linearly dependent on time and is proportional to their initial concentration, and to the transfer coefficients of the respective odorants. *Harrison* and *Hills* [13.197] further incorporated the effects of the gas flow into their model. They observed that with a longer mastication time, the degree of flavor release becomes very sensitive to the gas flow. According to these authors, this observation may partly explain the differences in perception between individuals. *Harrison* [13.195] also considered the effect of salivary flow on the release of aroma, thereby showing the great importance of the level of salivary flow on the release processes in the vapor phase. In the same study, *Harrison* et al. [13.200] further developed a computer simulation describing flavor release from solid foods in the mouth. Saliva flow, mastication, and swallow-

ing were incorporated into their model. These authors showed that the initial rate of flavor release primarily depends on the mass transfer coefficient of the respective odorant and on breakage mechanisms of the food which depends on the food's structure and composition. They also specified that an individual's mastication and swallowing pattern greatly influenced the rates of flavor release at longer times.

For foods in which the interactions exist between aroma compounds and macromolecules, the release rates mainly depend on the affinity constant. It has been demonstrated that most of the time, the limiting step for aroma release is the mass transfer across the interface liquid-vapor [13.199, 204].

Molecular Models

Molecular modeling provides information on the conformation and the mobility of macromolecule chains (such as polysaccharides) and on the characteristics of their binding sites. These models use data obtained by spectral methods such as X-ray diffraction or NMR spectroscopy to determine inter-atomic distances and call on NMR, infrared or Raman spectroscopies to know the value of the bond angles. Molecular modeling reveals deformations not detectable by use of spectroscopy, or hydrogen bonds that are difficult to observe in the presence of a solvent. It also enables to quantify the importance of each type of interaction. The combined use of spectral techniques and modeling leads to obtain additional information on the nature of the interactions between host and ligand, the latter may be an aroma compound. Modeling is widely used to better understand the interactions between active principles and macromolecules, especially the interactions with polysaccharides and proteins.

By varying the value of the bond angles, for example between glucosyl residues, and searching the energy minima, it is possible to determine the most stable conformations. Molecular modeling demonstrated the high flexibility of polyosides in solution [13.205]. *Neszmélyi* and *Hollo* [13.206] have shown by a combination of NMR spectroscopy and molecular modeling that oligomers or polymers of α 1-4 glucose have a high mobility in solution. It was also observed that molecules such as β-cyclodextrins can deform when these molecules are in solution [13.207]. Cyclodextrin deformation during the inclusion of a ligand in the cavity was also visualized by molecular modeling. The importance of the deformation is related to the volume of the ligand [13.208, 209]. The conformation of the complexes between amylose and fatty acids was also determined using this technique [13.206, 210]: the lipids are included in a helix of seven glucose units per turn. The interior of the amylose single helix, somewhat hydrophobic, is sufficiently large to be able to accommodate a linear hydrocarbon chain, although charged headgroups can disrupt the helical conformation. These results agreed with the chemical shift changes observed by NMR signals due to amylose molecules. Molecular modeling further allows to highlight that fatty acids are included at the end of the helices, the polar head remaining outside [13.210].

Lichtenthaller and *Immel* [13.211] were interested in the hydrophobicity of polysaccharides. They showed that the internal cavity of β-cyclodextrins is hydrophobic and that secondary hydroxyl groups, located at one end of the molecule, form a highly polar ring while the other end bordered with primary hydroxyl groups, is less hydrophilic. The determination of these characteristics allows a better understanding of the nature of interactions and in this aim, the macromolecule ligand complexes were also modeled. In the case of complex p-iodoaniline/β-cyclodextrin or butane-1,4-diol/β-cyclodextrins, it is clear that the hydrophobic ligand areas are included in the cavity of cyclodextrins [13.212].

Among food proteins, β-lactoglobulin is the most widely studied for its interactions with aroma compounds involving hydrophobic interactions and hydrogen bonds in relation to the perception of flavor [13.111, 113]. This protein interacts with many flavors such as aldehydes, ketones and esters. It has been shown that in the same chemical class of aroma compounds the affinity for β-lactoglobulin increases with the length of the carbon chain, except for terpenes [13.213]. With the help of the 3D-QSAR (Quantitative Structure–Activity Relationship) molecular modeling, *Tromelin* and *Guichard* [13.214] suggested the existence of several types of binding, confirming the presence of at least two binding modes and highlighting the role of hydrogen bonds. *Guichard* [13.111] made the assumption that during the aroma release process in the mouth, not only are free aroma compounds released but also those reversibly bound by the protein, pointing out the fact that flavor perception is only affected if strong binding occurs.

Recently, *Golebiowski* et al. [13.215] compared molecular modeling approaches (pharmacophore, docking and molecular dynamics) to biophysical data (fluorescence spectroscopy). The combination of molecular modeling and fluorescence spectroscopy is used to study the affinity of an odorant binding protein towards various odorant molecules. These authors were interested in the interactions between odorants and proteins involved in the perception of smell and they focused on the capability of molecular modeling to rank odorants according to their affinity with the protein, which is involved in the sense of smell.

13.5 Flavor Release or Retention

13.5.1 Partition Coefficients

Vapor–Liquid Equilibrium (or Solid)

In a vapor-liquid system, the component i is present in both phases. When the system is at equilibrium, the chemical potentials in the two phases are equal: $\mu_i^{\text{liq}} = \mu_i^{\text{vap}}$.

What is written as

$$\mu_i^{0\,\text{liq}} + RT \ln a_i = \mu_i^{0\,\text{vap}} + RT \ln \frac{p_i}{p^0} , \tag{13.6}$$

$$\ln \frac{p_i}{p^0 a_i} = \frac{\mu_i^{0\,\text{liq}} - \mu_i^{0\,\text{vap}}}{RT} . \tag{13.7}$$

The second member of this equation is constant at a given temperature, the activity of component i in the solution and its partial pressure are proportional.

In the case of an ideal solution, $a_i = x_i$, and the partial pressure of component i follows Raoult's law

$$p_i = P_i^{\text{S}} x_i , \tag{13.8}$$

where P_i^{S} is the saturated vapor pressure of pure component i. It only depends on the temperature and nature of i.

If the vapor is an ideal gas, the relationship between the partial pressure of the component i and its molar fraction in this phase follows the law of Dalton

$$p_i = y_i P_{\text{T}} , \tag{13.9}$$

where y_i is the molar fraction of the constituent i in the vapor phase and P_{T} the total pressure in the system (Pa).

For a real solution, where $a_i = x_i \gamma_i$, and if the vapor is perfect, the vapor-liquid equilibrium of component i follows Henry's law

$$p_i = P_i^{\text{S}} x_i \gamma_i . \tag{13.10}$$

This law is applicable only for pressures less than 10 times the atmospheric pressure and for temperatures far below the critical temperature of the solvent [13.216]. Henry's constant H_i is defined by

$$H_i = P_i^{\text{S}} \gamma_i . \tag{13.11}$$

From (13.9) and (13.10), the activity coefficient is defined by

$$\gamma_i = \frac{y_i}{x_i} \frac{P_{\text{T}}}{P_i^{\text{S}}} . \tag{13.12}$$

The vapor-liquid partition coefficient K_{mol} expressed as molar fraction of the constituent i is

$$K_{\text{mol}} = \frac{y_i}{x_i} . \tag{13.13}$$

The molar fraction x_i of the liquid phase is present in this equation, which requires knowledge of the total number of moles in this phase. However, the total number of moles is only accessible for simple media models where the molecular weights of each component are known. With complex food matrices, the results of the vapor-liquid partition coefficient are expressed in terms of mass fractions rather than molar fractions. The determination of the activity coefficient of aroma compounds in these matrices requiring knowledge of the partition coefficient expressed in molar fraction is not accessible. The thermodynamic constants H_i and K_{mol} represent the volatility of component i. When the component i is at infinite dilution, constant H_i, K_{mol} and γ_i do not vary for a given temperature and a given pressure.

Liquid–Liquid Equilibrium

In a liquid-liquid system containing an organic phase and an aqueous phase, immiscible or partially miscible, the partial pressures for each phase are

$$p_i^{\text{org}} = P_i^{\text{S}} x_i^{\text{org}} \gamma_i^{\text{org}} , \tag{13.14}$$

$$p_i^{\text{aq}} = P_i^{\text{S}} x_i^{\text{aq}} \gamma_i^{\text{aq}} . \tag{13.15}$$

At equilibrium, the chemical potentials in each phase are equal, then

$$P_i^{\text{S}} x_i^{\text{org}} \gamma_i^{\text{org}} = P_i^{\text{S}} x_i^{\text{aq}} \gamma_i^{\text{aq}} . \tag{13.16}$$

The partition coefficient P_i of the liquid-liquid component i expressed as a molar fraction is given by the following equation

$$P_i = \frac{x_i^{\text{org}}}{x_i^{\text{aq}}} = \frac{\gamma_i^{\text{aq}}}{\gamma_i^{\text{org}}} . \tag{13.17}$$

The partition coefficient P_i is defined for the same molecule in the two phases. The liquid-liquid partition coefficient P_i', expressed in concentration terms, is given by

$$P_i' = \frac{C_{\text{org}}}{C_{\text{aq}}} , \tag{13.18}$$

where C_{org} is the solute concentration (mol/L) in the organic phase at equilibrium, and C_{aq} is the solute concentration (mol/L) in the aqueous phase at equilibrium.

In the case of two partially miscible phases, a portion of each liquid phase dissolves in the other phase, the partition coefficient is then apparent because it takes into account the liquid-liquid partition of the solute in each phase. At infinite dilution, the coefficient of liquid-

liquid partition of the component i is constant in this range of concentration. Solute-solute interactions are then negligible and the nature of the solvent-solute interactions does not change.

13.5.2 Mass Transfer

The dynamic aspect of the release of aroma compounds is not considered in the partition study at equilibrium. Aroma compounds are distributed between the matrix and the gas phase: measurement of the transport phenomena is a central preoccupation because flavor is one of the key factors determining food quality and acceptance [13.217]. The distribution of compounds in the food requires their diffusion in the different phases and their transfer from one phase to another. Transfers of small molecules (water, aroma compounds) can occur between homogeneous or heterogeneous (in composition or in physical state) phases. The measurements of diffusion and transfer are not always easy [13.218].

Diffusion

In macroscopically immobile media, the matter migrates due to the propagation of the molecular agitation [13.219]. Molecular diffusion is defined as a transfer matter within a stationary system. It is due to a chemical potential gradient, pressure gradient or temperature gradient.

In a steady state, the Fick's law expresses the amount of diffusing matter m_i of component i through the surface section S, in a direction normal to the surface, for a time t and a distance dx

$$J_i = \frac{\mathrm{d}m_i}{\mathrm{d}t} = -SD_i\frac{\mathrm{d}C_i}{\mathrm{d}x}\,, \tag{13.19}$$

where J_i is the flux of component i (mol/s), D_i is the diffusion coefficient (m^2/s) and dC_i/dx the concentration gradient of component i in the distance x.

The diffusion coefficient D_i of a component i in a given system depends on the mass, on the shape and on the molar volume of the component, on the viscosity of the solution and on the temperature of the system [13.33].

If the conditions, at a given point, vary upon the time or in a unidirectional way, the applied Fick's law in the case of a steady state is as follows

$$\frac{\mathrm{d}C_i}{\mathrm{d}t} = D_i\frac{\mathrm{d}^2C_i}{\mathrm{d}x^2}\,. \tag{13.20}$$

There are many solutions to this equation given by *Crank* [13.220] upon the initial conditions and the limits.

Transfers Between Different Phases

A transfer between two phases through a planar interface can occur in a multiphase system:

- From the vapor phase to the liquid phase (or solid)
- From the liquid phase to the liquid phase (or solid).

For example, in the case of a transfer of aroma compound through the vapor-matrix interface between the matrix and the vapor phase, the global mass transfer coefficient k (m/s) can be determined by

$$k = \frac{J_i}{A\left(C_{\mathrm{matrix}} - C_{\mathrm{vapor}}\right)}\,, \tag{13.21}$$

where J_i is the flow of component i (mol/s), C_{matrix} and C_{vapor} the concentration of aroma compounds at a given time (mol/m^3) in the matrix and in the vapor phase, respectively, and A is the interfacial area between the vapor and matrix phases (m^2).

The aroma compounds are predominantly lipophilic. According to *McNulty* and *Karel* [13.192], the transfer of these compounds towards the vapor phase is performed in two steps: first the aroma compounds must cross the lipid phase – aqueous phase interface and after the aqueous phase – vapor interface. The calculation of transfer rates between the two corresponding phases allows quantifying the transfer of compounds at the interfaces. Transfer rates are determined by following the evolution of the concentration of the compound in one or in the two phases over time.

These transfer rates depend on the nature of the aromatized phase and on the aroma compound. The transfer rate of the linear alcohols from C_3 to C_8 of sunflower oil towards the water increases with the length of the carbon chain but decreases if tristearin is used [13.192]. *Castelain* et al. (1994) [13.221] showed that the transfer of hydrophobic compounds is faster from water to oil than from oil to water. *Salvador* et al. [13.159] determined that the transfer of the hydrophilic compound, diacetyl, is faster from oil towards the vapor phase than from water towards the vapor phase.

This thermodynamic and kinetic knowledge is useful to the study of physico-chemical interactions between aroma compounds and constituents of food matrices and release phenomena.

13.6 Preservation of Food Quality and Perspectives

The objective is to use these physico-chemical parameters (saturation vapor pressure, water solubility, hydrophobicity) to predict the behavior of aroma compounds in a food matrix. It is therefore useful to try to link the results of sensory evaluation to vapor partition coefficients matrix of aroma compounds P_i and to mass transfer coefficients k. These physico-chemical parameters determine not only the potential amount of aroma compounds available in the gaseous state to be perceived by the receptors of the olfactory epithelium but also the rate to obtain the thermodynamic equilibrium.

The distribution of an aroma compound in food depends on its affinity for the different phases, and its ability to be released into the vapor phase [13.222]. This distribution, responsible for the availability of aroma compounds, will modify sensory perception at sniffing or during consumption.

A food is a very complex matrix containing mostly lipids, proteins, carbohydrates and emulsifiers from lipid or protein types, thickeners and water. The use of these components can modify the structure of the food, which will eventually induce a difference in sensory perception. The release of aroma compounds may also be reduced by the ability of certain food components to delay the mobility of the molecules towards the surface, which results from a high viscosity or a barrier due to the structure (gel with a three-dimensional (3-D) network).

For example, the impact of the size of fat globules on the sensory perception of model cheeses containing sodium caseinate as emulsifier (11−22%) was highlighted by *Dubois* et al. [13.223]. The intensity of the note due to garlic diallyl sulfide decreases as the size of fat cells decreases, this decrease is correlated to a reduction of this compound in the vapor phase. Molecules used to stabilize emulsions such as proteins or polysaccharides could also bind to aroma compounds and influence their distribution within the emulsion (within the droplets, continuous phase and oil-interface water), which can affect the availability of flavor molecules [13.141, 224]. *Charles* et al. [13.162] also showed an effect of the size of the oil droplets on the perception of volatile compounds in a salad dressing. The release of aroma compounds, and therefore, their perceived intensity, are more important for the more hydrophilic compounds (acids, alcohols, acetoin, diacetyl) in the presence of large droplets. Because of a decrease of the viscosity of the salad dressing with the droplet size increase, the release of the compounds is then facilitated. *Miettinen* et al. [13.163] conducted sensory evaluation by sniffing on emulsions based on 5 and 50% rapeseed oil. They observed that an effect of droplet size on the perception of linalool is more important for small droplet sizes, obtained by increasing the pressure during homogenization. But, for the same concentration of fat, the type of emulsifier does not affect sensory perception.

The stability of aroma compounds plays an important role in food quality, but it is not easy to control. A way to preserve the sensory perception, and thus to retain aroma compounds inside the food, is encapsulation. Encapsulation is often a relatively effective method to limit the degradation of aromas during processes and storage of the product. Encapsulation also allows to limit or prevent undesirable events such as the aroma-aroma interactions and reactions induced by light and/or oxygen [13.225]. The method of encapsulation of sensitive substances consists of two steps: the first is the production of an emulsion composed of the couple *aroma-lipid compound* in a solution of a dense material that forms the *wall* such as polysaccharides or proteins. The second step is the drying or cooling of the emulsion to stabilize the capsule [13.226]. The stability of the encapsulated flavor compound depends on the nature of the *core*, the nature of the *wall*, the encapsulation method, the interactions and the storage conditions. The encapsulation size can vary from a few millimeters to less than a micrometer, which gives it the name of *microencapsulation*. Microencapsulation is a technique that is increasingly being developed and widely used in the pharmaceutical, cosmetic and food industries [13.227]. Microencapsulation comprises an active substance coating with a uniform continuous or composite layer. There are several microencapsulation techniques: chemical (coacervation, co-crystallization, molecular inclusion) and/or mechanical (spray drying, freeze drying, extrusion). Coatings, especially emulsified coatings, can be used as microencapsulation agents for aroma compounds, this gives them the name of *active* packaging [13.228].

This microencapsulation in edible films prevents the release of aromas and protects them from oxidation [13.227]. There are a few studies on this topic, *Kim* et al. [13.229] studied the encapsulation of orange essential oil in films based on sodium caseinate, whey and soy proteins. They found that soy protein was more efficient to retain the orange essential oil with 87.7% retention, followed by sodium caseinate with 81.5% retention and ultimately whey protein being the least efficient with 72.7% retention. The retention of aroma model in maltodextrins, gum acacia, corn syrup, modified starch, soy and whey proteins was proposed by *Dronen* [13.230]. Similarly, the retention of several aroma compounds in various starch matrices

was studied by *Boutboul* et al. [13.87]. More recently *Rodrigues* and *Grosso* [13.231] compared microencapsulation with Cashew gum and arabic gums to protect the aroma of coffee extracts.

Homogeneous films consisting of only one substance had good barrier properties and good mechanical properties, but rarely both simultaneously. Emulsified films for which the lipids are dispersed in a continuous matrix of hydrocolloids have the advantage of simplifying the manufacturing and application processes [13.232]. That is why the desired properties are often reached by using the combination of different materials. Thus, hydrocolloids form interchain interactions to create a network responsible for the mechanical strength and lipids have a water barrier role or a carrier role to encapsulate the hydrophobic volatile compounds.

Lately, *Ciriminna* and *Pagliaro* [13.233] opened the route to sustainable fragrances and aromas using the sol-gel microencapsulation of odorants and flavors: sol-gel encapsulation technology is a promising method to encapsulate fragrance and aroma chemicals. It allows an effective control of biomolecules, drugs or essential oils released. The sol-gel entrapment of aroma in the inner pores of amorphous sol-gel SiO_2 allows the practical utilization of chemically unstable essentials oils.

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14. Models of the Oral Cavity for the Investigation of Olfaction

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In this chapter, we will briefly describe the complexity of the main mechanical, biochemical and physicochemical phenomena that occur in the mouth during food consumption using examples. To better understand the reactions occurring in the mouth during food consumption, in vitro systems called model mouths were developed to simulate food consumption and thus answer some of the more fundamental questions regarding olfactory perception. This chapter provides examples of the applications of the model mouth in performing oral functions, such as mastication, saliva production and airflow, as well as swallowing, while the released volatile compounds are measured. The recent model mouth designs represent the actual occurrence of food consumption under oral conditions in a more accurate way. We believe that this type of methodology will be even more commonly applied in the future to improve the knowledge in this field.

14.1 Oral Food Processing and the Effect on Olfaction

During food consumption, various oral processing steps are followed. Initially, the solid food is ingested, then masticated, and mixed with saliva to form a bolus. The food particles are transferred into different locations in the mouth, until they are ready to be swallowed [14.1, 2]. During the complex process of oral food consumption, the physical properties of food are modified and, as a consequence of these diverse steps as a whole, the perception of flavor and texture are affected. Therefore, the release of flavor compounds from the food and their delivery to receptors are key factors leading to flavor perception.

In the last twenty years, several devices have been proposed to simulate the various steps and parameters involved in this process [14.3, 4]. These simulators were developed to study the breakdown of food and/or the release of volatile compounds from food products of various textures. Due to the vast complexity of the mouth's functionalities, the development of mouth model had to be focused on a limited number of factors to simulate a specific process for the study under consideration.

14.1.1 Main Oral Functions

The major in-mouth phenomena that occur after the ingestion of solid food are mastication, salivation, the formation of the bolus, the transport of particles into different locations in the mouth and swallowing [14.1, 2]. Mastication is the action of breaking down food and preparing a food bolus for swallowing. During this step of oral food processing, the physical properties of the food are modified and the perception of flavor and texture are affected. The release of aroma and taste compounds from the food and their delivery to receptors are key factors leading to flavor perception. The breakdown of food into particles, associated with the effect of temperature, increases the surface area of the food that is exposed to saliva and air. This facilitates the dissolution of taste compounds within saliva and

the release of volatile compounds into the gas phase of the *mouth space*, leading to flavor sensations. During this process, variations in the texture of the food and the bolus are constantly perceived and the chewing pattern is consequently adjusted according to the peripheral feedback. In addition, the masticatory pattern and the characteristics of saliva may vary considerably between individuals [14.2].

Food oral processing is divided into the three following phases: I) ingestion, II) rhythmic sequences of mastication and, III) swallowing and clearance. The properties of food (flavor and texture) are perceived during these three consecutive stages. The temporal perception of flavor and texture are important determinants in the acceptability and choice of food by the consumer, with direct consequences for his nutritional status. The breakdown patterns are specific to the food under consideration. Whereas solids must be fragmented by the teeth and be softened by and mixed with saliva to form a cohesive bolus [14.5], liquids are nearly ready to be swallowed [14.6]. Furthermore, the type and extent of oral forces change throughout the process according to the physical state of the bolus that is perceived by mechanoreceptors. Subsequent motor actions are then adapted through feedback-control mechanisms. In the case of brittle solids, the first transformation that occurs in the mouth consists of the fragmentation of the food into smaller particles. The breakage function depends mainly on the anatomical characteristics of the subjects [14.7]. The breakage function also depends on the resistance of the food to be broken [14.8]. Saliva also plays a role in the formation of a bolus from brittle food because it allows the particles to cohere due to its viscosity. Cohesive foods, such as meat and some cheeses, do not break into separate particles; instead, they are softened by chewing [14.9]. In particular, cheeses with a low lipid-to-protein ratio result in harder boli than those with a high ratio. For this type of food, the salivary intake into the food matrix is an important factor in determining the texture of the bolus: the higher the salivary intake, the softer the bolus. In the case of semisolids, the main change in food properties that occurs is the reduction of viscosity due to shearing forces, the temperature change, dilution, or chemical degradation induced by saliva.

Texture, which is dependent on the physical properties of food, is perceived through mechanoreceptors that are distributed in the oral cavity. The masticatory process can be considered as a combination of compressive and shearing activities. Studies of the mechanical properties of food have indicated that chewing and the mandibular movements occurring during the breakdown of solid food are strongly affected by the food's texture [14.10]. Thus, the hardness of the food has an effect on the number of chewing strokes necessary to trigger a swallow. The harder the food, the more chewing strokes are needed. Increasing the hardness of the food also increases the extent of the masticatory motions, thereby increasing the forces applied to the food matrix [14.11]. Compression has been shown to be dominant during the early stages of biscuit breakdown, and shearing was found to be dominant during the subsequent stages of mastication [14.12]. Food texture also affects the salivary flow rate because the chewing forces developed during mastication and the salivary flow rate, particularly the parotid gland secretion, are linked. The hardness of solid foods is therefore positively correlated with the parotid gland flow rate.

In addition to the initial hardness of the food, the progressive comminution and softening of food during mastication also affects oral physiological parameters [14.13]. Generally, the force generated by jaw muscles decreases during bolus formation due to sensory feedback between the intraoral mechanoreceptors and the muscles involved in mastication. Oral physiology and textural perception are tightly linked due to the sensory feedback between the chewing behavior and the food texture during food consumption. Moreover, tongue movements, temperature and the salivary composition are also important for textural perception because these oral parameters can modify the matrix structure of the food during in-mouth processing and consequently the sensory evaluation of texture.

In the case of brittle food (biscuits and chips), its texture is determined by the ability of the matrix to form particles, the rate of particle size reduction and the resulting particle size distribution. The level of lubrication of the particles, provided by salivation, is of obvious importance in forming a cohesive bolus prior to the swallowing step [14.5]. For nonbrittle foods, such as meat and meat products, different correlations between the oral physiological parameters and texture perception were found [14.14]. For example, the work and efficiency of chewing were found to be relevant parameters in predicting textural perception. Subjects with low efficiencies presented shorter chewing time and reached the maximal intensity of tenderness earlier. However, other works have shown that subjects presenting a lower chewing efficiency required a longer chewing time to form a bolus [14.15].

14.1.2 Release and Perception of Flavor in Oral Processing

Flavor perception during food consumption is determined by the nature and amount of volatile and non-

volatile compounds and the availability of these compounds to the sensory system as a function of time. The extent and rate of the release of flavor compounds and their subsequent transport to the receptors depend on the process of breaking down the food matrix through mastication. The progressive breakdown of the food matrix, the salivary volume and the swallowing process are important factors in the release of both nonvolatile and volatile compounds, but differences between the two compound categories can be observed.

In general, the quantity of nonvolatile compounds in saliva increases at the beginning of the chewing process, reaches a maximum, and then decreases more or less rapidly until the end of mastication. For example, the salivary concentration of nonvolatile compounds that were released from a piece of chewing gum peaked within the first minute of mastication [14.16]. The kinetics of the release of nonvolatile compounds from a model cheese were related to various chewing parameters, such as the rate, duration, and efficiency of mastication, which were closely linked to each other and to the salivary flow rate [14.17–19]. The release of sodium from the salt in model cheeses was highly affected by textural and compositional factors, and the oral physiology of individuals. For example, rapid sodium release was linked to increased bite force and slower sodium release was linked to a prolonged chewing sequence, whereas a faster perception of saltiness was related to the masticatory performance and bite force. As the area of surface contact increases during chewing, more taste papillae are stimulated and the perceived intensity increases. The compositional factors of the model cheese matrices, particularly the fat and water contents, had strong impacts on both the release of sodium in the mouth and the perception of saltiness. Increasing the fat content and reducing the water content were both found to be related to a global decrease in the release of sodium and an increase in the perceived saltiness. This result suggests that the water content affects the initial release of sodium, whereas the effect of the fat content is more pronounced in the perception of saltiness during the chewing process. However, the effect of each compositional parameter on the release of sodium differs according to the duration of chewing, and globally some of the oral parameters that affect the release of sodium are different from the parameters that affect the perception of saltiness. Thus, the significant distinction between the time of sodium release and the time of the perception of saltiness can be explained by the differential effects of both the oral and matrix parameters.

Mastication also plays a key role in the temporal pattern of the release of volatile organic compounds (VOCs), whereas processes such as swallowing and the flow of nasal air determine their subsequent delivery to receptors located in the nose. The process of mastication involves the gas-phase transfer of VOCs from the mouth to the pharynx, where they are swept through the upper airways to the nose by the air that is expired from the lungs. As is the case for nonvolatile compounds, the release of VOCs in the mouth depends on both the food characteristics and the oral parameters. For example, for cheese products, an increase in the masticatory rate increases the overall aroma release. The level of the effect of the masticatory rate differs for compounds depending on their varying mass-transfer coefficients. Most of the chewing parameters are positively correlated with a high concentration of volatiles in the nose, mainly due to the increase in the surface area due to the sample breakdown [14.20, 21]. Volatile compound release was also shown to depend on the interaction between the food matrix composition and the chewing behavior. The seal between the mouth and the pharynx opens intermittently during mastication according to the air movement in and out of the mouth [14.22]. During jaw closure, the volume of the mouth decreases, which may push some air out of the mouth into the pharynx [14.23]. However, oral behaviors vary highly according to the subject, leading to interindividual differences in the patterns of volatile compound release. For example, during the consumption of a sweet mint tablet, swallowing events were the main contributors to menthone release. The tongue and jaw movements did not induce menthone release in all of the subjects, indicating that the interindividual differences in terms of the quantity of menthone released arose from the percentage of degradation of the sweet mint tablet [14.24].

In the case of chewable products such as cheeses, it was shown that subjects differentially adapted their chewing behaviors in forming a swallowable bolus [14.25] and that this phenomenon was an important source of interindividual variability. Food texture affects both oral behavior and flavor release. An increase in firmness induced an increase in the chewing duration and the amount of saliva that was incorporated into the food bolus, which led to an increase in the total amount of released VOCs. The release rate of the volatile compounds differed according to their physiochemical properties. A higher fat content led to a larger amount of product remaining in the mouth after swallowing, resulting in the release of a smaller amount of volatile compounds and a prolonged persistence of aroma in the breath. The polarity of the volatile compound affects its release rate. A larger amount of a more polar compound, ethyl propanoate, was released during the masticatory step, whereas more nonan-2-one

was released during the post-swallowing step and it was more persistent in the mouth, due to its higher hydrophobicity [14.26].

The release and perception of flavor are also affected by the composition of the food and the quantity of saliva, which vary greatly among individuals. Saliva is a complex viscous aqueous medium containing minerals and a wide variety of organic molecules, including proteins with various properties, such as enzymatic conversion, binding, and transport. Saliva plays an important role in the in-mouth breakdown process, mainly due to its hydrating, lubricating and hydrolytic capacities. Moreover, saliva dilutes the food in the mouth, thus decreasing the amount of volatile compounds that are released to the air. Saliva actively contributes to the formation of a bolus that can be swallowed and to the release of active compounds, including taste and aroma compounds, during chewing and swallowing [14.2]. Thus, saliva plays a major role in flavor perception [14.27]. Volatile compounds can interact differentially with the components of saliva [14.28]. Three types of behavior were reported based on the physicochemical properties of the volatile components: The partition coefficient of a group of compounds is not affected by mucin; mucin decreases the partition coefficient of the second group, mainly through hydrophobic interactions between saliva proteins and VOCs; and mucin decreases the partition coefficient of the last group but that process is affected by the presence of salt and sugar. Different salivary enzymatic activities are suspected to have a nonnegligible effect on taste and aroma perception. Regarding volatile compounds, their flavor can be altered by the salivary enzymatic activities [14.29, 30]. For example, ester hydrolysis by salivary extracts was found to alter flavor in model systems. The addition of human saliva to white wine led to significant changes in the volatile compound profiles. In particular, the levels of esters and fused alcohols were reduced by 32% and 80%, respectively, whereas in contrast, the levels of 2-phenyl ethanol and furfural were increased by 27% and 155%, respectively [14.31]. Regarding nonvolatile compounds, the in-mouth amylase activity, which hydrolyses bonds within amylose and amylopectin, can quickly lower the viscosity of starch in starch-thickened foods [14.32]. This enzymatic activity can reduce the perception of saltiness in starch-thickened foods as the structure of the product is changed during the enzymatic process. Thus, a direct relation was found between the level of α-amylase activity in saliva and the perception of saltiness in starchy matrices [14.33]. As another example, human salivary lipase is suspected to affect the perception of fat but this conclusion is subject to controversy. However, recent studies showed that human salivary lipase plays a significant role in the perception of fat [14.34, 35].

14.2 Simulation of Oral Processing

As described previously, human olfaction is a complex process that involves a wide array of factors that interact to affect the final perception of flavor. The main factors include mastication and the mixing of the bolus by the teeth and tongue, temperature and hydration, salivary enzymatic reactions, and airflow. Simulating in vivo oral functions using model mouth devices poses a challenge and in some cases, the actual behavior cannot be fully reproduced. However, the large deviations in the masticatory and swallowing patterns among individuals and the differences in the flow pattern and composition of saliva created the need to apply model mouths in investigations. The main advantage of these models is the ability to isolate a single parameter and study its effect on food breakdown and volatile compound release. This chapter describes the development of oral processing models in the field of volatile compound release, from the early simple devices to the recent ones. Moreover, the model-simulated oral functions and the research applications of the model mouth in food science are mentioned.

14.2.1 History of Model Mouth Designs

More than twenty years have passed since the development of the early models to simulate oral food processing and the release of VOCs. The models incorporated the following functions:

- Mastication and food breakdown
- Salivary mixing
- Airflow
- Body temperature
- VOC sampling.

The aim of each model is to determine the complexity of the model's features. The earliest models focused on sampling the VOCs that had been released into the headspace at certain intervals. A 21 flask containing a 50 ml sample and 10 ml of artificial saliva was used

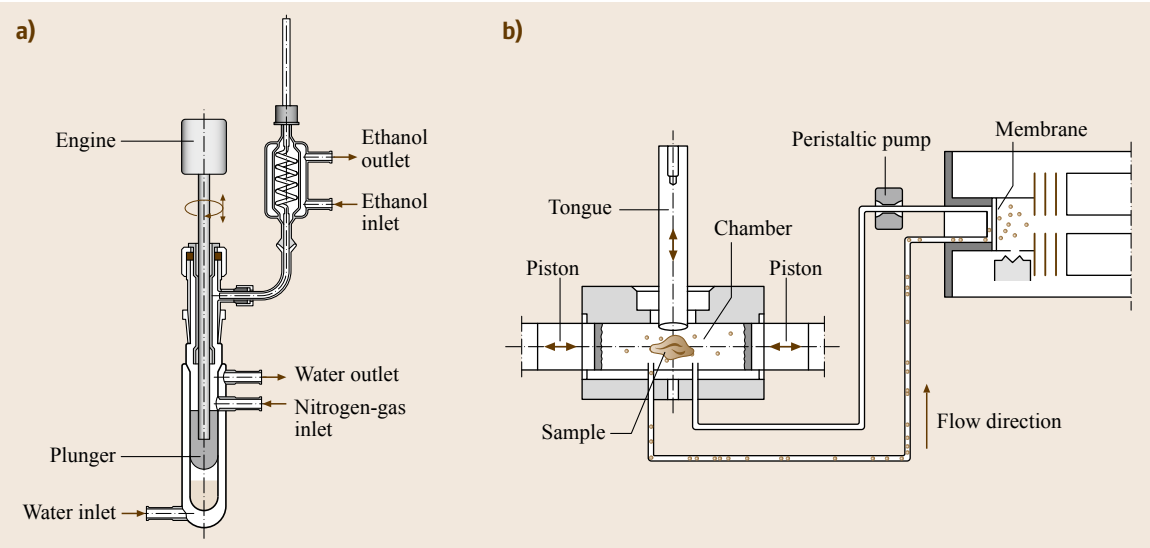

Fig. 14.1 Examples of model mouth designs to simulate oral food processing and VOC release **(a)** after [14.36], **(b)** after [14.37]

to study the release of VOCs from alcoholic beverages (old malt whiskey) [14.38]. Oral mastication in this model was mimicked by shaking a flask containing glass beads. The headspace was sampled after equilibrium was reached, at 45 min. Another device was developed [14.39] and was later used in the investigation of flavor release from dressings [14.40]; this device consisted of a temperature-controlled sample flask (70 ml) and an oscillating plunger moving vertically (4 cycles/min) to evaluate the effect of mastication on the release of VOCs from food samples (Fig. 14.1a). The samples were mixed with artificial saliva that was prepared using the major minerals and proteins that exist in human saliva. Certain VOCs were inserted into solid food (rehydrated diced bell peppers) or liquid food matrices (cream dressings or sunflower oil). The VOCs released from the food samples were trapped in a Tenax trap with a nitrogen gas flush and were later analyzed using gas chromatography with flame-ionization detection (GC-FID).

The first model mouth used to perform dynamic headspace analysis of VOC release was the so-called *retronasal aroma simulator* (RAS) [14.41]. Its design was based on a modified blender; nitrogen was purged over the sample in this blender while the temperature was held at 37 °C. The headspace was sampled at certain time points, which allowed the authors to plot the temporal release curves of several VOCs and to calculate the volatility rate constants ($k \times 10^{-5}\,\mathrm{min}^{-1}$). However, this model mouth lacked the proper dimensions and chewing geometry of the human mouth. Artificial saliva was incorporated into the device immediately before the sample was introduced, unlike in the more advanced apparatus [14.42] in which artificial saliva was pumped continuously into the glass reactor. Inside was a stirrer made of a six-star impeller, which mixed the liquid food. This model was the first to use a computer-controlled system to regulate the flow rates of the air and salivary inlets and the sample outlet. This system yielded VOC release data with a highly satisfactory level of reproducibility. The problem with this model was the nonproportionality of the conditions relative to those of a human mouth. For example, the stirrer speed of 450 rpm and the salivary flow rate of 175 ml/min deviated strongly from the real-life parameters. Later, the accuracy of the masticatory compressive and the shearing strengths was increased by using two horizontal pistons with wavy surfaces to simulate the irregularity of teeth shape and by applying one vertical piston to mimic the tongue's actions [14.37]. Together, the pistons chewed solid food (chewing gum) under the control and direction of the user via compressed air. Another unique feature of this model was the ability to measure the released VOCs online using a membrane-inlet mass spectrometer (MIMS) to simulate monitoring of their release during eating.

Despite the progress made in the development of model mouth systems, there were challenges to overcome to obtain a better resemblance to a real human mouth. The main problems with the models described above were the limited number of oral functions that could be represented simultaneously and the fact that the dimensions used were quite different from those of an actual oral environment.

Table 14.1 Comparison of functionalities between model mouth devices

Model mouth device	*Van Ruth* and *Roozen* [14.36]	*Roberts* and *Acree* [14.41]	*Woda* et al. [14.43]	*Arvisenet* et al. [14.44]	*Salles* et al. [14.45]	*Benjamin* et al. [14.46]	*Ishihara* et al. [14.47]
Airflow	Nitrogen 20 ml/min	Air or nitrogen 1200 ml/min	–	Helium not indicated	Air 30–50 ml/min	Air 1000 ml/min	–
Salivation	Constant volume	Constant volume	Small spurts	Constant volume	Constant flow rate	Constant flow rate	Constant flow rate
Mastication	Plunger screw (Compression and shearing)	Blender (Shearing)	Disks	Plunger (Compression and shearing)	Upper and lower jaws (Compression and shearing)	–	Flat plunger (Compression and shearing)
Heat	Water jacket	Water coils	Internal heater	Water jacket	Water jacket (initially) Thermofilm (now)	Water jacket	External regulation
Teeth	–	–	Shaped surfaces	Sharp shape	Human molar reproduction	–	–
Tongue	–	–	–	–	Hard cylinder (conically ended)	Soft ball	–
Measure of volatile compounds	Chemical traps	Chemical traps SPME	–	Chemical traps SPME	Connection with APCI or PTR-MS	Connection with PTR-MS	–
Measure of nonvolatile compounds	–	–	–	–	Possible by saliva sampling or introduction of sensors at different times	–	–
Food texture range	Soft–medium	All	Medium–hard	Medium–hard	All	Soft	All

14.2.2 Recent Developments in Model Mouth Devices

To address the previously mentioned limitations of the model mouth, more sophisticated and functional devices have recently been developed. This section describes several models that were designed to simulate the mastication of solid food through teeth and jaw movements and the mixing of liquid food through the pre-swallowing tongue pressure while monitoring the release of volatile compounds. A comparison of the functionalities of some model mouth devices is presented in Table 14.1.

The ability of the model to perform proper masticatory actions is well correlated with the exposure of the surface area of the bolus particles and their proper mixing with saliva. Both processes have a significant impact on VOC release [14.48]. The masticatory function of the model can be evaluated by comparing the sizes of the food particles generated with those generated by the human mouth using image analysis. The artificial mouth presented in [14.44] was developed to study the VOC release from apples crushed at different frequencies of compressive movements, speeds of rotational movement and periods of mastication. The apparatus was composed of a sample container (600 ml) and a notched plunger that rotated vertically and horizontally according to controlled motors (Fig. 14.2a). The apple samples were mixed with artificial saliva and the VOCs were extracted using solid-phase microextraction (SPME) fibers before analysis using gas chromatography (GC). The authors found that both the duration of mastication and the speed of rotational movement affected the intensity of the released aromas due to the different sizes of tissue obtained. Hence, any oxidative and enzymatic reactions that occurred accordingly affected VOC release. The same device was used to compare the aroma extract obtained from bread mastication to the aroma perceived in the human mouth [14.19]. It was found that saliva and water differentially affected the way the bread was commuted into small particles. Due to the presence of salivary proteins, saliva played a significant role in the release of VOCs according to their physicochemical properties.

A novel chewing simulator that integrated most of the main functions of the human mouth has recently been developed (Fig. 14.2b) [14.45]. This device includes a fixed upper jaw and a mobile lower jaw with teeth that reproduce the molar motif and can generate shearing forces of up to 250 N. The compressive and shearing movements are controlled in angular and vertical planes by a computer. Another unique feature of this model is that an inert material called polyetheretherketone (PEEK) was used to build the apparatus to avoid

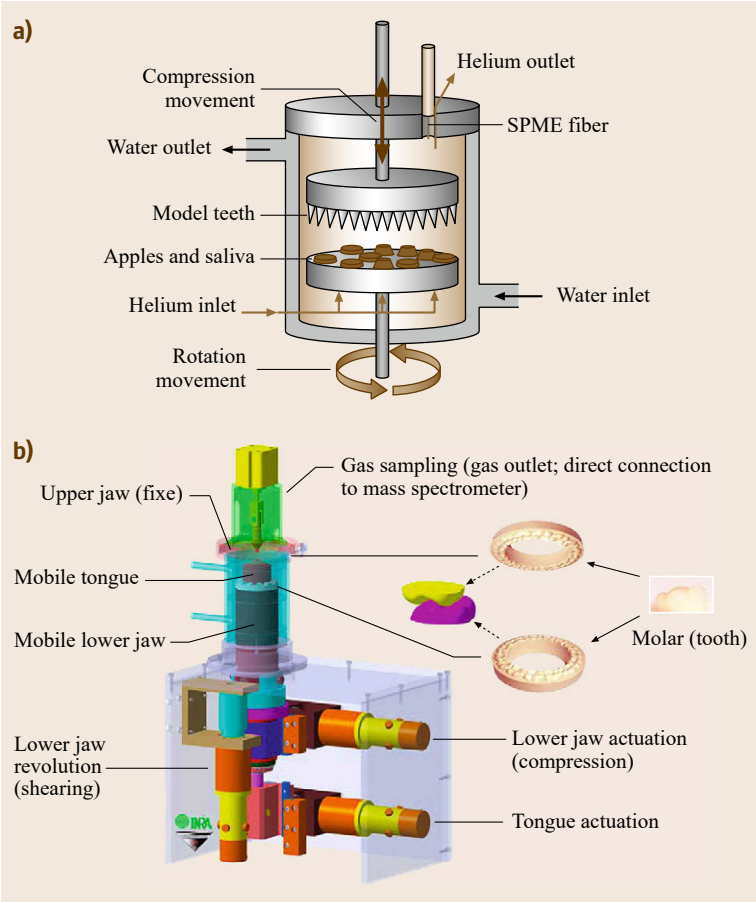

Fig. 14.2 Examples of recent model mouth designs to simulate solid food mastication and VOC release (**a**) after [14.44], (**b**) after [14.45]

the loss of VOCs through adsorption to the material. The operational volume resembles that of the oral cavity (100 ml), and artificial saliva is pumped into the chewing cell using a controlled flow system to mimic the salivary flow rate in the mouth under nonstimulated and stimulated conditions (0–5 ml/min). The masticatory capability of the simulator was tested using peanuts under oral environmental conditions. The results indicated that the course of natural food breakdown could be reproduced if the appropriate shearing forces and angles were applied as a function of the mechanical properties of the food sample. This chewing simulator was used to study the brittle behavior of cereal food products under masticatory conditions. The study reported that food fragmentation is followed by significant agglomeration after less than ten chewing cycles. Both phenomena were correlated with the magnitude of force applied and the evolution of the force. Moreover, through artificial mastication of the products, the chewing simulator was also able to discriminate products under dry masticatory conditions. The results showed a qualitative agreement with human mastication and textural properties of naturally chewed food [14.49].

The release of VOCs into the headspace is monitored in real time through a direct connection to an atmospheric pressure-ionization mass spectrometry device (API-MS) [14.50]. In particular, the temporal pattern of volatile compound release was found to change according to the oral parameters and the physico-chemical properties of the compounds.

Other devices were more dedicated to the study of food-bolus formation and the changes that occur during the chewing process. The artificial masticatory advanced machine (AM2) was developed to mimic the process of bolus formation in the mouth [14.43]. The main aim was to simulate the preparation of a food bolus in the model that had properties similar to those produced by natural mastication. The masticatory chamber is a cylindrical cavity. The two ends of the chamber are formed by the stationary *maxillary disk* and the moving *mandibular disk*. The mandibular disk can move back and forth along and rotate around the central axis of the cylinder. The authors obtained a good level of consis-

tency between the in vivo and in vitro results for the breakdown of peanuts and carrots using different chewing cycles, with good repeatability. The in vitro and in vivo boluses displayed the same median particle size distributions for each food. The in vitro and in vivo boluses obtained at different times during the chewing process were also similar when the in vitro mechanical parameters were adjusted [14.51].

The simulation of liquid and semisolid food processing in the mouth has not progressed much, nor has the simulation of the tongue's role in volatile compound release. A mouth simulator has been specifically developed for semisolid foods, for which the oral processing is dominated by the effects of tongue movements [14.52]. The system, which is equipped with an electric motor to rotate the sample using a mixing vane, as well as a video camera, laser, optical sensor and temperature probe, is able to measure changes in viscosity due to temperature, shear, dilution and structural breakdown and to mimic the mixing pattern of semisolids and saliva in the mouth. Changes due to mixing were analyzed using a reflectance sensor (online) and image processing (offline). This study showed that enzyme-induced structural breakdown has a dramatic effect on the viscosity of starch-based semisolid products in time scales that are relevant to those of in-mouth processing.

A simpler mouth model was developed to quantify the salt release from food structures, such as biopolymer gels, after they were compressed [14.53]. The model consists of a jacketed vessel fitted with an impeller and a conductivity probe. To measure the results of diffusion, the sample is caged in the liquid phase and subjected to low shear, whereas to measure the results of compression, the sample was subjected to cyclic compressions using a texture-analyzer probe. The authors observed that salt release was affected by both the type of gelling agent used and the temperature. In particular, the compression of the gel only affected salt release when fractures occurred, which was interpreted as being a consequence of the increased surface area. A mechanical simulator was developed to mimic the action of the human jaw in the presence or absence of artificial saliva for both soft and harder foods [14.47]. The simulator consists of a cylindrical chamber composed of acrylic resin and a flat plunger with a 50 mm diameter for simulating compression and shearing simultaneously. This device was used to prepare a model bolus from various gel samples and to subject them to dynamic viscoelasticity measurements to investigate the rheological properties regarding the gel composition and the level of added saliva. As an example of its application, a model bolus prepared from a binary gel (mixture of gellan gum and psyllium seed gum) using this device showed weak-gel rheological behavior and had greater structural homogeneity than that derived from gellan gum gel. Moreover, the dynamic viscoelasticity parameters of the binary gel were less dependent on the level of saliva. The authors also reported that the greater structural homogeneity of the model bolus formed from composite gels with various physical properties were related to their greater miscibility with saliva [14.54].

Another artificial mouth has been developed to study the in-mouth processing of soft foods [14.55]. The system is composed of a 150 ml closed double-jacketed vessel with a usable volume of 100 ml. The shear rate applied to the studied mixture can be modulated using a marine propeller driven by a viscosimeter. The temperature is controlled at 35 °C and the redox potential and sodium concentration of the saliva are continuously recorded during processing by the artificial mouth. For example, one application was studying the effect of the saliva/cheese ratio and the cheese composition on salt release using pooled raw human saliva previously collected from different people, and following the changes in the composition of the saliva using different types of sensors. Regarding salt release during cheese digestion in the artificial mouth, good correlations with the sodium concentration were observed using a single sodium sensor and an array system that combined chloride and sodium detectors. By comparing domestically prepared soft cheese samples treated with deionised water and pooled human saliva in the artificial mouth system, it was found that a larger amount of sodium was released from the water-treated samples, whereas in general, a smaller amount of sodium was released from the saliva-treated cheeses. This slower release was attributed to the partial absorption of sodium by the saliva during the initial stages of digestion.

Recently, an innovative and dynamic model mouth has been developed to investigate whether the intraoral pressures produced by the tongue affect the release of VOCs [14.46]. The tongue is known to play an important role in manipulating and transporting the bolus within the mouth and lubricating it with saliva while applying pressure against the hard palate [14.2]. This model mouth incorporates some of the main human oral features to allow a better understanding of the pattern of VOC release in the mouth.

The model includes several parts that function simultaneously. The volume of the main chamber replicates that of the oral and nasal cavities in which the VOCs are released from a food bolus. Within the chamber, an artificial tongue composed of glass and silicone rubber masticates the sample. Various materials for constructing the tongue were considered to find materials

with the viscoelastic properties of human muscle and inertness against the absorption of VOCs. Finally, glass material was chosen for the experiments on the release of volatile compounds, because it responds well to the pressures exerted by the elastic silicone rubber. The forces and pressures generated by the tongue are measured using two sensors: a compression load cell to assess force and a pressure transducer. The tongue movements are controlled by a computer-driven actuator and follow different mathematical movement patterns (sine wave, pulse and ramp). Figure 14.3 illustrates the pressure patterns generated by the artificial tongue made of glass and silicone rubber compared to the human tongue. The patterns for both materials follow similar curves with an average maximal pressure and duration of 20–30 kPa and 0.4–0.8 s, respectively. These values correspond to real values measured in participants while they swallowed liquids [14.56, 57]. The chamber has temperature-control circulation using a jacketed cylinder and the artificial saliva flows into the bottom and is mixed with the food. At the same time, a flow of air carries the VOCs from the headspace to the PTR-MS instrument for online detection. Thus, this model mouth can simulate a very rapid process, such as the consumption of liquid food. In the next sections, several applications of the model will be discussed.

The oral food processing is not complete without considering the swallowing phase and the intense VOC release that appears after. It was found that the majority of the VOCs are present in the thin layer coating the throat after swallowing and are carried into the nasal cavity by the exhaled air [14.58, 59]. To simulate the dynamic conditions of VOC release from liquid foods after swallowing, a model artificial throat was designed [14.60]. The system consists of vertical glass tubing that splits into the two following parts: the upper part into which the sample, artificial saliva and the cleaning solution are poured; and the bottom part from which the liquids are drained (Fig. 14.4). In the middle, there is a piece of Viton rubber that can be opened or closed using a clamp to mimic the swallowing behavior and the closure of the pharynx by the velum. A thin layer of liquid remains on the rubber surface and is responsible for the continuous release of volatile compounds. The VOCs are monitored online using an atmospheric pressure chemical ionization gas-phase analyzer (APCI-GPA). Testing the hypothesis that the release of the majority of VOCs is enhanced after swallowing yielded comparable results in the artificial throat and in humans. Compared with the results obtained using static headspace analysis or a model mouth, the relative amounts of VOCs released were much lower for the artificial throat due to the short measurement time, and closer to human values. However, the model system cannot fully simulate the events that occur in the human throat. One of the main differences between the two systems is the force that drives the swallowing, which is gravity in the artificial throat and pharyngeal peristalsis in the human throat. Therefore, the intensity of VOC release cannot be expected to be the same. In the future, the artificial throat model will undergo some modification, such as the addition of tidal airflow to simulate breathing patterns, control of the temperature and humidity of the air and the choice of material for the tube.

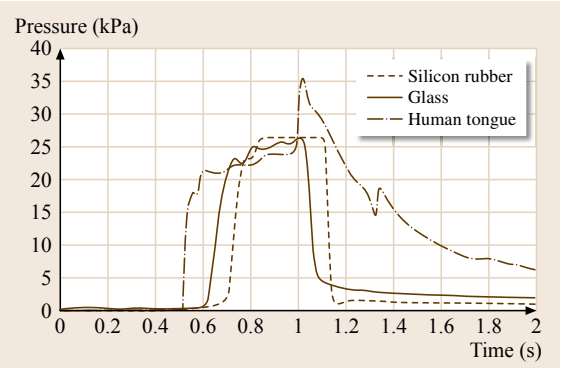

Fig. 14.3 Pressure pattern comparisons between the model mouth and human swallowing (after [14.46])

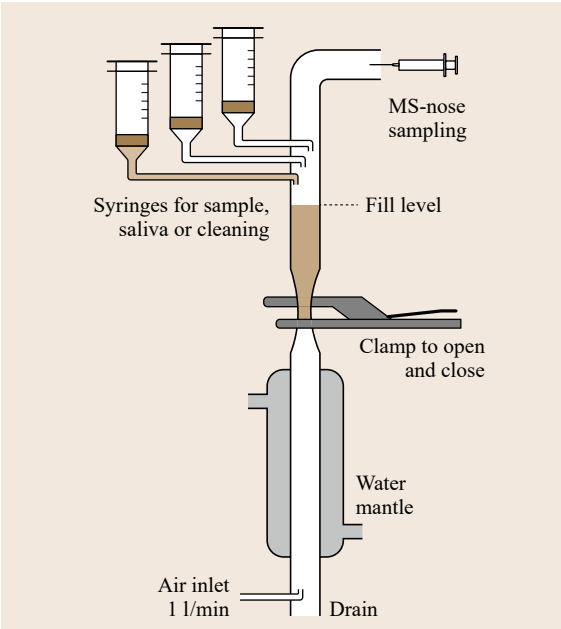

Fig. 14.4 Schematic overview of the artificial throat (after [14.60])

14.2.3 Simulation of the Oral Conditions and Oral Processing

In the section concerning the main oral functions, the high level of complexity within the mouth during oral food processing was explained. Understanding the role of each factor within the mouth and its effect on the release of flavor compounds is nearly impossible. However, a model mouth system has the clear advantage of allowing the analysis of a single parameter. The following oral functions and conditions are discussed in this section: mastication, mixing with saliva, temperature and airflow.

The mastication of solid and liquid food using the teeth and tongue has been investigated using several model mouths. One of the earlier models of *van Ruth* and *Buhr* [14.61] was applied to masticating rehydrated diced bell peppers using a plunger to make up and down screw-like movements. The intensity of the VOCs in the headspace was higher after mastication compared to that of nonmasticated material, which emphasizes the effect of the exposure of new surface areas and the efficacy of mixing food with saliva. The increased release of VOCs after mastication was also strongly perceived by assessors in terms of the odor intensity. The role of mastication for different types of foods was demonstrated using a more advanced mouth model [14.62]. The chewing efficiency of this device greatly resembled that of the panelists according to the size distribution of peanut particles that were produced at different force intensities and masticatory frequencies. The combined effect of similar mandibular, tongue and teeth shearing forces, shear angles and oral dimensions were found to be the key elements for a suitable model to simulate solid-food mastication. The structure and composition of the food affect the pattern of VOC release. This effect was clearly observed using the model mouth system. Using this device, significant differences in the release of VOCs from chewing gum compared to olives were reported.

The effect of mastication on liquid foods was studied using different mouth models [14.61, 63]. Similar to the case with solid foods, mastication generally enhances the release of volatile compounds from liquids. The turbulence from mixing and the changes in the extent of the interfacial surface area facilitate the diffusion of the VOCs into the headspace. The gas–liquid interface was increased two-fold by increasing the stirring rate from 100 to 400 rpm, which corresponded to a three-fold increase in the released VOCs [14.42]. The effects of tongue pressure and mastication on the release of VOCs in the mouth are very difficult to evaluate. However, a recently developed model mouth with an artificial tongue provides useful knowledge on the possible effects of the human tongue [14.46]. The authors applied a range of actual tongue intraoral pressures for various periods while monitoring the released VOCs online using a proton transfer reaction mass spectrometer. The findings validated the results regarding the enhanced VOC release after the mastication of solid and liquid foods. The tongue was found to create more turbulence in the liquid and more changes to the interfacial surface area when it remained in the liquid longer. The effect of the tongue on VOC release was observed as a clear peak after each masticatory cycle, following a different pattern according to the physicochemical properties of the VOC (Fig. 14.5). The location of the tongue in relation to the liquid and its direction of movement also affected the release of volatile compounds due to possible changes in the diffusion of VOCs from the liquid to the interface and then into the headspace (Fig. 14.6). The tongue is covered by a thin layer of liquid after mastication, which supports the release of more VOCs.

Finding that the composition and flow of saliva affected the release of volatiles in the mouth raised questions as to the extent of the impacts and the underlying mechanisms. Once again, the model mouth can be a suitable system in which to isolate the saliva parameter in food processing. Most of the model mouth systems use artificial saliva containing the major minerals (sodium, chloride, calcium, potassium and phosphate), proteins (mucin) and enzymes (e.g., amylase and lipase). The final composition should be close to that of human saliva and should have similar physical properties, such as the viscosity, pH and ionic strength.

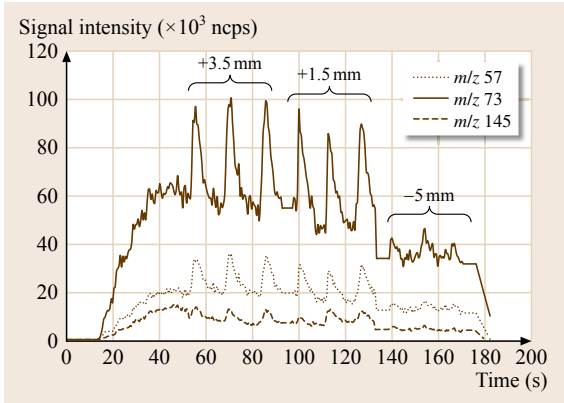

Fig. 14.5 Release curves for three volatile organic compounds (1-butanol (m/z 57), ethyl butyrate (m/z 89) and ethyl hexanoate (m/z 145)) masticated by the tongue in downward direction and different initial tongue positions from the aqueous solution surface (plus position relates to above the surface and minus position to below the surface) (after [14.64])

Among the effects of saliva on VOC release that are found in the model mouth are the dilution effect, interactions with salivary proteins and enzymes and to some extent, the possibility of *salting-out* [14.40, 65, 66]. For example, the effect of the amylase in saliva on VOC release is demonstrated by the higher odor intensity when the food contains starch. The degradation of starch by amylase leads to the release of volatile compounds that were trapped in the food complex, regardless of whether the amylase is human or porcine. VOCs are not equally affected by the presence of saliva; the effect depends on the polarity of the compound. Hydrophobic VOCs can be retained by interactions with proteins and hydrophilic VOCs can be retained by being diluted in a liquid system. An interesting finding was obtained when artificial saliva was compared to human saliva using the dynamic model mouth system with a flavored liquid sample [14.64]. These two types of saliva showed similar VOC release patterns, which supported the usage of artificial saliva as a proper alternative to human saliva. However, when saliva was replaced with water or artificial saliva lacking mucin, the extent of VOC release was higher. The authors attributed the increase in the extent of VOC release to the lower viscosities of the mixtures lacking the mucin that was present in the other types of saliva. The more viscous samples tended to adhere better to the tongue and the glass of the chamber, forming new surfaces that were exposed to the headspace. The salivary flow rate into the model chamber can be controlled at various rates to simulate a large variety of flows due to individual differences and the types of food. Consequently, the change in the flow rate corresponds mainly to the dilution effect of the sample.

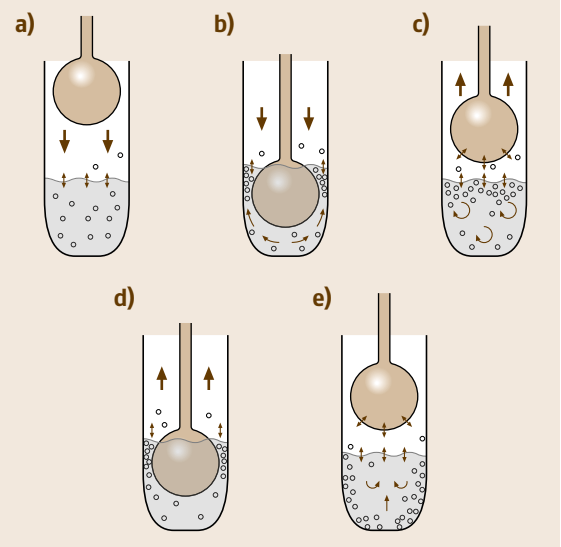

Fig. 14.6 Schematic illustrations of the artificial tongue masticating the sample at forward **(a–c)** and backward **(d–e)** movement directions (after [14.64])

The transfer of VOCs from the food to the oral and nasal cavities also depends on the respiratory rate of the lungs and the timing of the opening of the velum. To mimic the real situation of airflow, the model mouth design should consider the displacement of the volume of air in the headspace with a new supplement of air after each swallowing event, or in the case of solid food, with small volumes of air during the masticatory process. Most models used a continuous flow rate to simplify the experiment while ignoring the high level of complexity in oral physiology concerning the airflow. The model mouth designed by *Rabe* and collaborators [14.42] is most likely the one that most closely simulated the normal airflow patterns. In this model, the airflow is controlled by valves that introduce air at certain time points in correlation with the stirring and saliva-flow activities. Increasing the rate of airflow resulted in a higher flavor intensity due to the enrichment of the VOC content in the headspace. The effect of the airflow is highly dependent on the headspace volume. In the case of a large headspace volume, the enrichment process is slow when the air exchange occurs too rapidly relative to the rate of the mass transfer of VOCs.

The oral temperature is another important parameter that affects the release of VOCs. The rapid change in the temperature of a food sample once it was introduced into the mouth was measured in a model mouth system using a thermocouple sensor that was within the chamber [14.64]. In less than 70 and 100 s, oil- and water-based samples, respectively, reached body temperature (Fig. 14.7). The relationship between the temperature (T) and the partition coefficient (K) of VOCs can be described by the following equation

$$\frac{\mathrm{d}\ln K}{\mathrm{d}T} = \frac{\Delta H^\circ}{RT^2}\,, \tag{14.1}$$

where ΔH° (kJ/mol) is the enthalpy of vaporisation of the VOCs and R is the universal gas constant. The higher the temperature becomes, the more volatile compounds are released into the headspace due to the lower ΔH°. The change in the temperature of the sample that occurs during oral processing also affects the solubility of the VOCs. Large hydrophobic VOCs were found to be less soluble at cool temperatures than were hydrophilic compounds that interacted with water through hydrogen bonds [14.64]. Moreover, the viscosity of the medium is temperature-dependent, which is more pronounced in oily systems. The viscosity of oil decreases five-fold from 60 to 4 °C, which affects the molecular and eddy diffusion of the VOCs.

Another oral phenomenon that is difficult to mimic in in vitro devices is the mucosa covering the oral surface. The artificial devices reported to date are composed of metal, glass or chemical polymers that do not confer the same mechanical, biochemical and physicochemical properties as the human mucosa. The dissimilarities have many consequences at different levels. The structure of the oral mucosa in different regions of the mouth varies considerably and consequently, the water-absorptive capacity varies according to the mouth region. The thickness of the salivary film varies in different regions of the mouth, depending on the proximity of the minor and major salivary glands. The salivary pellicle is a film that coats the oral surfaces and functions as a moisture retainer, a protective barrier, a lubricant and a determinant for microbial colonization. The pellicle is a multilayered film that is initially formed by the selective adsorption of salivary molecules to oral surfaces, followed by homo- or heterotypic complexing of these molecules with other molecules in the ambient saliva. The salivary components that adsorb to the oral mucosal epithelial cells comprise the mucosal pellicle. The forces that mediate the interactions between the salivary molecules and the epithelial cell surface most likely include noncovalent interactions involving electrostatic and hydrophobic forces. The oral mucosal pellicle that is formed by the selective adsorption of saliva to the epithelial cell plasma membrane cannot be closely reproduced in the in vitro surfaces but has many effects on the partitioning of the volatile and nonvolatile stimulatory compounds within different phases of the residence within the oral cavity and on the food-breakdown mechanisms involved in oral processing, such as tribological factors, resistance to breakdown and the deformation of the bolus. Therefore, the artificial saliva formulation is important in properly reproducing the oral phenomena through in vitro processes.

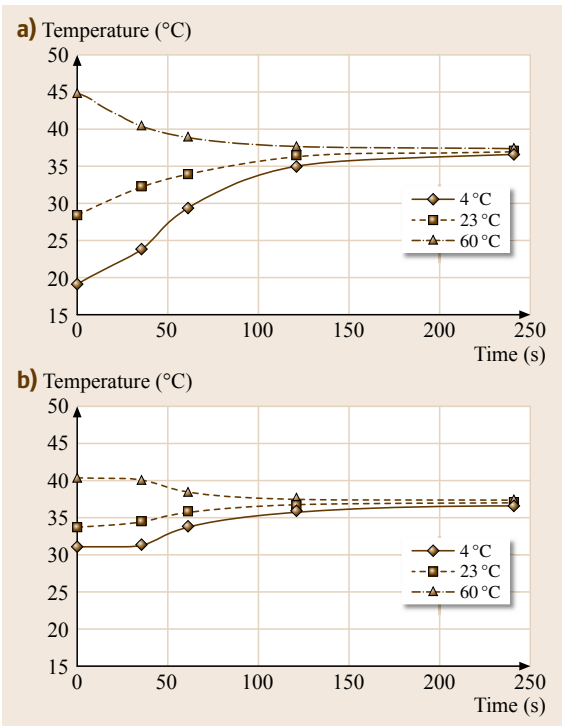

Fig. 14.7 Temperature curves in the model mouth of **(a)** aqueous solution and **(b)** oil samples versus mastication time period. The sample temperature was 4, 23 and 60 °C (after [14.64])

However, exactly reproducing human saliva is particularly difficult because of the high level of complexity of this biological fluid, its unstable character, its interindividual variability and the high cost of human salivary ingredients. The previous observations showed the importance of the physical properties of saliva in oral processes. The properties and composition of saliva are subject to significant subject variability, which is difficult to mimic using artificial solutions. Artificial saliva formulations that satisfy the viscosity requirement for the use in a masticator apparatus designed to prepare food boluses have been proposed [14.67]. The properties and composition of saliva also affect the in-mouth release of volatile compounds, which is another reason that a relevant formulation of artificial saliva is important. This statement is supported by various examples. The significant differences in the volatility of compounds that occurred when artificial saliva or water was added indicated that the saliva replacement was inadequate for studies of volatile compound release [14.68]. The salivary components differentially interact with the volatile compounds according to their physicochemical properties, leading to changes in their volatility [14.28]. The enzymatic activities of saliva can modify the composition of the released volatile compounds [14.29–31].

The human sensory system can be mimicked by electronic systems that are coupled to the mouth simulator for the detection and quantification of the released compounds. A connection to an API-MS or PTR-MS device allows the online recording of the real-time release of volatile compounds during the artificial chewing of food [14.61, 69]. However, the limitation of such detection systems is the level of sensitivity because the human olfactory system is much more sensitive and detects active odorants at concentrations at which no electronic system can detect them. There are very few reports concerning the in vitro detection of the release of nonvolatile taste compounds during oral processing. Simple sensor systems, such as pH, conductivity

and sodium probes, or more complex sensor systems, such as electronic tongues, can be implemented in artificial mouth devices. Electronic tongues [14.70], which are considered artificial gustatory sensors, consist of sensor arrays and pattern-recognition systems. These systems generally aim to discriminate and analyze food and beverages [14.71, 72], but they generally need a large volume of liquid sample, are limited in sensitivity and the obtained results are poorly correlated with the taste attributes described and rated by a sensory panel because they consist only of the simultaneous measurements of chemical components by sensor-array systems. More recently, cell-based sensors have been developed, which have some advantages, such as fast response, excellent selectivity, high sensitivity [14.48], and the ability to respond specifically to compounds of a given basic taste. The use of such sensors coupled with a mouth-simulator device appears to be a promising way to mimic taste perception.

14.2.4 Model Mouth Applications

Model mouth systems have a wide range of applications in the fields of food science, nutrition, pharmacology and medicine. The following are a few examples of their applications in completed studies and future possibilities for their use:

1. Characterizing the release of VOCs from food samples according to composition. For example, the strong retention of hydrophobic VOCs in emulsions and oil-based systems was easily discerned using the model mouth [14.64].
2. Differentiating between food products as a function of the pattern of flavor release in the mouth. The release of aromas from red and white wines was compared under oral conditions using artificial saliva and a model mouth system [14.31].
3. Performing release-pattern assessments of different VOCs from food matrices during oral processing using a model mouth system.
4. Investigating oral food-processing behavior of food systems such as emulsions. Its ability to apply several oral parameters simultaneously while monitoring the release of flavor makes the model mouth a powerful research tool. For example, the model mouth can be used to gain more knowledge on the food-structure/flavor-release relationship [14.73]. This relationship was examined in two types of emulsion systems: primary and multilayered emulsions. The stability of the primary emulsion was more affected by changes in pH and saliva composition (e.g., the content of mucin) than was that of the multilayered emulsion, which also exhibited enhanced VOC release (Fig. 14.8). A multilayered emulsion consisting of two layers of pectin and β-lactoglobulin tended to better resist oil-droplet flocculation during consumption.
5. Comparing flavor release in in vitro experiments using the model mouth and in in vivo trials using participants. The results of such research can be used to evaluate the accuracy of the model mouth and to improve the elements that differ significantly [14.74].
6. The model mouth can be utilised in applications other than flavor release, such as taste analysis, salt-diffusion measurements and food-texture optimisation.
7. The model mouth can be used as a predictive tool. The oral parameters can be decoupled because each parameter is individually controlled. The model mouth could also be useful in testing mathematical models based on in vivo-acquired data.

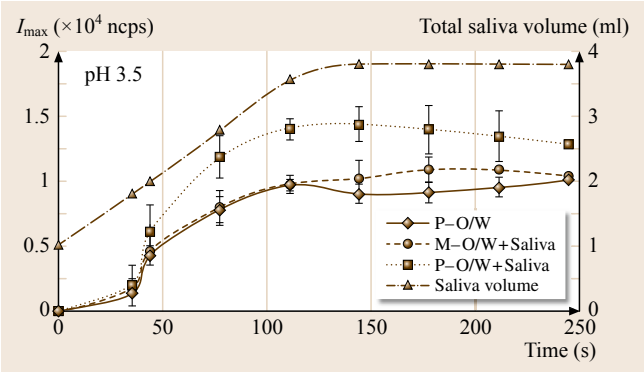

Fig. 14.8 The maximum signal intensity after mastication (I_{max}) of ethyl hexanoate from multilayer oil-in-water (M-O/W) emulsions at pH 3.5 as a function of saliva addition (after [14.73])

14.3 Conclusions

This chapter described an important tool for investigations of the oral processing of food. Using a model mouth system provides the researcher with control over different parameters that affect this complex process while investigating their impact on olfactory perception. The chapter includes a short background on the main oral functions and their influence on the release of VOCs. The history of model mouth devices and the

recent improvements are considered in the chapter, together with details on certain oral functions that can be simulated by these models. Lastly, the applications of the model mouth in the field of food science are mentioned. Without doubt, research will be conducted in the future using such systems to improve the fundamental understanding of olfactory perception, the development of food products and quality assurance.

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15. Regulatory Oversight and Safety Assessment of Flavorings

Karl-Heinz Engel

This chapter serves as an example of governmental regulatory oversight and safety assessment of flavorings. In the European Union (EU), the regulatory framework for the use of flavorings in and on foods is provided by Regulation (EC) No 1334/2008. The Regulation provides for three basic regulatory tools: (i) a Union list of flavorings and source materials approved for use in and on foods, (ii) conditions of use of flavorings and food ingredients with flavoring properties in and on foods, and (iii) rules on the labeling of flavorings. The safety evaluation of flavoring substances has been performed using a group-based approach. The procedure is based on a decision tree that considers information on structure–activity relationships, metabolism, intake, and toxicity.

15.1 Regulatory Framework

In the European Union (EU), the regulatory framework for the use of flavorings in and on foods is provided by Regulation (EC) No 1334/2008 of the European Parliament and of the Council of December 16th, 2008 [15.1]. This Regulation repealed Council Directive 88/388/EEC of 22 June 1988 on the approximation of the laws of the Member States relating to flavorings for use in foodstuffs and to source materials for their production [15.2] and Commission Directive 91/71/EEC of 16 January 1991 laying down rules for labeling of flavorings [15.3]. The Regulation applies to flavorings, food ingredients with flavoring properties, to food-containing flavorings and/or food ingredients with flavoring properties, and to source materials for flavorings and/or source materials for food ingredients with flavoring properties.

A flavoring or any food in which such a flavoring and/or food ingredients with flavoring properties are present, must not be placed on the market if their use does not comply with this Regulation. Flavorings or food ingredients with flavoring properties to be used in or on food must meet the following general conditions: (i) They do not, on the basis of the available scientific evidence, pose a safety risk to the health of the consumer, and (ii) their use does not mislead the consumer.

The Regulation provides for three basic regulatory tools:

- A Union list of flavorings and source materials approved for use in and on foods.
- Conditions of use of flavorings and food ingredients with flavoring properties in and on foods.
- Rules on the labeling of flavorings.

15.1.1 Categories of Flavorings

Flavorings are products not intended to be consumed as such, which are added to food in order to impart or

modify odor and/or taste; they comprise the following categories:

- Flavoring substances: defined chemical substances with flavoring properties
- Flavoring preparations: products, other than flavoring substances, obtained from food by appropriate physical, enzymatic or microbiological processes either in the raw state of the material or after processing for human consumption by one or more of the traditional food preparation processes listed in Annex II of Regulation (EC) No 1334/2008 [15.1] and/or obtained from material of vegetable, animal, or microbiological origin, other than food, by appropriate physical, enzymatic, or microbiological processes, the material being taken as such or prepared by one or more of the traditional food preparation processes listed in Annex II of Regulation (EC) No 1334/2008 [15.1].
- Thermal process flavorings: products obtained after heat treatment from a mixture of ingredients not necessarily having flavoring properties themselves, of which at least one contains nitrogen (amino) and another is a reducing sugar; the ingredients for the production of thermal process flavorings may be food and/or source material other than food.
- Smoke flavorings: products obtained by fractionation and purification of a condensed smoke yielding primary smoke condensates, primary tar fractions, and/or derived smoke flavorings as defined in Regulation (EC) No 2056/2003 [15.4].
- Flavor precursors: products, not necessarily having flavor properties themselves, intentionally added to food for the sole purpose of producing flavor by breaking down or reacting with other components during food processing; they may be obtained from food and/or source material other than food.
- Other flavorings: flavorings that do not fall under one of the abovementioned definitions; an example is the so-called *rum ether*.

15.1.2 Establishment of a Union list

One of the basic differences between Regulation (EC) No 1334/2008 [15.1] and the former Directive Council Directive 88/388/EEC [15.2] is the fact that flavoring substances may only be placed on the market and used in or on foods if they are included in the so-called Union list; a list of flavoring substances that are authorized to the exclusion of all others. The procedure to establish such a list had been laid down in Regulation (EC) No 2232/96 of the European Parliament and of the Council of October 28, 1996 [15.5]. Member States were requested to notify to the Commission a list of flavoring substances which at that time were legally accepted on their territory. The resulting register of about 2800 substances notified by the Member States was adopted by Commission Decision (1999/217/EC) of February 23, 1999 [15.6]. The measures for the evaluation program were laid down by Commission Regulation (EC) No 1565/2000 of 18 July 2000 [15.7]. In the light of the large number of substances in the register, it was decided to make use of already existing safety assessments. Flavoring substances that had been considered as being safe at the current levels of intake either by the Scientific Committee on Food of the European Commission (SCF), the Experts on Flavoring Substances of the Council of Europe (CEFS) or by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) before 2000 did not need to be re-evaluated. Flavoring substances classified after 2000 by JECFA as presenting no safety concern at the current level of intake had to be considered by the SCF (subsequently replaced by the European Food Safety Authority (EFSA)); on the basis of these considerations it was decided whether the outcome of the performed safety evaluations was acceptable or whether further evaluation is necessary. The remaining flavoring substances had to be evaluated by the SCF or the EFSA; the principles underlying the employed safety assessment procedure are outlined in detail in Sect. 15.2. The Union list of flavoring substances has finally been adopted by Commission Implementing Regulation (EU) No 872/2012 of October 1, 2012 [15.8] and introduced in Annex I of Regulation (EC) no 1334/2008 [15.1].

15.1.3 Specific Conditions of Use for Flavorings and Food Ingredients with Flavoring Properties

In addition to a *positive list* containing flavoring substances authorized to the exclusion of all others, the Regulation 1334/2008 [15.1] also provides for *negative lists*. These contain flavoring substances or flavoring source materials which shall not be used as such or set specific conditions for the use of flavorings and food ingredients with flavoring properties.

The CEFS has evaluated flavoring source materials since 1970 and identified a number of substances occurring naturally in flavorings and food ingredients with flavoring properties that may raise safety concerns [15.9]. Since 1999, the SCF and later the EFSA have expressed opinions on these substances. Substances for which the toxicological concern raised by the CEFS was confirmed are regarded as undesirable substances. These substances shall not be added as such

Table 15.1 Substances that shall not be added as such to food (after [15.1])

Agaric acid	Menthofuran
Aloin	4-Allyl-1,2-dimethoxybenzene (methyleugenol)
Capsaicin	Pulegone
1,2-Benzopyrone (coumarin)	Quassin
Hypericine	1-Allyl-3,4-methylene dioxy benzene (safrole)
β-Asarone	Teucrin A
1-Allyl-4-methoxybenzene (estragol)	Thujone (alpha and beta)
Hydrocyanic acid	

Table 15.2 Examples of maximum levels of undesirable substances in certain foods

Name of the substance	Compound food in which the presence of the substance is restricted	Maximum level (mg/kg)
Estragol[a]	Dairy products	50
	Processed fruits, vegetables, nuts, seeds	50
	Fish products	50
	Nonalcoholic beverages	10
Methyleugenol[a]	Dairy products	20
	Meat preparations and meat products	15
	Fish preparations and fish products	10
	Soups and sauces	60
	Ready-to-eat savories	20
	Nonalcoholic beverages	1
Pulegone	Mint/peppermint-containing confectionery	250
	Micro breath freshening confectionery	2500
	Chewing gum	350
	Beverages:	
	Mint/peppermint-containing nonalcoholic	20
	Mint/peppermint-containing alcoholic	100
Coumarin	Traditional and/or seasonal bakery ware containing a reference to cinnamon in the labeling	50
	Breakfast cereals including muesli	20
	Fine bakery ware, with the exception of traditional and/or seasonal bakery ware containing a reference to cinnamon in the labeling	15
	Desserts	5

[a] The maximum levels shall not apply where a compound food contains no added flavorings and the only food ingredients with flavoring properties which have been added are fresh, dried, or frozen herbs and spices. After consultation with the Member States and the Authority, based on data made available by the Member States and on the newest scientific information, and taking into account the use of herbs and spices and natural flavoring preparations, the Commission, if appropriate, proposes amendments to this derogation.

to food and are listed in Annex III, Part A of Regulation 1334/2008 [15.1] (Table 15.1).

Undesirable substances may be present in flavoring preparations and food ingredients with flavoring properties, which are traditionally used as foods or food ingredients. Maximum levels have been established for the presence of these undesirable substances in those foods, which contribute most to the human intake of these substances. They are listed in Annex III, Part B of Regulation 1334/2008 [15.1]. Table 15.2 shows examples in terms of the compound foods in which the presence of the substance is restricted and the respective maximum levels.

Source materials that shall not be used for the production of flavorings and food ingredients with flavoring properties are listed in Annex IV, Part A of Regulation 1334/2008 [15.1]. At present, only one source material is listed: tetraploid form of *Acorus calamus* L.

For flavorings and food ingredients with flavoring properties produced from certain source materials, conditions of use have been established in Annex IV, Part B of Regulation 1334/2008 [15.1]. For example, flavorings and food ingredients with flavoring properties produced from St. John's wort (*Hypericum perforatum* L.) may only be used for the production of alcoholic beverages.

15.1.4 Rules on the Labeling of Flavorings

Regulation 1334/2008 [15.1] does not differentiate between *nature-identical* flavoring substances (i. e., those obtained by chemical synthesis or isolated by chemical processes and chemically identical to a substance naturally present in material of vegetable or animal origin) and *artificial* flavoring substances (those obtained by chemical synthesis but not chemically identical to a substance naturally present in material of vegetable or animal origin) as previously defined in Council Directive 88/388/EEC [15.2].

There are, however, specific requirements for the use of the term *natural*:

- *Natural flavoring substance* means a flavoring substance obtained by appropriate physical, enzymatic, or microbiological processes from material of vegetable, animal, or microbiological origin either in the raw state or after processing for human consumption by one or more of the traditional food preparation processes listed in Annex II of the Regulation (Table 15.3). Natural flavoring substances correspond to substances that are naturally present and have been identified in nature.
- The term *natural* for the description of a flavoring may only be used if the flavoring component comprises only flavoring preparations and/or natural flavoring substances.
- The term *natural flavoring substance(s)* may only be used for flavorings in which the flavoring component contains exclusively natural flavoring substances.
- The term *natural* may only be used in combination with a reference to a food, food category, or a vegetable or animal flavoring source if the flavoring component has been obtained exclusively or by at least 95% by w/w from the source material referred to.
- The term *natural* food(s) or food category or source(s) *flavoring with other natural flavorings* may only be used if the flavoring component is partially derived from the source material referred to, the flavor of which can be easily recognized.
- The term *natural flavoring* may only be used if the flavoring component is derived from different source materials and where a reference to the source materials would not reflect their flavor or taste.

Table 15.3 List of traditional food preparation processes (after [15.1])

Baking	Grinding
Chopping	Heating
Coating	Infusion
Cooking	Maceration
Cooling	Microbiological processes
Cutting	Mixing
Distillation/Rectification	Peeling
Drying	Percolation
Emulsification	Pressing
Evaporation	Pressure cooking[c]
Extraction[a]	Refrigeration/Freezing
Fermentation	Roasting/Grilling
Filtration	Squeezing
Frying[b]	Steeping

[a] Including solvent extraction in accordance with Directive 88/344/EEC
[b] Up to 240 °C at atmospheric pressure
[c] Up to 120 °C

15.2 Safety Assessment Procedures

15.2.1 Evaluation of Flavoring Substances Included in the Union List

The measures for the adoption of an evaluation program were laid down in Commission Regulation No 1565/2000 [15.7]. In order to make the evaluation process as efficient as possible, a group-based approach should be followed. The flavoring substances contained in the register were divided into 34 structurally related chemical groups; substances within a group are considered to have some common metabolic and biological behaviors. The following information had to be provided:

- Purity and chemical specification
- Natural occurrence in foods
- Total amount of the substance that is added to foods in the EU
- Normal and maximum use levels of the substance in defined food categories, if available
- All relevant toxicological and metabolic studies on the substance or closely related substances.

The principles of the evaluation were based on an opinion expressed by the SCF on December 2, 1999 [15.10]. The SCF considered a procedure developed by JECFA at the 44th meeting [15.11] and

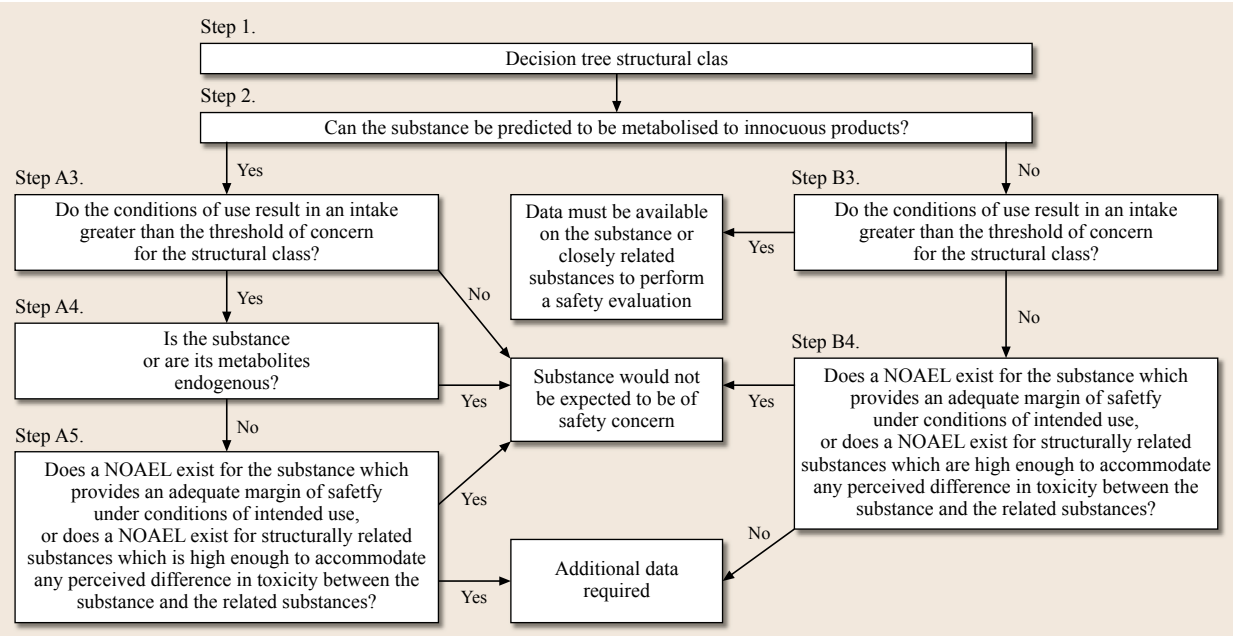

Fig. 15.1 Procedure for safety evaluation of chemically defined flavoring substances (NOAEL: no observed adverse effect level)

subsequently applied in an adjusted version to the evaluation of a large group of chemically defined flavoring substances [15.12–14] as the most updated and systematic approach. The procedure is based on a stepwise decision-tree approach that considers information on structure–activity relationships, metabolism, intake, and toxicity (Fig. 15.1).

The first step is the assignment of a flavoring substance to one of three classes (I, II, III) for which thresholds of concern (human exposure thresholds) have been specified. Class I contains flavoring substances that have simple chemical structures and efficient modes of metabolism, which would suggest a low order of oral toxicity. Class II contains substances with structural features that are less innocuous, but are not suggestive of toxicity. Class III includes flavoring substances with structural features that permit no strong initial presumption of safety, or may even suggest significant toxicity [15.15]. The thresholds of concern for these structural classes (1800, 540, and 90 $\mu g/(person\,day)$, respectively) are derived from a large dataset derived from subchronic and chronic animal studies. They were calculated using a 100-fold safety factor from the 5-th centile of either the no-observed-effect-levels (NOELs) from chronic studies or of one-third of the NOELs from subchronic studies [15.12, 16].

The next steps in the decision tree address the following questions:

- Can the flavoring substance be predicted to be metabolized to innocuous products?
- Do the conditions of use result in an intake greater than the threshold of toxicological concern for the structural class?
- Is the flavoring substance or are its metabolites endogenous?
- Does a no-observed-adverse-effect level (NOAEL) exist for the flavoring substance or structurally related substances, which provides an adequate margin of safety under the conditions of intended use?

The decision tree shown in Fig. 15.1 corresponds to the decision tree as applied by JECFA [15.12–14], except that the option to accept flavoring substances with the only argument that their estimated intake is lower than the threshold of concern of 1.5 $\mu g/(person\,day)$ was not adopted by the SCF [15.10]. In addition, the SCF also emphasized that flavoring substances should be examined for structural alerts of potential genotoxicity.

The intake assessment plays an important role in the application of the Procedure. As a default, the so-called *maximized survey-derived daily intake* (MSDI) approach, which is based on annual production volumes, was used [15.17]. However, when the EFSA Panel examined the information provided by the European Flavor Industry on the use levels of flavoring substances in various food categories, it became ob-

vious that the MSDI approach in a number of cases would grossly underestimate the intake by regular consumers of products flavored at the use levels reported by the Industry. Therefore, the intakes were also estimated using the *modified theoretical added maximum daily intake* (mTAMDI) approach, which is based on normal use levels reported by Industry and consumption data for certain food categories [15.17]. The mTAMDI value was not considered in the Procedure but was only used as tool to prioritize the flavoring substances according to the need for a refined intake screen and the request for more precise data.

The flavoring substances have been assessed in so-called flavoring group evaluations (FGE). The following types of conclusions can be found:

- Based on the default MSDI approach, the candidate substance, which was evaluated through the Procedure, would not give rise to safety concern at the estimated level of intake arising from the use as flavoring substance.
- Based on the mTAMDI approach, the estimated intake of a flavoring substance is above the threshold of concern for the respective structural class. In this case, more reliable exposure data are required. On the basis of such additional data, the flavoring substance should be re-evaluated using the Procedure; subsequently, additional toxicological data might become necessary.
- Additional toxicological data are required.
- Additional specification data, for example, regarding geometrical and/or optical isomers, are required.

Substances shown to be genotoxic in vitro and in vivo are toxicologically not acceptable and were deleted from the register; an example is pentane-2,4-dione [15.18, 19]. For substances shown to be genotoxic in vitro, further in vivo genotoxicity data are required.

The α,β-unsaturated aldehyde and ketone structures are considered as structural alerts for genotoxicity. The EFSA Panel noted that there were limited genotoxicity data on these flavoring substances but that positive genotoxicity studies were identified for some substances in the group. Flavoring Group Evaluation 19 (FGE.19) contains 360 flavorings substances from the EU register being α,β-unsaturated aldehydes or ketones and precursors, which could give rise to such carbonyl substances via hydrolysis and/or oxidation [15.20]. These substances were divided into structurally related subgroups, representative substances were selected, and the Flavoring Industry has to provide additional genotoxicity data. If on the basis of these data, a genotoxic potential can be ruled out, the substances are merged with structurally related substances in other FGEs and evaluated using the Procedure.

Guidance documents, such as a scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment [15.21] and genotoxicity test strategies for substances belonging to FGE.19 [15.22], and a scientific report regarding minimum criteria for the acceptance of in vivo alkaline Comet Assay reports [15.23] are available.

15.2.2 Evaluation of Newly Submitted Flavoring Substances

The established Union list is open-ended and capable of being amended in the light of scientific and technical developments. The European Commission has requested EFSA to elaborate a guidance document for the risk assessment of flavorings newly submitted after the adoption of the Union list [15.24]. The EFSA Panel considered that the data required for the risk assessment of new flavorings should build upon the experience gained in the course of the evaluation of flavoring substances included in the Union list. Therefore, a general principle of this guidance is that flavorings which can be assigned to one of the existing flavoring group evaluations (FGEs) on the basis of structural and metabolic similarities should be evaluated according to the scientific principles and to the group-based approach underlying the former evaluation program (Fig. 15.2).

In addition, the guidance provides a procedure for the evaluation of flavoring substances that cannot be assigned to one of the existing FGEs. The scheme outlined in Fig. 15.3 shows that the type of data required depends on the following:

- Whether there are experimental data available for the substance to demonstrate that the metabolites can be considered innocuous, and

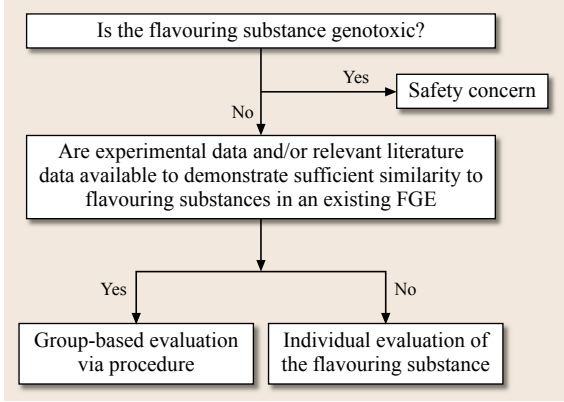

Fig. 15.2 Overall strategy for the risk assessment of flavoring substances

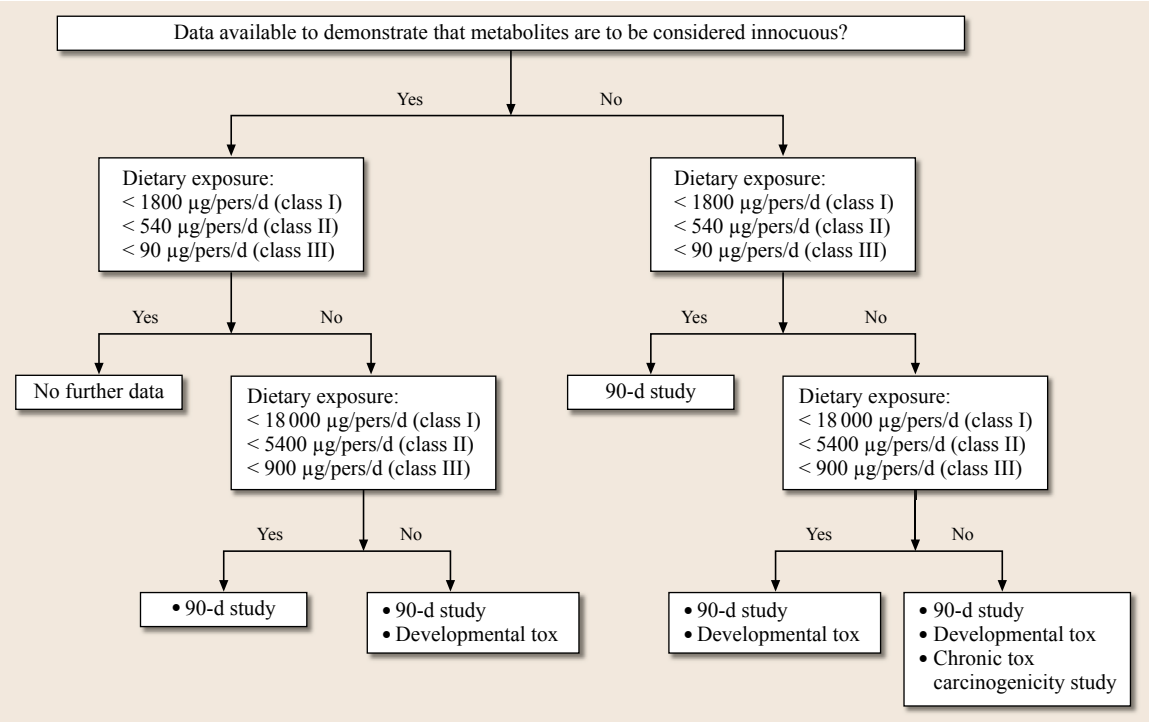

Fig. 15.3 Individual evaluation of the flavoring substance

- Whether the chronic dietary exposure, based on added use levels, is below or above the threshold of concern of the structural class to which the flavoring substance belongs.

The dietary exposure assessment plays an important role in the guidance document. The applicant needs to provide:

- Normal and maximum occurrence levels as added flavoring substance
- Normal and maximum occurrence levels of the substance from other sources, e.g., as natural constituent, as substance developed through the processing of foods, as carry-over originating from their use in animal feed or as residues of packagings
- Normal and maximum combined occurrence levels of the substance, taking into account all sources.

In addition, the applicant needs to indicate the non-food uses of the flavoring substance.

For the assessment of dietary exposure, a new approach called *added portions exposure technique* (APET) has been introduced [15.24]. The APET is calculated based on the occurrence levels provided by the applicant in a list of food categories and subcategories corresponding to the CODEX General Standard for Food Additives [15.25] used by JECFA to develop the SPET technique [15.26]. The APET is calculated by summing the highest potential dietary exposure within each of the two groups (*Beverages* and *Solid foods*). Such an estimate, based on daily consumption of one single standard portion of beverage and one single portion of solid food, is likely to provide a conservative assessment of long-term average dietary exposure for consumers of flavored products. The APET is expressed in mg/kg bw per day. For an adult, a body weight of 60 kg is considered and the portions are those established by the JECFA when developing the SPET technique (FAO/WHO, 2008). A case study on the use of the APET technique to estimate total dietary exposure to flavoring substances has been provided [15.27].

15.3 Flavorings Other than Flavoring Substances

In addition to flavoring substances, Article 9 of Regulation (EC) No 1334/2008 of the European Parliament specifies the following categories of flavorings for which an evaluation and approval are required:

- Flavoring preparations obtained from material of vegetable, animal, or microbiological origin, other than food.
- Thermal process flavorings for which ingredients for their production are source materials other than food and/or for which the conditions of their production and/or the maximum levels of undesirable substances set out in Annex V of Regulation 1334/2008 [15.1] are not met. These conditions are as follows:

  1. The temperature of the products during processing shall not exceed 180 °C.
  2. The duration of the thermal processing shall not exceed 15 min at 180 °C with correspondingly longer times at lower temperatures, i. e., a doubling of the heating time for each decrease of temperature by 10 °C, up to a maximum of 12 h.
  3. The pH during processing should not exceed the value of 8.0.

  The maximum levels for 2-amino-3,4,8-trimethylimidazo [4,5-f] quinoxaline and 2-amino-1-methyl-6-phenylimidazol [4,5-b] pyridine are 50 µg/kg.
- Flavor precursors obtained from source material other than food.
- Other flavorings.

For these categories of flavorings, the respective information that has to be supplied with an application for authorization is described in the EFSA guidance document [15.24].

15.3.1 Smoke Flavorings

For smoke flavorings as defined in Regulation (EC) No 2056/2003 [15.4], also a Union list has been established which contains authorized smoke-flavoring primary products for use as such in or on foods and/or for the production of derived smoke flavorings [15.28]. The specific requirements for the evaluation of smoke flavoring primary products have been compiled in an EFSA guidance document. They comprise data on the manufacturing process, the identity of the primary product, and toxicological data [15.29]. Quality criteria for validated analytical methods for sampling, identification, and characterization of primary smoke products have been laid down in Commission Regulation (EC) No 627/2006 of April 21, 2006 [15.30]. For assessment of the dietary exposure to smoke flavoring primary products specific methods have been developed [15.31]. The safety assessments of eleven smoke-flavoring primary products evaluated by EFSA have been summarized in an overview [15.32].

15.4 Outlook

The implementation of the Union list constitutes a basic change in paradigm regarding the use of flavoring substances in foods in the EU. On the one hand, a list of authorized substances will be helpful because it may increase consumers' confidence and may serve as a reliable platform for the involved stakeholders. On the other hand, it remains to be seen how far innovations and research will be hampered by this new economically relevant hurdle. It will also be interesting to follow which role the regulatory oversight implemented in the EU will play in the light of other internationally applied approaches and whether it will serve as model for regions in which the respective regulatory tools are being developed.

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16. Odors in Paper and Cardboard Packaging

Michael Czerny

Packaging materials based on paper are often used for packing foods, for example, flour, spices, rice, noodles and frozen products. Like all materials used for packing, cardboards and papers have to fulfill legal requirements in order to avoid the transfer of undesirable and harmful packaging constituents to foods. These legislations also cover the deterioration in the organoleptic properties of the packed food. Thus, methods and standards have been established in order to evaluate the sensory impact of packaging on foods.

This chapter reviews several human-sensory tests that are often applied for the evaluation of the organoleptic – in particular olfactory – quality of cardboards and papers. The standards enable a fast and reliable detection and characterization of packaging odors, but sources of odors are difficult to identify. Therefore, instrumental–analytical methods like gas chromatography in combination with mass spectrometry (GC/MS) and/or olfactometry (GC/O) are helpful tools for the identification of undesirable odorants. Based on the chemical structures, pathways and precursors, contamination sources can be detected and strategies for reduction or elimination of packaging odor can be developed.

16.1 Paper

16.1.1 History

The native papyrus plant was the first raw material used for manufacturing paper-like sheets by the Egyptians already in 4th century BC. Thin strips of pith from the plant stems were laid crosswise over each other and were then hammered and pressed together. The starch-containing sap that came out of the pith helped to strengthen the structure [16.1]. Paper akin to the paper we know today was first manufactured more than 2000 years ago. The Chinese court official and inventor of this paper was Ts'ai Lun who developed the manufacturing process in 105 AD. He used waste textiles, tree bark, and fishing nets and processed these into a thin, smooth sheet. Up to the middle of the 8th century, this valuable paper manufacturing process was kept secret. Then, a lost Chinese military campaign resulted in the secret passing to the Arab world. Subsequently, other Arab campaigns saw paper-making spread from the East to the West.

In the 13th century, the technology arrived in Europe. The first paper mill in Europe is recorded as being in Valencia (Spain) and the first mill in Germany was built in Nuremberg in 1390 [16.2, 3]. The invention of the paper machine in 1799 allowed the rapidly growing demand for paper to be met and the first paper manufacturers were established [16.1]. Paper is nowadays manufactured from plant fibers by a process of felt formation and gluing. By varying a wide range of parameters during the manufacture, more than 3000 different types of paper can be distinguished. The worldwide production of paper and cardboard amounted to 400 million metric tons in 2012 [16.4]; the regional distribution is displayed in Fig. 16.1.

16.1.2 Industrial Paper Manufacture

Paper manufacturinginvolves several steps and types of mechanical and chemical treatment of wood, the main raw material source. In the chemical process, wood chips are boiled in a solution of sulfur-containing acid or alkali [16.1]. The lignin almost completely dissolves and the fibers readily separate. The result is brown chemical pulp that is used, among other things, for making corrugated board. For paper and cardboard manufacture, the pulp must then be bleached (the second manufacturing step) in order to meet optical requirements (degree of whiteness).

In the mechanical process, mechanical pulp is recovered. The pulping process weakens the lignin and the fibers readily separate [16.1]. The mechanical pulp and chemical pulp are also called primary fibers. Secondary fibers can be recovered from waste paper. This pulp is suspended, purified, and milled. Depending on the type of paper, various amounts of raw materials (primary and secondary fibers) and additives are combined to give the paper improved quality and stability [16.5].

The porridge-like feed has a water content of 99% and is passed to the paper machine where the fiber suspension is drained through a moving screen and forms a layer. This removes a large amount of the water. The resulting paper web has low strength and a residual moisture content of about 80% and is transferred onto an absorbent mat. Press rolls remove water under pressure and the residual moisture is decreased to 60–65%. The paper web is dried in a labyrinth-type, steam-heated drying cylinder. The strength increases and the residual moisture content decreases to 5–8%. If no further finishing is required to improve the surface quality for printing, the paper is smoothed using steel rollers. Finally the paper is rolled up ready for forwarding to industry. Coating with compounds or polymers is applied if a high printing quality of papers is required [16.5].

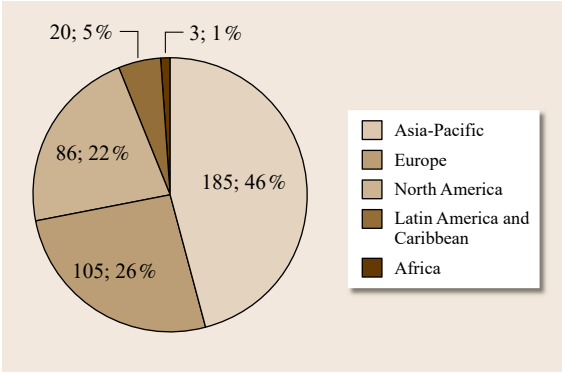

Fig. 16.1 Regional distribution of paper and cardboard production (in million metric tons and percent) in 2012 (after [16.4])

16.1.3 Classification of Paper and Cardboard

Paper is often used as a general term for paper, board and cardboard. Paper and board can be distinguished by their mass per unit area. According to DIN 6730 [16.6], paper has a grammage of $7\,g/m^2$ up to $225\,g/m^2$. Above this value, the material is called board. The term cardboard is not officially defined. However, Ref. [16.7] states that cardboard has a mass per unit area between that of paper and board (Table 16.1). The manufacturing processes for these three materials are, however, essentially based on the same principle [16.5].

Cardboard is widely used as a packaging material. It has considerably greater weight than paper and this gives it greater stiffness and strength. These are key properties when selecting a suitable packaging material.

Cardboard has a multilayer structure but consists of the same materials as paper. The different raw materials (mechanical and chemical pulp) give rise to different types of cardboard designated by letter/number coding as described in DIN 19303 (Table 16.2). The codes have three letters/numbers. The first letter designates the surface coating, the second letter designates the pulp used and the digit at the end designates the color of the reverse side [16.8].

Table 16.1 Classification of paper, cardboard and board according to [16.7]

	Grammage (g/m^2)
Paper	8–150
Cardboard	150–450
Board	> 450

Table 16.2 Codes of cardboard categories (after [16.8])

	Code	Explanation
1. Coating	A	Cast-coated
	G	Pigment-coated
	U	Uncoated
2. Materials	Z	Chemical pulp treatment, bleached
	N	Chemical pulp treatment, unbleached
	C	Mechanical pulp treatment
	T	Cardboard with recycled paper
	D	Cardboard with recycled paper, grey middle, and bottom layer
3. Colour	1	White
	2	Cream-colored
	3	Brown

A typical example of cardboard used by the food industry is GC1 which is a pigment-coated cardboard made of mechanical pulp with a white reverse side. The sole difference between GC1 and GC2 cardboard is the application of color on the reverse side (Table 16.2).

16.2 Legal Basics: Food Legislation

Many different dry foods (e.g., flour, spices, sweets, rice, noodles, and sugar) as well as foods with high fat content (e.g., chocolate, frozen products) are packed in paper or cardboard. These packaging materials might contain compounds which can migrate into the packed food resulting in a contamination of foods with negative effects for the food itself and the human health. In order to protect both foods and consumers from negative impacts, the legislative authorities of several countries established statutory requirements for packaging, paper, and cardboard.

16.2.1 Legislation in Europe

In Europe, there is no legislation specifically covering the use of paper, board, and cardboard. Merely the general suitability of packaging for food is described in Regulation (EC) No 1935/2004 [16.9]. Article 1, Paragraph 2 of this regulation defines materials and objects as materials and objects (. . .) that are finished products, which:

- Are intended to be brought into contact with food or
- Are already in contact with food and were intended for that purpose or
- Can reasonably be expected to be brought into contact with food or transfer their constituents to food under normal or foreseeable conditions of use.

General requirements and interactions between the packaging and food are specified in Article 3, Paragraph 1. Accordingly, materials and objects must be manufactured in compliance with good manufacturing practice such that, under the normal or foreseeable conditions of use, they do not transfer their constituents to foods in quantities which could:

- Endanger human health or
- Bring about an unacceptable change in the composition of food or
- Bring about deterioration in the organoleptic characteristics thereof.

Beside this legislation, Regulation No 10/2011 [16.10], which is a specific measure of Regulation (EC) 1935/2004 [16.9], contains a positive list of monomers and additives which can be used in manufacturing of food packaging.

Even though food legislation in Europe is in the process of being harmonized, it is still necessary to have valid legislation at a national level. Taken Germany as an example for a member country of the EU, the German Food and Feed Code (Lebensmittel- und Futtermittelgesetzbuch, LFGB) covers all stages of food manufacturing processes and focuses on food safety [16.11]. Just like at a European level, German food legislation contains no specific regulations for cardboard packaging for foods. The German Food and Feed Code (Article 30, Paragraph 1) prohibits consumer products to be manufactured or treated in such a way that when used as intended or foreseen, they are harmful to health due to their material composition and in particular due to toxicologically active substances or due to contaminants. Article 31 contains regulations for the migration of substances into foods covering the health risk, the quantity of compound transfer, and the avoidance of negative impact on aroma, taste, and appearance of food resulting from packaging including paper and cardboard. Both Articles are adopted from Regulation (EC) No 1935/2004 (Articles 2 and 3) [16.9].

For materials that are not regulated by Regulations (EC) No 1935/2004 and No 10/2011 there are no statutory regulations in Germany but rather recommendations of the German Institute for Risk Assessment (Bundesinstitut für Risikobewertung, BfR). Recommendation XXXVI covers papers, cardboards, and boards and lists all raw materials for paper production, auxiliary materials and finishing materials that may be used for the manufacture which are not regulated by EU legislation [16.12].

16.2.2 Legislation in US

The Food and Drug Administration (FDA) is responsible for the approval and monitoring of foods in the USA. The FDA regulations on paper and cardboard are given in the Code of Federal Regulations, Title 21 (Food and Drugs) [16.13]. Only general requirements are given in part 174.5 of the title such as good manufacturing practice or the organoleptic inertness of a packaging material.

16.3 Analytical Methods

The legal basics described in Sect. 16.2 demand that paper-based packaging is manufactured such that emitting compounds do not negatively impact – besides health risks – the packed food. In order to evaluate the presence of sensorial-active compounds in paper and cardboards and their transfer to foods, several test methods have been established. In general, two approaches are applied for the detection and description of the organoleptic food characteristics:

i. Human-sensory tests with sensory panels consisting of usually trained assessors which evaluate packaging or packed foods by use of their senses (in particular smell and taste) and
ii. Instrumental–analytical tests that focus on the identification of packaging compounds by means of chromatographic and detection tools.

All reported compounds responsible for negative sensory sensations in paper and cardboard are volatile and – in case of a transfer – the odor of foods is affected. Tastants seem to play a limited or no role in deterioration because much higher amounts of tastants in comparison to odorants are necessary in order to influence the sensory food quality. Regarding hexanal – a typical autoxidation product of unsaturated carbon acids – and sucrose as examples, the thresholds (10 and 8200 µg/l water, respectively) differ by the factor 820 [16.14, 15]. Therefore, instrumental-analytical methods aim at the elucidation of the volatile fraction by gas chromatography/mass spectrometry (GC-MS).

The following sections describe, in particular, the sensory methods which have been established in the EU and United States. The procedures are very similar and easy to apply in most cases for testing the sensory quality of paper and cardboard as well as their potential for odor transfer to foods. Instrumental-analytical methods and isolation techniques aiming at the identification of odorants are reported subsequently.

16.3.1 Sensory Methods

According to DIN EN ISO 5492:2009, sensory analysis is defined as *the science involved with the assessment of the organoleptic attributes of a product by the senses* meaning that the sensory characteristics are evaluated by sight, smell, taste, touch, and sound [16.16]. Chemical and physical stimuli emitted from foods or materials are incorporated and detected by receptors in the relevant sensory organs, converted into stimulus patterns and transmitted to the central nervous system. Regarding smell as an example, volatile and odor-active compounds evaporate from paper or cardboard into the air which is inhaled by a human. The volatiles reach the olfactory receptors located in the nose and a signal pattern is sent to the brain, which is perceived as an odor (Chap. 27).

Sensory analysis allows the characterization of a product and – in consequence – the determination of its sensory quality. Standards with defined procedures for the characterization of the sensory quality of paper and cardboard by a sensory panel have been established. Requirements for test facilities, test execution and training and selection of assessors have been defined in additional standards in order to objectify the results of sensory tests. Table 16.3 lists several standards which have particular relevance to the sensory evaluation of paper and cardboard packaging.

DIN EN 1230

DIN EN 1230 is split into two parts. The first part (DIN EN 1230-1) concerns the perception of the intrinsic odor of paper and cardboard when these materials are

Table 16.3 Standards applied in sensory tests for the evaluation of paper and cardboards

Standard	Title
DIN EN 1230-1:2010	Paper and board intended to come into contact with foodstuffs – Sensory analysis – Part 1: Odour
DIN EN 1230-2:2010	Paper and board intended to come into contact with foodstuffs – Sensory analysis – Part 2: Off-flavor (taint)
ISO 13302:2003	Sensory analysis – Methods for assessing modifications to the flavors of foodstuffs due to packaging
ASTM E619-09	Standard practice for evaluating foreign odors in paper packaging
DIN EN ISO 8586:2012	Sensory analysis – General guidelines for the selection, training, and monitoring of selected assessors and expert sensory assessors
ISO 8589:2007	Sensory analysis – General guidance for the design of test rooms
DIN EN ISO 4120:2007	Sensory analysis – Methodology – Triangle test
DIN EN ISO 5495:2007	Sensory analysis – Methodology – Paired comparison test
DIN EN ISO 10399:2010	Sensory analysis – Methodology – Duo-trio test
DIN ISO 8587:2010	Sensory analysis – Methodology – Ranking
DIN EN ISO 13299:2010	Sensory analysis – Methodology – General guidance for establishing a sensory profile

used for food packaging [16.17]. The second part of this standard (DIN EN 1230-2) describes a procedure for testing the transfer of flavors from paper and cardboard to foods [16.18]. Both standards can only be applied to paper and cardboards packaging. Sensory panels trained according to standard DIN EN ISO 8586 [16.19] and test rooms according to ISO 8589 [16.20] are again required for the tests.

DIN EN 1230-1. This standard is applicable to all types of papers and cardboards including coated and/or printed materials which are intended to come into contact directly or indirectly with food. The test procedure describes the sampling of defined paper or cardboard size (6 dm^2). The samples are inserted into glass jars (volume: 500 ml), closed with lids and stored at a temperature of 23 °C (±2 °C) for 20–24 h in the dark. The odor of the samples and a blank sample (glass jar without sample) is finally evaluated by a sensory panel which consists of at least eight trained assessors. They have to open the lid of the jar, sniff the samples, and score the intensity of the overall odor on a five-point scale (Table 16.4).

The description of the odor using attributes can be indicated from score 2 onward. The arithmetic mean is calculated from the single evaluations and rounded to 0.5 score units.

DIN EN 1230-2. In contrast to DIN EN 1230-1, the transfer of paper and cardboard constituents which migrate via the gas phase into foods is tested by DIN EN 1230-2 [16.18]. All types of papers and cardboards including coated and/or printed materials, which are intended to come into contact with food are investigated.

The standard requires a food that has to be tested in addition to paper and cardboard. If feasible, the same kind of food intended for packaging with the test paper or cardboard should be used as test sample. If this is not possible, a suitable food model (test sample) which exhibits low odor intensity should be selected. Some recommendations for food models are specified in the standard, for example, butter or margarine (simulating meat and cheese), cookies (dry and nonfat foods), and water (milk products and other beverages).

The food (or test sample) and the packaging are inserted into a glass jar by avoiding any direct contact. Figure 16.2 displays an example of such a setup which separates food from packaging: saturated magnesium nitrate or sodium chloride solutions are added to the glass jar in order to adjust humidity to 53 or 75%, respectively. A Petri dish containing the test sample is placed above the solution. A grid is inserted above the dish and the packaging sample is placed on the grid.

In parallel, a blank sample is prepared using the setup but without any food or test sample. The glass jars are stored at 23 °C (±2 °C) for 44–48 h in darkness. Differences in both the test and the blank samples are evaluated by a sensory panel using a triangle test according to DIN EN ISO 4120 [16.21]. A triangle series consists of three test vessels, one vessel with sample A (e.g., test sample) and two vessels with sample B (blank sample) (Fig. 16.3).

The assessors have to taste the samples and to detect the sample which is differing in the overall sensory sensation from the other. Based on the number of assessors and correct answers, significant differences of both samples and consequently a transfer of packaging odor to foods can be determined.

ISO 13302

Standard ISO 13302 [16.22] covers in contrast to DIN EN ISO 4120-1 and 4120-2 besides paper and card-

Table 16.4 Five-point scale used for the evaluation of odor intensity of paper and cardboard according to DIN EN 1230-1 (after [16.17])

Score	Intensity
0	No perceptible odor
1	Just perceptible odor
2	Moderate odor
3	Moderate to strong odor
4	Strong odor

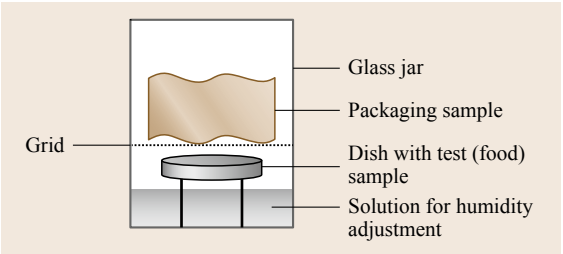

Fig. 16.2 Example of an experimental setup for testing odor transfer of paper and cardboard into foods according to DIN EN 1230-2 (after [16.18])

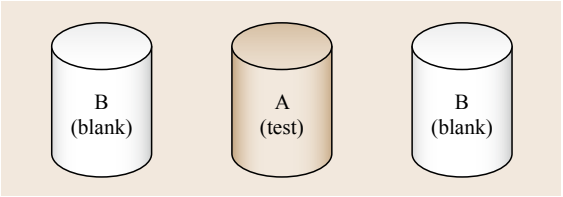

Fig. 16.3 Experimental setup of a triangle test according to DIN EN ISO 4120 (after [16.21])

board all materials for packaging foodstuffs including, for example, plastics, foils, kitchen utensils, etc. The procedures of the standards is however similar. Sensory panels trained according to standard DIN EN ISO 8586 are again required for the tests [16.19]. The actual food should be used for testing but storage (temperature, contact time, etc.) should comply with the conditions which are applicable for a food-packaging system. Although Annex C of the standard provides more information regarding food models and specifications on storage temperature, the storage conditions of the relevant foods packed in paper-based materials are identical with DIN EN ISO 4120-2.

The inherent odor of a packaging or the effect of odor transfer to a food can be evaluated based on the overall odor intensity using, for example, the scale listed in Table 16.4. In addition to a triangle test (DIN EN ISO 4120) [16.21], further sensory tests can be applied in order to determine modifications in the flavor of foods:

- Paired comparison test (DIN EN ISO 5495) [16.23]: the test compares one test and one blank sample by asking the assessors: *Which sample is tainted*?
- Duo trio test with constant reference (DIN EN ISO 10399) [16.24]: the test consists of a blank sample which is known to the assessors (reference) and additionally of another blank sample and the test sample (both not known to assessors). The assessors have to detect the sample deviating from the reference among both unknown samples.
- Ranking test (DIN ISO 8587) [16.25]: the test is applied if three or more samples are compared and the taint is known. Several samples – blank sample may be included – are ranked according to the intensity of the off-flavor.

Affected foods are then determined by application of statistical methods.

ASTM E619 – 09

The American standard ASTM E619 covers a wider range of paper products including [16.26]:

> *. . . all paper packaging products and . . . auxiliary components, such as coatings, inks, and adhesives, as well as plastic materials used in conjunction with paper.*

Several preparation methods (direct examination of packaging (with/without confinement), moistening methods, test of odor transfer) are described in detail and suggested for use in dependence on the material. A sensory panel with trained assessors is used and requirements for test rooms are specified. The odor intensity of the samples (paper, auxiliary components, test food used for transfer experiments) should be scored on five-point scales (Table 16.5).

The arithmetic mean of the individual scorings is calculated. After intensity scoring, the assessors should characterize the perceived off-odor.

DIN EN ISO 13299:2010

The sensory test methods described in Sect. 16.3.1, *DIN EN 1230–ASTM E619 – 09* are easy to perform and provide results within a short period (usually one day). However, more detailed information on taints is sometimes helpful regarding odor characteristics and their intensities, for example, by establishing a sensory profile according to DIN EN ISO 13299:2010 [16.27]. This approach might be used, for example, during development and optimization of (new) packaging materials.

The conventional profile is among the four different types of profiles, which is applied most frequently. Training of the panel assessors according to DIN EN ISO 8586 [16.19] is a prerequisite for performing the test. The odor attributes of packaging are identified by the panel in a first session. An already existing terminology or a packaging-specific terminology which is later on applied to the material is used for the evaluation of the characteristic descriptors. An appropriate intensity scale like an ordinal scale (series of numbers related to intensity) or an interval scale (unstructured lines) is selected in a second step. Finally, the intensities of each characteristic descriptor is evaluated on the selected scale. The arithmetic mean is calculated and the result can be displayed as a spiderweb diagram as displayed in Fig. 16.4.

16.3.2 Instrumental–Analytical Methods

Sensory methods enable a fast and reliable detection and characterization of packaging odors. However, sources of packaging odor are difficult to identify via sensory approaches because no information on the nature of the odorants responsible for a smell is determined. Knowledge on chemical structures of odorants would allow the identification of pathways, precursors

Table 16.5 Five-point scales used for the evaluation of odor intensity of paper and cardboard according to ASTM E619-09 (after [16.26])

Intensity	Score of scale	
	A	B
None	1	0
Very slight	2	0.5
Slight	3	1
Moderate	4	2
Strong	5	3

and contamination sources and finally the development of strategies for reduction or elimination of packaging odor. Several research groups aimed therefore at the elucidation of the composition of the volatile fraction in particular by gas chromatography in combination with MS.

Isolation of Volatile Compounds

A prerequisite for the analysis of volatile compounds by GC is their isolation from paper and cardboard packaging. In general, two isolation principles are applied: (i) extraction from the headspace and (ii) extraction by solvents.

Several of these methods have been established successfully within the last decades. The methods described subsequently are isolation techniques which have been applied to paper and cardboard.

Solid-Phase Micro Extraction. Solid phase micro extraction (SPME) is a headspace preparation technique [16.29]. Although a direct extraction from liquids (including nonvolatiles) is also possible, the headspace approach is applied most frequently. The basic principle of SPME is the ad- or absorption of headspace volatiles to polymers or sorbents coated onto fused silica. The fiber is connected to a steel needle and both, fiber and steel needle, are inserted into a septum-piercing needle. By connection to a needle holder, the fiber at the tip of the steel needle can be moved through the piercing needle and exposed to the gas phase above a sample that should be analyzed.

To achieve this aim the sample to be analyzed is placed into a vial, sealed with a cap with a septum and volatiles are allowed to evaporate until equilibrium. The piercing needle is subsequently penetrated through the septum of the cap, the fiber is exposed to the sample headspace and the volatiles are ad-/absorb to the coating. After extraction, the fiber is removed into the piercing needle and the piercing needle is also removed from the septum. Then, the needle is introduced, for example, into a heated split/splitless injector, the fiber is exposed, and the volatiles are thermo-desorbed and analyzed by GC.

SPME is a rapid isolation technique that requires no solvents. The fiber can be used up to 100 times depending on the ab-/adsorbent and the chosen desorption conditions. However, volatiles with lower volatility are discriminated when using this approach.

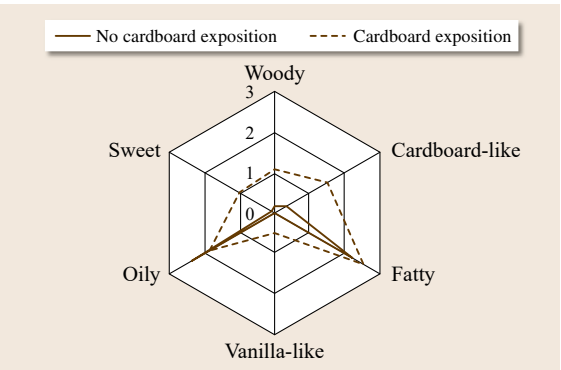

Fig. 16.4 Conventional profile of sunflower oil with and without cardboard exposition (test conditions according to DIN EN 1230-2 (after [16.21])) (figure adapted from [16.28])

Simultaneous Distillation–Extraction. Extracts containing the volatile fraction can also be obtained by extracting them from paper and cardboard with a solvent. Solvents with a low boiling point (e.g., *n*-pentane, diethyl ether, dichloromethane) are usually chosen due to the fact that the extract has to be concentrated for GC analysis by distilling off the solvent; accordingly, volatiles with a boiling point lower than the solvent are lost. However, nonvolatiles like fat are also extracted and a separation of the nonvolatiles that would cause problems during GC is required.

In 1964, *Likens* and *Nickerson* developed an isolation technique that allows the extraction exclusively of the volatile fraction [16.30]. This simultaneous distillation–extraction (SDE) technique uses a special glass tool. A sample is added to a flask which contains water and this flask is then connected to the device. After boiling, the mixture of water vapor and evaporated volatiles is led into a mixing chamber. In another flask, which is also connected to the glass tool, an organic solvent immiscible with water is evaporated by boiling and transferred into the mixing chamber. The vapors are condensed and the volatiles are extracted from the aqueous into the organic phase. Due to their differing density, water and organic solvent are returned in the course of the respective distillation processes to the corresponding flask. The organic extract is subsequently concentrated and used for GC analysis.

SDE allows a relatively fast isolation of volatiles but thermal treatment of the samples during boiling may adulterate the composition of the volatile fraction due to generation of compounds from precursors or degradation of volatiles by chemical reactions that are thermally enhanced.

Solvent-Assisted Flavor Evaporation. The solvent-assisted flavor evaporation (SAFE) technique is a method for the isolation of volatiles from a solvent extract by mild distillation in high vacuum [16.31]. Low boiling solvents are commonly chosen and solid materials can be extracted by, for example, stirring prior to

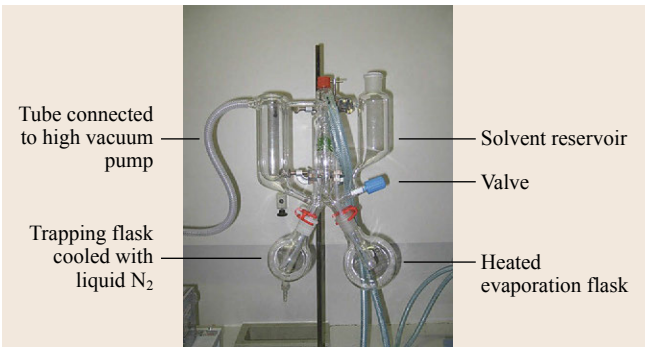

Fig. 16.5 Photograph of glass device used for solvent-assisted flavor evaporation (SAFE) technique according to [16.31]

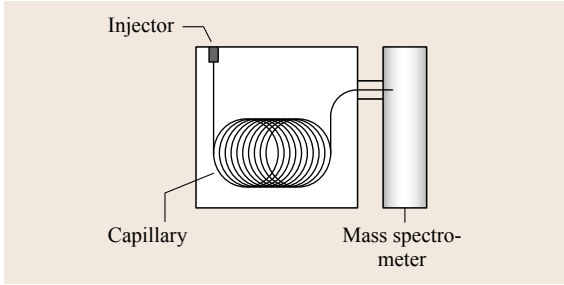

Fig. 16.6 Schematic setup of a gas chromatograph with mass spectrometer

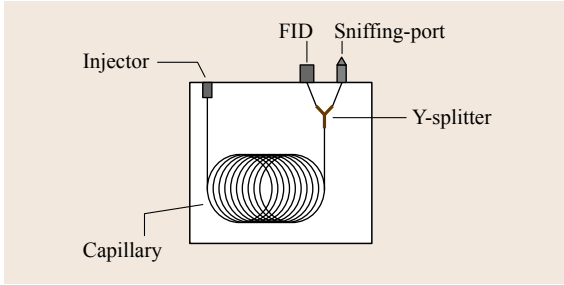

Fig. 16.7 Schematic setup of a gas chromatography/olfactometry system

distillation. In several cases, liquids (e.g., juices, beer) can be distilled directly without any additional pretreatment.

The glass device used for distillation is displayed in Fig 16.5. It is set to a vacuum of about 10^{-1}–10^{-2} Pa by a high-vacuum pump. The left trapping flask is cooled with liquid nitrogen and the right evaporation flask is heated to 40–50 °C depending on the solvent used for extraction. The organic extract is filled into the reservoir and dropped via the valve into the evaporation flask. Solvent and volatiles evaporate and they are transferred into the trapping flask where they are condensed and frozen. The flask is removed, the frozen distillate is thawed and finally concentrated.

The SAFE technique is a very gentle isolation method because the thermal impact is low. Disadvantages are the at times relatively high time required for the distillation process, the loss of volatiles with a boiling point lower than that of the solvent during concentration of the distillate, and the discrimination of higher boiling compounds [16.31, 32].

Gas Chromatography/Mass Spectrometry

GC/MS is a standard method for the separation of a volatile mixture and the identification of volatile compounds. A GC/MS system (Fig. 16.6) consists of an injector (e.g., split/splitless, on-column) into which a solvent extract from, for example, paper and cardboard is injected or an SPME fiber with ab-/adsorbed volatiles is inserted (Sect. 16.3.2, *Isolation of Volatile Compounds*). The injector is coupled to a capillary column [16.33].

By applying a carrier gas flow (e.g., helium) to the injector and in consequence to the capillary column, the volatiles are transferred onto the capillary column and are separated by the chromatographic process (whereby a temperature program is required). The end of the capillary column is installed into a mass spectrometer in which the separated volatiles are transferred. In the electron impact mode (MS-EI) the compounds are ionized and fragmented by an electron beam. The ions are recorded by a detector resulting in characteristic mass spectra (fragmentation patterns) of each volatile [16.34]. Commonly, a comparison of the mass spectrum and the retention time with the properties of the supposed reference compound confirms the structure of the volatile.

GC/MS analysis can be performed with extracts of the cardboard material and by using SPME.

Gas Chromatography/Olfactometry (GC/O)

Analysis of volatiles by GC/MS reveals the chemical structures of compounds. Volatiles vary however in their odor activity by a magnitude of at least 10^9 [16.18] and no information on odor activity of a single compound can be obtained. Therefore, the human sense of smell was introduced to analysis and gas chromatography/olfactometry (GC/O) was established in the 1980s.

In GC/O analysis, a regular gas chromatograph with injector and capillary column is used. In contrast to, for example, GC/MS, the compounds eluting at the end of the capillary column are commonly split by a Y-splitter into two capillaries leading to a detector (flame ionization detector (FID), MS) and a heated sniffing-port [16.33] (Fig. 16.7). An experienced assessor sniffs the compounds of an extract separated by GC at the sniffing port and records the perception of odor-active

compounds in the gas chromatogram or by a voice-recording system. In order to estimate the relative contribution of each detected odorant, aroma extract dilution analysis (AEDA) can be performed [16.33]. The extract is diluted stepwise in a defined ratio (usually 1 : 2, v/v) with the extraction solvent, and GC/O analysis is repeated with each dilution until no odor is perceived. Flavour dilution (FD) factors of the odorants which are defined as the highest dilution in which a compound is still perceived can be determined and the relative impact of the odor-active substance to the odor can be assessed.

Two-Dimensional Gas Chromatography/ Mass Spectrometry/Olfactometry

Identification of some odorants can be achieved by GC/O in combination with a mass spectrometer. However, many odorants are trace compounds with odor thresholds within or below the ppb range [16.18] and identification by GC/O/MS often fails because mass spectra of co-eluting nonodor-active compounds are obtained. A purification and separation of odorants is therefore required prior to identification experiments. Fractionation of the extract by, for example, column chromatography on silica gel can be applied but it is time- and material consuming. Two-dimensional gas chromatography in combination with mass spectrometry and olfactometry (2D-GC/MS/O) is an analytical alternative [16.35]. For an extensive description of the methodology please refer to Chaps. 19 and 17 in this book. Here the main features are only briefly depicted in the following:

The system consists of two gas chromatographs which are connected via a cryo trap (Fig. 16.8). The first GC oven is equipped with an injector and a capillary column. The end of the capillary is split into a sniffing port and a detector (flame ionization detector). The compounds of an extract are analyzed by GC and odor-active compounds, which are detected in a first GC run, are transferred in a second run via the transfer unit onto the cyro trap. After trapping and thermodesorption, the compounds are analyzed after separation on the capillary column of the second GC oven. This column differs from the column of the first GC in polarity. Column combinations of a polar (FFAP, first GC) and nonpolar column (DB-5, second GC) are often chosen. Thereby, further separation of the transferred compounds is achieved. Again, the analyzed compounds are split at the end of the column and led to a mass spectrometer and a sniffing port at which odorants are detected by sniffing. The detection of odor-active compounds by sniffing and recording of mass spectra in parallel provides the desired information on the chemical structure of odorants. Analyses of reference compounds under the same GC conditions finally confirm the results.

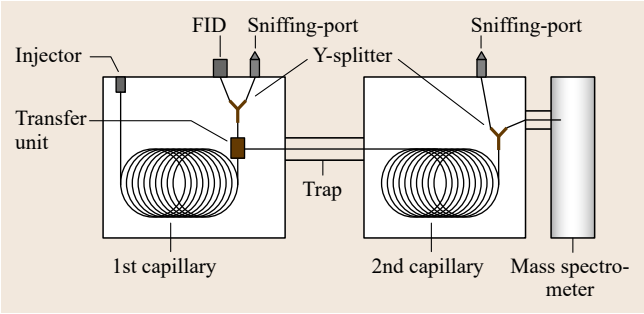

Fig. 16.8 Schematic setup of a 2-D gas chromatography-mass spectrometry/olfactometry system

16.4 Off-Odorants in Paper and Cardboard Packaging

The volatile composition of paper and cardboard is very complex. *Donetzhuber* et al. [16.36] identified more than 200 compounds in commercial samples from European manufacturers and further investigations increased the number of volatiles reported until today to a total of 291 volatiles [16.28, 36–46]. These volatiles belong to different substance classes, in particular aromatic compounds, aldehydes, alcohols, ketones, esters and aliphatic hydrocarbons (Table 16.6).

However, only some publications are available focussing on the elucidation of odor-active compounds in paper and cardboard by GC/O approaches or considering volatile concentration in relation to odor threshold.

Fresh cardboard is usually not odorless and exhibits a weak odor. It is therefore not surprising that various volatile compounds have been identified [16.41]. In particular unsaturated aldehydes like (*E*)-non-2-enal, (*E*, *E*)-nona-2,4-dienal, (*E*, *E*)-deca-2,4-dienal, and oct-1-en-3-ol with fatty and mushroom-like notes were considered as contributors to cardboard odor due to their determined concentrations in relation to their known high odor activities. Thereby, unsaturated fatty acids from wood resins were assumed as precursors of the odorants, which are generated by autoxidation.

Analysis of commercial cardboard by GC/O after SPME extraction demonstrated that additional saturated and unsaturated aldehydes with a (*E*)-2 configuration (C_4–C_9) can be odor-active constituents [16.42]. Application of SAFE for volatile extraction and AEDA further revealed vanillin (vanilla-

Table 16.6 Volatile compounds of commercial paper and cardboard (after [16.28, 36–46])

Substance class	Number of compounds
Aromatic incl. cyclic compounds	51
Aldehydes	46
Alcohols	35
Ketones and ethers	34
Aliphatic hydrocarbons	33
Esters and acids	31
Miscellaneous	28
Terpenes	19
Heterocyclic compounds	14
Total	291

like), γ-nonalactone, δ-decalactone, γ-dodecalactone (coconut-like), 2-methoxyphenol (smoky, vanilla-like), 3-propylphenol (leather-like, ink-like), 4-methyl-, and 4-ethylphenol (horsestable-like, faecal) as odorants with high intensities [16.28].

Coating of papers with latex can cause an odor which is not defined precisely but described as characteristic and unique for latex [16.43]. Analysis of such papers coated with styrene-butadiene latex in comparison with off-odor-free papers by GC/FID and GC/MS evaluated 4-phenyl cyclohex-1-ene as the constituent solely present in the papers affected with *latex odor*. The compound was proposed as a product of a side-chain reaction during latex manufacturing (Diels–Alder condensation of styrene and buta-1,3-diene, Fig. 16.9). An interesting GC/O approach was applied in this study in order to clarify the odor impact of the compound: a flame ionization detector (FID) of the GC/FID system was used as sniffing port after extinguishing the flame. 4-Phenyl cyclohex-1-ene was the only compound which exhibited the typical *latex odor* during sniffing. Quantification of the odorant in several raw latices revealed concentrations of 35–331 mg/kg which were far above the determined odor threshold of 4-phenyl cyclohex-1-ene (10 µg/kg water, 5.2 mg/kg latex coated paper). The odorant was confirmed in another investigation as the key odorant of latex coated and unprinted paper by GC/O analysis using extracts obtained by SDE [16.46]. Optimization and control of paper coating with latex is required in order to minimize or reduce the formation of the off-odorant.

Off-set printing was reported as another source of cardboard odor [16.44]. GC/O analysis clearly confirmed the presence of several odor-active unsaturated aldehydes and ketones (e.g., oct-1-en-3-one, (*E*, *E*)-nona-2,4-dienal, (*E*, *E*)-deca-2,4-dienal). Autoxidation of unsaturated fatty acids at the cardboard surface during off-set ink drying was elucidated as the crucial step in odor formation. Adaption of the drying process (temperature, time) can be a strategy in order to minimize or to avoid autoxidation and consequently odor.

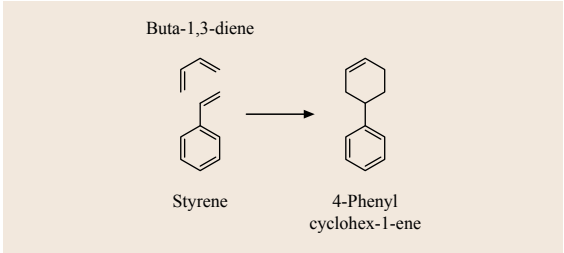

Fig. 16.9 Diels–Alder condensation of buta-1,3-diene and styrene leading to 4-phenyl cyclohex-1-ene

Cardboard produced by using recycled paper-based materials can be affected with chemical, musty and sour odor [16.45]. Oct-1-en-3-one (mushroom-like), oct-1-en-3-ol (mushroom-like), benzaldehyde (bitter almond-like), an unspecified 2-nonenal isomer (leaf-like, fatty), acetophenone (sweet, honey-like), 3-methylbutanoic acid (musty, sewer-like, sweaty, pungent) and an unspecified methylguaiacol isomer (phenolic) were identified by GC/O analysis in such cardboard with high odor intensities. It was concluded that off-odor of recycled cardboard can be correlated with aromatic odorants (benzaldehyde, acetophenone, methylguaiacol) in combination with lipid autoxidation products (oct-1-en-3-one, oct-1-en-3-ol, 2-nonenal).

Microorganisms are another potential source for off-odors in unprinted paper and cardboard. In particular, the introduction of closed water circuits in paper mills caused odor problems by a massive growth of anaerobic bacteria (*Bacillus* and *Clostridium* ssp.) [16.46]. They are able to metabolize cellulose and starch into short-chain fatty acids like butanoic acid, 2-/3-methylbutanoic acid and pentanoic acid with cheesy and sweaty odor notes causing off-odors. Biocides and slime control agents were successfully applied in order to reduce the germ count and to eliminate these unpleasant notes [16.47].

16.5 Summary

Paper and cardboard used as food packaging must be manufactured such that the organoleptic properties of foods are not negatively affected. Several sensory tests have been developed in order to detect and to characterize paper and cardboard odor on an objective basis. These methods are rapid and easy to apply but it is often difficult to obtain information on odor sources. In contrast, gas chromatography/olfactometry offers this possibility. By detection and identification of the odor-active compounds, the odor of paper and cardboard is characterized on a molecular level, odorant pathways and sources can be elucidated and measures can be taken for odor reduction and control.

Sources and odorant pathways causing paper and cardboard odor have been elucidated using GC/O. They are summarized as follows together with possible avoidance strategies (given in brackets):

- Formation of 4-phenyl cyclohex-1-ene in a chemical side-reaction of styrene and buta-1,3-diene during paper coating (optimization of latex coating resulting in 4-phenyl cyclohex-1-ene concentrations which are not relevant for odor).
- Autoxidation of unsaturated fatty acids to yield highly odor-active unsaturated aldehydes and ketones during off-set printing (replacement of unsaturated fatty acids with another agent, oxygen reduction, temperature control).
- Introduction of recycled paper odorants during paper processing (pre-selection of contaminated recycled paper).
- Microorganisms bio-converting cellulose and starch into odor-active short-chain fatty acids (use of biocides).

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Part C Analytics

Part C Analytics, Sensor Technology and Human-Sensory Evaluation

The previous sections provide detailed information about the broad importance of odorants in diverse aspects of human life. Commonly, numerous volatiles can be detected in our environment, from the air we breathe to the food we consume, and beyond. However, when it comes to odorants, the analytical scientist often faces the problem that the target substances are present at only low concentrations amongst countless odorless volatiles. In the early days of odorant analysis, this problem was partly addressed by recovering the molecules from large sample sets, e.g., by distilling or extracting liters of a sample and then tediously separating the respective molecules into their single constituents, thereby applying extremely laborious and time-consuming methodologies. A major problem in these analyses was to establish which molecule or fraction should be focused on and to determine how to avoid degrading target substances or generating artifacts during the diverse recovery, enrichment, and separation steps. With the advent of novel analytical techniques, primarily gas chromatography (GC) and mass spectrometry (MS), but also fast and gentle techniques capable of handling minimal sample quantities but still generating meaningful structural information, a new era in odorant analytics dawned. In this respect, especially the idea of combining human olfactory skills with instrumental methodologies led to a veritable revolution in resolving such molecules. Nowadays, modern odorant analytics is capable of tracing odorous contaminants, elucidating odorous constituents in complex odor mixtures or amongst an abundance of non-odorous molecules, assigning stereo-chemical characteristics within the course of a GC separation, or monitoring compounds while they are generated, released, exhaled, or captured, in several cases even in real time. This knowledge is then implementable in testing tools and protocols that link human smell perception with the data recorded by the analytical device, to elaborate the real sensory meaning and impact of what is measured by machines.

Humans do not function like machines, and their sensing does not follow the same relationships, such as a linear behavior, which are the basic principles of instrumental detectors. However, even if we are not yet at the stage of having developed a universal artificial nose that can sniff out what is important in our lives with the same speed and accuracy as the human organ, the analytics that can be achieved today is still highly impressive, as will become apparent in the following section.

17. Gas Chromatography–Mass Spectrometry in Odorant Analysis

Sung-Tong Chin, Graham T. Eyres, Philip J. Marriott

Precise odorant analysis by gas chromatography (GC) with olfactometry (O) methods, especially for complex sample mixtures, relies firstly upon achieving the best possible chemical separation of the volatile components. Odorants are often sampled from the headspace into the GC, since this is where our perception of the product is usually conducted, but the total sample or a liquid extract (e.g., from a wine sample) may also be introduced into the GC. Multidimensional GC (MDGC) methods achieve higher resolution than single column GC. For complex mixtures heart-cut MDGC, where discrete subsamples of eluate are transferred from the first column separation for analysis on a second column, significantly improve resolution of the components in the heart-cut fraction. Alternatively, comprehensive two-dimensional GC (GC × GC) improves resolution for all components in the injected material, but here the second column must complete analysis of each fraction very quickly e.g., within 5 s. Both approaches have been widely studied for aroma extracts and essential oils. Integrating simultaneous olfactometry – providing sensory assessment of a compound – and mass spectrometry (MS) – providing identification of the compound – in the MDGC experiment is important. At the end of the second column, split flow of effluent to O and MS allows effective dual detection of the resolved components. This is best conducted by the heart-cut MDGC approach. Novel methods usually based on MDGC can also be used to re-combine different odor-eliciting compounds, to study synergistic effects.

Understanding flavor perception firstly requires identification of the volatile compounds responsible for the characteristic aroma properties of the food or beverage sample. Amongst these compounds are those that impart specific aroma identified with specific products. These are commonly known as character-impact odorants. Odorant analysis has traditionally relied on gas chromatography (GC) as the separation tool of choice for the analysis of volatile compounds. GC incorporates high-resolution gas phase separation of analytes using an appropriate stationary phase coated capillary column and elution in a carrier gas stream to a detector. Sample introduction may be achieved by liquid injection of a sample or by analysis of a headspace gas above a sample of interest via split, splitless or thermal desorption. Detectors may have a universal response to all compounds, such as the flame ionization detector (FID), or they may be selective, where a given element provides a specific mode of response. For example, selective detection is found for halogens (electron capture detector), sulfur (flame photometric detector), nitrogen (nitrogen-phosphorus detector), amongst others. For analysis of odorants, the two key detectors are (i) the mass spectrometer that provides identification of compounds through mass spectrum interpretation (usually MS database matching with database of reference spectra), and (ii) sensory evaluation of eluted peaks by human assessors via olfactometry.

Hyphenating a high-resolution GC separation with detection of human assessors, known as GC-olfacto-

metry (GC-O), has been an indispensable tool to identify the odorants in sample mixtures [17.1, 2]. In this technique, the human assessor provides the most sensitive and selective detector for the chemical property of interest – odor activity. Various GC-O protocols such as odor intensity, dilution-to-threshold, or detection frequency, may be applied in assigning the relative importance to odor-active compounds eluting individually from a GC separation [17.1, 3–8].

A common limitation in odorant analysis is the incidence of overlapping chromatographic peaks arising due to insufficient resolution in complex samples. This presents considerable challenges in determining the chemical compounds responsible for a perceived odor activity. Multidimensional gas chromatography (MDGC) has evolved in the past few decades, involving the incorporation of multiple separation dimensions (more than one GC column) to enhance the resolving power of the analysis. This chapter describes the advent of various MDGC approaches for odorant analysis and their applications.

17.1 Rationale for Multidimensional GC

Overlapping of chromatographic peaks is mainly due to insufficient resolution power in a given separation method; this is increasingly important for a complex sample. The power of mass spectrometry (MS) is often unable to provide an unambiguous mass spectrum for each compound present in grossly co-eluted peaks [17.9], even by utilizing spectral deconvolution algorithms. In this circumstance it is very challenging to precisely assign a chemical compound to the odor perception, particularly when a trace odor-active compound co-elutes with a matrix background of an abundant odor-inactive peak. The technology of achieving individual component separation and odor assessment still plays an important role in odor analysis. Combining olfactory detection with simultaneous mass spectrometry and retention index estimation is a powerful identification strategy for the characterization of an unknown odor.

To simplify the complexity of an odor extract, various sample preparation and fractionation procedures are commonly applied prior to GC-O analysis. These are typically low resolution methods such as acid/base chemical isolation [17.10, 11], flash sorbent/agar fractionation [17.12–16], and normal or reversed-phase liquid chromatography (LC) [17.16–18]. Multiple fractions are generated from the original aroma extract corresponding to the process condition (pH, polarity, ion exchange ligand), which then require further analysis. Such methods have been reviewed elsewhere [17.19–21], and often exhibit the possibilities of analyte losses or artifact formation caused by hydrolysis and oxidation of analytes during the fractionation process [17.2, 21]. These techniques are also incompatible with headspace aroma extraction techniques, as the isolated odorants are normally thermally desorbed from the sorbent medium directly into the GC inlet. The pre-fractionation processes to simplify the aroma extract do not suit analysis by solid phase microextraction (SPME), which is widely used in odor analysis.

An alternative approach is to improve resolution using MDGC. The essence of MDGC is the incorporation of multiple gas phase separations by using more than one GC column organized in a sequential arrangement. The two columns typically have orthogonal separation mechanisms (different column selectivities) which are referred to as *dimensions* of separation. A transfer process between the analytical columns serves to effectively decouple individual retentions and transfer target analytes from the first dimension to the second dimension. This results in greater resolving power, and significantly higher peak capacity without artifact generation. Development of MDGC has undergone a recent resurgence through development of precision devices such as microfluidic switches, better gas flow control and improved fast responding detection methods [17.22].

Essentially, the two main approaches for online MDGC involve (a) subjecting a target portion(s) of chromatographic effluent from a first dimensional column to a secondary column separation (conventional heart-cut MDGC), or (b) applying the two-column separation advantage to the entire sample via a fast, continuous modulation process (comprehensive two-dimensional GC; GC × GC).

17.2 Offline MDGC-O

Offline two-dimensional (2-D) GC involves separation using two columns in two discrete analytical systems. In principle, offline MDGC uses high resolution GC as a preparative primary separation dimension to isolate and simplify the complexity of a target fraction, in contrast to other pre-fractionation tools described

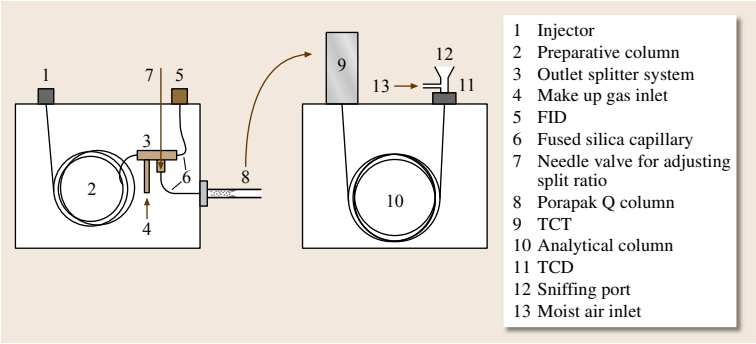

Fig. 17.1 Schematic diagram of an offline MDGC system (after [17.23])

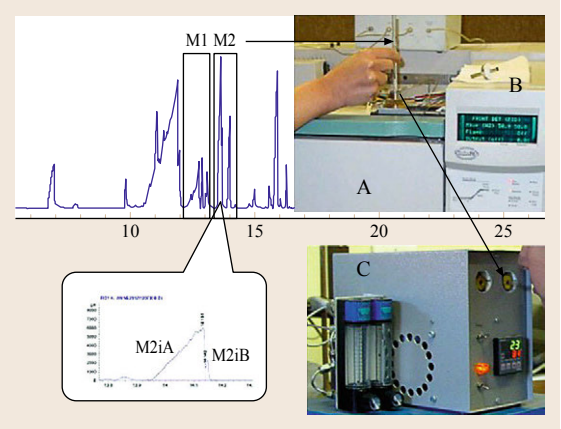

Fig. 17.2 Heart-cutting and offline olfactometry for identifying the fraction of a chromatogram responsible for a coffee odor. Sections of the chromatogram are captured from the GC effluent on a MCT (*A*). The trap rests on the flame tip of an inactive FID during collection. The FID ion collector is removed prior to heart-cutting (*B*). The odor is thermally desorbed from the trap with 20 ml/min nitrogen gas flow in an offline olfactometer (after [17.24])

above. An additional sorbent trapping medium is commonly required to perform a *heart-cut* operation by trapping the target region(s) eluting from the first dimensional (^{1}D) GC separation. Subsequently, trapped analytes are either solvent eluted or thermally desorbed from the absorbent medium for introduction into the second dimensional (^{2}D) GC separation. Although manual operation may be implemented in some cases, this method offers the convenience of subjecting the trapped region(s) to another individual chromatography and/or detection system when a flow switching ancillary device is unavailable and/or online hyphenating configuration is unachievable, such as in the case of nuclear magnetic resonance (NMR) spectroscopy. Nevertheless, losses of target analytes during the trapping procedure cannot be neglected, whilst contamination of the trapping medium also needs to be considered to ensure the sample has the highest possible purity.

Miranda et al. [17.25] reported the identification of odor-active compounds from the headspace of banana passa using offline MDGC-O/MS analysis. An open-tubular capillary (1 m × 0.33 mm id) coated with DB-210 phase with a CO_2/acetone cold trap was applied as the trapping medium at the olfactory outlet of a ^{1}D separation. After five analyses collecting the most potent aroma chromatographic regions, the most odor-active compound, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (sweet, caramel), was identified through its MS library matching. Similarly, *Rogerson* et al. [17.26] proposed a GC fractionation approach to investigate the sweet aroma descriptors in Portuguese wines from the Douro region. The target odor region from the ^{1}D separation was collected by the condensation of sniffing port effluent into a 2 ml vial filled with 10 µl of diethyl ether at −20 °C. A norisoprenoid compound identified as 2,6,6-trimethylcyclohex-2-ene-1,4-dione with a sweet, honey aroma character was determined to be the key contributor to Port wine aroma.

Figure 17.1 illustrates the configuration of an offline MDGC arrangement reported by *Nishimura* [17.23], which included the application of a Porapak Q column (6 mm O.D. glass tube) as the trapping medium and a thermal desorption cold trap injector (TCT) for analyte desorption to ^{2}D analysis. The oxygenated hydrocarbon fraction of the extract from Japanese fresh ginger rhizomes was analyzed for the chiral composition of characteristic odorants in fresh ginger samples. After ^{1}D separation in a Wax megabore column, each enantiomer of linalool, 4-terpineol, isoborneol and borneol was successfully trapped and resolved in the chiral CP-Cyclodextrin-2,3,6-M-19 column. The odor character of each enantiomer was confirmed in ^{2}D analysis with simultaneous thermal conductivity detection (TCD) and olfactory assessment.

Recently, a multichannel open tubular silicone rubber trap (MCT) was described for its trapping capability under room temperature conditions for headspace sam-

pling of milk and wine volatiles (Fig. 17.2) [17.24, 27]. Discrete portions of the chromatographic profile, either individual peak(s), region(s) or combinations of both can be recaptured with a second MCT during a subsequent run that can be further thermally desorbed for direct aroma evaluation or into GC–MS with simultaneous olfactometry for odorant identification. An aroma region of several minutes allows sniffing of each aroma fraction by six panelists. Olfactory results suggested that a synergistic combination of 2-heptanone and 2-nonanone could be responsible for a pungent cheese, sour milk-like aroma [17.27]. The manual method should be suitable for automation with appropriate MDGC method development. This approach was also applied to the investigation of the compounds responsible for coffee aroma character in *Pinotage* wine from several South African wine cellars [17.24]. Synergistic aroma effects were evaluated utilizing the recaptured fractions, released in a controlled manner for offline olfactory evaluation, and for qualitative analysis using GC × GC–TOFMS for compound separation and identification. This approach permitted correlation of odor activity with specific identified compounds. Combined furfural and 2-furanmethanol resulted in a synergistic effect for the occurrence of coffee odor in *Pinotage* wine, which is a desired effect that is deliberately obtained from toasted oak wood. It was demonstrated that neither of the individual compounds in isolation was responsible for the characteristic aroma.

17.3 Online MDGC-O

Most modern MDGC techniques involve online transfer of heart cut regions using a valve or flow-switching device, such as a Deans-switch system. Microfluidic flow technology offers better operational inertness and precision in the flow switching and solute modulation processes [17.22, 28–32]. Figure 17.3 illustrates a schematic configuration of an online MDGC system mounted with a Deans-switch interface (DS) and a cryogenic trap (CT). The peak broadening due to solute dispersion as a consequence from ^{1}D chromatography can be minimized by cryofocusing at the ^{2}D inlet, giving the best performance of multidimensional analysis with automated control. In addition, fast oven programming can be offered by a dual-oven low-thermal mass (LTM) GC system with greater flexibility of independent temperature control implemented in both separating dimensions [17.33, 34]. The emergence of integrated MDGC-O/MS further extends the analytical efficiency with operational flexibility, which is effective for rapidly characterizing the aroma compounds and identifying taints for odor quality issues [17.9, 35].

For odorant analysts, MDGC-O/MS remains the method of choice to detect odor-active compounds in aroma extracts, supported by retention indices, and structural or element-specific information with other detectors. Based on the Siemens SiCHROMAT-2 double-oven GC and a Deans switch MDGC arrangement, *Nitz* et al. [17.36] described a system equipped with different injection modes, a micropreparative (μ-prep) module, a sniffing port and MS detection. Applications described by these authors are representative of many key aspects of flavor work, such as trace-level flavor compounds (methoxypyrazines and geosmin) as potential off-flavor compounds in various samples, and isolation of a terpene compound from parsley via μ-prep sampling after MDGC [17.37]. *Ragunathan* et al. [17.38, 39] demonstrated an MDGC system operating with valve-based effluent switching but configured with multiple parallel cryogenic traps that enabled multistage separation and Fourier transform infra-red (FTIR) and MS detection.

In the case of chiral flavor compounds, enantio-MDGC-MS provides a new approach for flavor-authenticity control [17.40–42]. Further, the combination of enantio-MDGC with isotope ratio MS (IRMS) [17.43, 44] allows evaluation of the origin of particular flavor compounds on not only the basis of their enantiomeric distribution but also consideration of the natural variation in isotope distribution for characterizing sample provenance. Constant-flow MDGC combustion/pyrolysis-IRMS was applied to the authenticity assessment of (*E*)-α(β)-ionone from six different

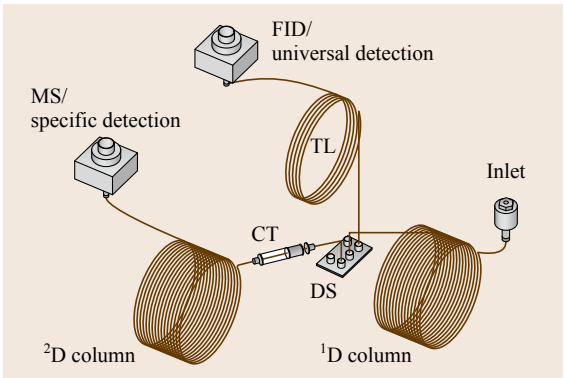

Fig. 17.3 Schematic diagram of heart-cut MDGC system

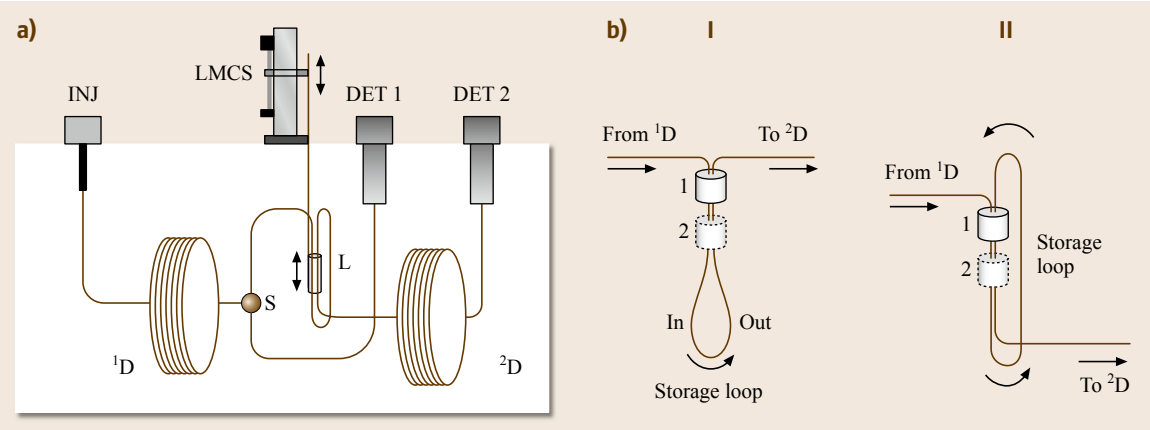

Fig. 17.4a,b Schematic diagram of **(a)** MDGC-DCSI-O system incorporating a looped column (L) through a LMCS. Target regions are selected, sampled, and passed to the second dimension (^{2}D) by action of the LMCS cryotrap. The splitter union (S) allows a small flow to pass to the first detector (DET 1) to allow precise selection of the target region. **(b)** Two different arrangements for the looped column modulator (after [17.9])

raspberry cultivars [17.45]. Characteristic authenticity ranges were accomplished by correlation of both $\partial^2 H_{V\text{-}SMOW}$ and $\partial^{13} C_{V\text{-}PDB}$ values.

An alternative online transfer mechanism incorporating MDGC was proposed by *Begnaud* et al. [17.46, 47] utilizing a double cool-strand interface (DCSI) arrangement incorporating the longitudinally modulated cryogenic system (LMCS) device, which required no additional pneumatic flow control (Fig. 17.4). This design was demonstrated to be useful in separation of chiral volatiles in malodor analysis with coupling to olfactory detection [17.46, 47]. An alternative loop arrangement (Fig. 17.4b) and the distinction between the two approaches is described in *Eyres* et al. [17.48] and *Ruhle* et al. [17.49]. *Eyres* et al. [17.48, 50] introduced a μ-prep technique based upon capillary MDGC utilizing the modified loop modulator interface as the transfer device to the ^{2}D separation, with a Deans switch to divert the flow to an external trapping assembly (xTA) at the outlet of the ^{2}D column, where the single analyte compound was collected with multiple injections until the desired amount was accumulated. It was demonstrated that sufficient quantities of the isolated analyte could be obtained to perform offline 2-D NMR analysis. This approach can be applied to resolve, isolate and identify pure volatile components from a complex sample matrix. This μ-prep system was also applied with x-ray analysis of isomeric products from catalysis reactions of phenyl acetylene with *para*-substituted aryl iodides [17.51].

17.4 Novel Approaches in Olfactometry

The most effective use of olfactometry in odorant analysis comes from a good understanding of the human assessor as the specific detector. Applying sound principles of sensory science to olfactometry experiments has the potential to provide the greatest understanding of odor perception [17.1]. In olfactometry experiments, a panel of multiple assessors is typically required, which can make a comprehensive investigation very time consuming. In an attempt to alleviate this limitation, a multisniffing system incorporating three olfactory ports with simultaneous MS detection was presented by *Debonneville* et al. [17.3]. A computer-controlled eight-way GC-O system was also developed that allows for an aromagram computed from eight panelists to be obtained simultaneously in a single GC analysis [17.52].

Sample representativeness is also an important issue to ensure any sample extract for analysis matches the original sample and that the sample preparation method does not result in artifact formation. A direct GC-O (D-GC-O) system using an uncoated deactivated fused silica column with no separation of analytes was proposed to evaluate the representativeness of an SPME extract desorbed into the GC injector [17.53].

Typical odorant analysis aims to achieve complete resolution of odor active compounds and with indi-

vidual olfactory assessment. However, the overall odor perception of a sample is the result of a complex combination of many interacting odorants and their proportional release from the original sample matrix [17.1]. It is also difficult to determine the recombined odor simply from individual separated compound(s), due to complex interactions and physiological effects [17.54]. Best practice in odorant analysis involves validation of the importance of the identified compounds by recombination studies to assess that the simulated model sample of the top odorants is similar to the original sample [17.55]. The contribution of individual compounds in the mixture may then be evaluated using omission experiments. A novel system for characterization of aroma mixtures in an online system was developed by *Johnson* et al. [17.56], termed GC recomposition-olfactometry (GC-RO). This system utilizes microfluidic flow-switching to heart-cut selected peaks and recombines them using a cryotrap prior to olfactory detection. The system was demonstrated by evaluation of an SPME extract of lavender flowers. Another system to investigate odor mixture interactions was presented by *Williams* et al. [17.57] where odorants from a GC separation were presented into an air stream with a constant background odorant, termed a GC-pedestal olfactometer (GC-PO). The advantage of these techniques is that interaction studies can be performed even when the odorants have not been identified.

17.5 GC × GC Approaches for Global Volatile Screening

Comprehensive flavor characterization studies often require a combination of analytical techniques utilizing an olfactory detector and chemical detectors in order to obtain the most precise interpretation. Comprehensive two-dimensional GC (GC × GC) is increasingly applied to odorant analysis in conjunction with dual detection devices (olfactometry and MS detection) to provide more accurate identification and improved sensitivity. *Breme* et al. [17.52] combined GC × GC coupled to time-of-flight mass spectrometry (GC × GC-TOFMS) analyses with the eight-way GC-O system for the identification of trace odorant constituents in an Indian cress sample. *Rochat* et al. [17.58] applied GC × GC-TOFMS for analysis of sulfur-containing compounds in roasted beef as key aroma components. Correlated with GC-SNIF (GC-surface of nasal impact frequency olfactometry), a number of sulfur compounds in the top note were confirmed, with some compounds identified for the first time.

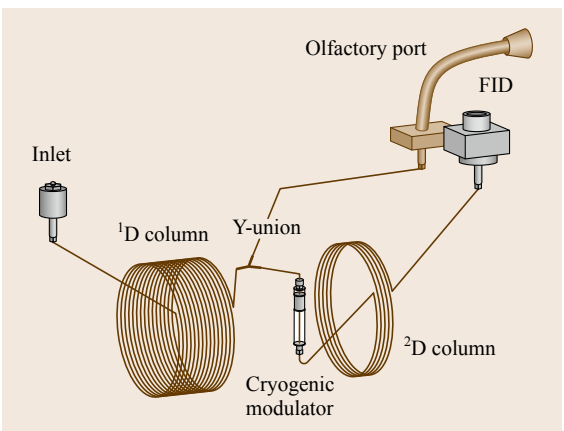

Fig. 17.5 Schematic diagram of GC × GC-FID/GC-O system

d'Acampora Zellner et al. [17.59] attempted to overcome imprecise interpretation of co-eluting peaks in the odor-active region from GC-O by coupling a sniff port with GC × GC. However, this technique is technically demanding, due to a number of very narrow modulated peaks (0.1–0.4 s peak width) which are too narrow for the typical breathing cycle of a human (3–4 s) [17.9]. Therefore, the application of H/C MDGC for the study of aroma-impact regions with olfactory detection remains important. *Eyres* et al. [17.60] studied the spicy character of hop essential oil by comparing data from GC-O with GC × GC-TOFMS to locate the odor-active regions. The co-eluting cluster of compounds responsible for a spicy odor could be resolved by H/C MDGC on ^{2}D, and generated discrete broader peaks (3–6 s) appropriate for olfactory assessment. Volatile constituents in wine and brewed coffee were analyzed using three different systems, the first incorporating both GC-O and GC × GC-FID as illustrated in Fig. 17.5, a GC × GC-TOFMS and a dual detection GC × GC–FID/FPD design [17.61]. Impact odorants were tentatively identified through data correlation of GC × GC contour plots across the results obtained using either TOFMS, or with flame photometric detection (FPD) for sulfur speciation (S_2^* emission). Interpolating the GC × GC data with those obtained from olfactory detection presents a great challenge in terms of method accuracy, robustness and simplicity. This highlights the requirement for an integrated MDGC-olfactometry arrangement.

17.6 Integrated MDGC-O Arrangement

Recently, a selectable 1-D/2-D GC–O/MS system was developed using multiple microfluidic Deans switches and LTM-GC [17.33, 34, 62–64]. The developed system offers the simplicity and flexible implementation of fast programmable 1-D or 2-D GC–MS operation, simultaneously with MS and olfactometry and element-specific detections such as nitrogen chemoluminescence detector (NCD), nitrogen phosphorus detector (NPD), pulsed flame photometric detector (PFPD) and sulfur chemiluminescence detector (SCD) to assure selection of a heart-cut region and correct identification of compounds of interest. Preparative fraction collection (PFC) was coupled to this system outlet for analysis of ultra-trace amounts of odor compounds [17.33]. The selectable system was applied for the assessment of sulfur compounds in whiskey samples by simultaneous heart-cuts of 16 fractions to the ^{2}D column in conjunction with retention time locking and sulfur specific detection and MS [17.63]. Such an approach was also validated and tested for its reproducibility and quantitative analysis of fragrance [17.62].

A dual switchable GC × GC/targeted MDGC analysis was proposed for study of aroma-impact compounds where GC × GC can be incorporated with MDGC to permit precise identification of aroma active compounds [17.66]. An integrated switchable MDGC system that allows targeted MDGC and GC × GC analysis to be performed in a single run with simultaneous MS and olfactory detection was developed as shown in Fig. 17.6 [17.65]. The system also utilized the techniques of online enrichment of target compounds by in-oven trapping [17.67] as well as cumulative solid phase microextraction sampling [17.68] to amplify the signal for improved sensitivity and identification. Utilizing the developed integrated system, olfactory data from 1-D GC-O was readily matched to MDGC-O/MS identification, with complementary GC × GC-FID analysis giving an overview of the sample's complexity. Any desired co-eluted regions from the ^{1}D column can be selected to permit targeted MDGC separation on a longer ^{2}D column than that employed for GC × GC operation. This workflow strategy is shown in Fig. 17.6b. This system results in better resolution and was proposed to be suitable for coupling with olfactometry or other detection methods that require slow acquisition rates, such as Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS). Analysis of several potent odor regions for wine and coffee samples as a complex problem was demonstrated as example applications [17.61]. Isomer identification is also feasible through verification by simultaneous matching of acquired mass spectral data, supported by both primary and secondary dimension retention indices data.

A variety of multicolumn methods have been developed to suit current volatile chemical analysis, with hybrid systems incorporating facets of both conventional H/C MDGC and GC × GC technologies. *Mitrevski* and *Marriott* [17.69] recently reported a hybrid (sequential) GC × GC-MDGC method for complex sample manipulation. This method was applied to separation

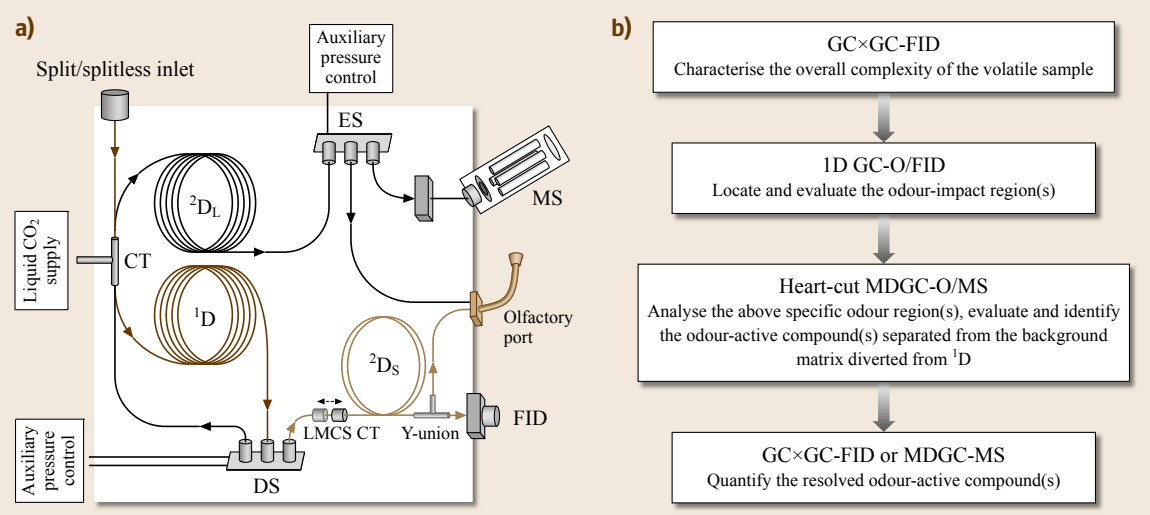

Fig. 17.6 Schematic diagram of **(a)** integrated MDGC/GC × GC-O/MS system; **(b)** Flow diagram of the strategy for odor-impact analysis in a complex sample using the integrated system (after [17.65])

and quantitative analysis of oxygenates in a thermally-stressed algae-derived biofuel oil without any pre-fractionation procedure. Profile reproducibility before and after heart-cut operations was demonstrated by extracting single components from a complex coffee volatile sample.

17.7 Current Challenges and Future Perspectives

Although current MDGC technology has greatly enhanced the reliability of compound identification owing to improved separation from the dominating background in analyzed samples, general acceptance of MDGC technologies is mostly restricted to the academic research sector, and is less commonly applied by industry to identify taints related to flavor and general profiling of aroma products. Through recent advances in development of standardized systems and approaches in online coupled systems, MDGC is a ready-for-adoption technology for widespread use in the industrial laboratory. Thus, a range of approaches should be validated as simpler automatic and less expensive solutions for odor analysis compared with more complex mass spectrometry methods that do not target complete component separation. MDGC methods can be conveniently adapted for rapid determination of aroma-related sensory problems in the industry.

Online interaction studies through recombination of odorant mixtures rather than evaluation of individual compounds is critically important in order to assess overall perception and to validate GC-O findings. This approach verifies significant perceptual interactions of volatiles which exist below their putative sensory threshold, representing an under-explored and promising research area in odorant analysis. Combining MDGC recomposition olfactometry methodology with other bioassays would generate useful information on the relationship between stimulus and perceived flavor response, or the pathway between these two parameters involving genetics, receptor binding, transduction and translation to cortical neurons.

17.8 Concluding Remarks

The resurgence of multidimensional gas chromatography techniques in conjunction with olfactory analysis provides a powerful approach to identify odorants in complex food and fragrance samples. There is great potential of MDGC approaches to enhance understanding of flavor perception through high-resolution odorant analysis utilizing innovations in advanced separation and detection systems. Improved separations in association with flavor/aroma analysis is especially bright, and continued innovation in this space assures that not only useful studies on fundamental human odor perceptions, but also contributions to chemical ecology research of other organisms with olfactory systems similar to that of humans will be enhanced.

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18. Odorant Detection by On-line Chemical Ionization Mass Spectrometry

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The nasal olfactory receptors allow us, as human beings, to detect and perceive odors almost instantaneously upon exposure and over a broad range of concentrations down to ultratrace levels. Translating this rapid and sensitive detection of odorant molecules to the analytical laboratory is a challenging, nontrivial endeavor that remains unachieved to date. On-line mass spectrometry based on chemical ionization (CIMS) comprises sophisticated analytical techniques that meet several of the key requirements in odorant detection, namely fast response times and direct analyses, trace level limits of detection, and a high sensitivity to a suite of odors or, more specifically, odorants. This chapter discusses on-line CIMS and its application in odorant detection in selected fields. The prominent CIMS techniques of selected ion flow tube mass spectrometry (SIFT-MS), proton transfer reaction MS (PTR-MS) and atmospheric pressure chemical ionization MS (APCI-MS) are considered, commencing with a brief introduction to their historical developments and a discussion of their operational features and suitability for odorant detection, followed by a review of their widespread applications in odorant measurements in diverse fields of study.

Chemical ionization mass spectrometry (CIMS) refers to mass spectrometric techniques that utilize specific reagent ions for the chemical ionization of neutral analyte molecules in the gas phase. Although chemical ionization is also employed in other systems such as gas chromatography MS (GC-MS) – in addition to the more frequently used electron ionization (EI) – CIMS in the context of this chapter refers to specific MS techniques that allow the ionization and detection of gas-phase volatile organic compounds (VOCs) directly and in a continuous manner, i. e., in real-time without the need of sample pretreatments such as enrichment, dehydration, or chromatographic separation.

It is noteworthy that none of the major on-line CIMS techniques discussed in this chapter were invented to specifically measure odor-active molecules, and their initial exploits were mostly in fields with only loose association per se to aroma or odor analysis. Their implementation for the specific purpose of odorant detection has been a by-product of their efficient and sensitive response to volatile compounds, regardless of the odor activity of the target molecules. The recognition of this application by researchers working in odor-related fields steered their early and successive development and increasing deployment for odorant detection in diverse areas of research.

This chapter commences with a retrospective of the development of CIMS and discusses the current predominant analytical CIMS tools that are in use for on-line odorant detection in different disciplines, focusing on their development and basic operating principles. The ensuing discussion moves on to the varying applications of these on-line CIMS techniques in individual fields of odor detection. Due to the nature of CIMS, whereby an extensive range of VOCs are detectable whether they are odorous or not, part of the discussion inevitably alludes to the detection of nonodorous molecules. Further, there are many studies that report on

the detection of odoriferous molecules without consideration of their odor-active properties. For the purposes of this chapter, which focuses on the detection of odorous molecules, these are broadly taken as being any compound that elicits an odor impression in the human nose at the typical concentrations encountered in the respective environment or application. This latter aspect is particularly important, since many molecules manifest odorous attributes only above a certain concentration threshold that might not be often reached in everyday scenarios.

Given the long history of CIMS and the rapid growth of related real-time tools over the last few decades, particularly in recent years, this chapter is by no means authoritative but aims to provide a broad overview of major past and present endeavors of diverse on-line CIMS techniques in the detection of odorants, and hints at prospective applications. It should be noted that the odorants treated in this chapter are discussed in terms of their detection by CIMS, but not at length on their (bio)chemical origins – or where necessary, only superficially – since this is the focus of other chapters of this book. Furthermore, since applications and best practices in the fields covered in this chapter are constantly being updated and novel developments are continually being introduced at a fast rate, the reader is advised to refer to the most recent scientific literature for the latest information on specific methods and topics.

Mass spectrometry, by definition, is the analytical procedure by which ionized target atoms or molecules are detected on the basis of the ratio of their mass to charge (m/z). In other words, the signal response elicited by an ion reaching the detector within the system is dependent on the m/z-related settings of the mass spectrometer. The ionized molecule that corresponds to this m/z signal – or spectrum thereof – is then identified according to known ionization pathways and fragmentation patterns, either from theoretical models or via analogous analysis of the pure compound. The abbreviation m/z appearing throughout this chapter represents the dimensionless quantity used almost universally as the independent variable in a mass spectrum. Odorant concentrations are presented in parts-per-unit-volume notation, typically parts per million (10^{-6}), parts per billion (10^{-9}), and parts per trillion (10^{-12}) by volume, or ppm_v, ppb_v and ppt_v, respectively. Although this unit of measure represents a volume mixing ratio (VMR) and, strictly speaking, is not a concentration, it is deemed most appropriate for all intents and purposes of the discussion on odor abundance and threshold/detection levels and is interchangeably referred to as *concentration*.

18.1 Techniques

Before discussing applications in odorant detection, the major on-line CIMS techniques currently in use for the detection of volatile (odorous) compounds are introduced, commencing with a brief history and subsequent outline of the operating principles of each technique, as well as presenting a discourse on the analytical advantages and shortcomings for odorant detection.

18.1.1 A Brief History of CIMS

Chemical ionization mass spectrometry was conceived half a century ago by the experimental work of *Burnaby Munson*, *Frank Field* and colleagues at the Humble Oil and Refining Co. in Baytown, TX, USA with their discovery of a technique in mass spectrometry that was *based on the formation of the ions of an unknown material by chemical reactions in the gas phase* [18.1]. The formation of product ions via chemical reactions inspired them to name this new technique *chemical ionization* [18.2]. In their seminal paper that introduced this new ionization method, *Munson* and *Field* showed that operating their mass spectrometer at high pressures of 1 torr ($\approx$ 133.3 Pa, or 1.33 mbar) and adding a reaction gas into the ionization chamber – in their experiments, methane – with a small amount of additives, or target compounds, led to the production of stable primary ions (CH_5^+ and $C_2H_5^+$) that did not react further with methane but did react by proton or hydride transfer with the organic additives to produce relatively clean and stable spectra of the latter, including appreciable amounts of quasi-molecular ions [18.1]. In comparison to the conventional method of electron (impact) ionization (EI), which yielded complex mass spectra due to extensive fragmentation upon ionization of the neutral target, this new technique was anticipated to be of especial relevance for both qualitative and quantitative compound analysis. This was the inception of chemical ionization mass spectrometry and the basis for the development of the on-line CIMS techniques of today. A thorough chronology of the initial experiments that led to the discovery of CIMS and its subsequent development is provided in the reminiscent article by *Field* [18.2]; further details of the ion chemistry observed in these early studies are recounted in a historic overview paper by *Munson* [18.3].

The subsequent developments that led to the invention of the derivative techniques at the focus of this chapter then diverged. In Boulder, CO, USA, experi-

ments carried out by *Eldon Ferguson*, *Fred Fehsenfeld* and *Art Schmeltekopf* at the Upper Atmosphere and Space Physics Division of the National Bureau of Standards – later to become the National Oceanic and Atmospheric Administration (NOAA) – in the mid-1960s led to the development of a technique known as *flowing afterglow* (FA) that was applied to investigate ion-molecule reactions in the Earth's upper atmosphere [18.4, 5]. The system comprised of a microwave discharge source to produce helium ions that were channeled via a nozzle into a flow tube in which neutral reactant gas could be added for subsequent ionization by He^+ ions [18.4]. The afterglow from which the name was derived referred to photoemissions that resulted from electron-ion recombination in the downstream plasma, which accordingly indicated the presence of ions in the flow tube [18.6]. A quadrupole MS coupled to the system allowed for mass spectrometric selection and subsequent detection of ions emerging from the flow tube and paved the way for studies on ion-molecule reactions under defined conditions. For further reading, a retrospective by *Ferguson* on the early development of FA and the first experiments on ion-molecule chemistry is available in the scientific literature [18.6].

Subsequent work by *McFarland*, *Albritton* and the aforementioned researchers at the Aeronomy Laboratory at NOAA in the early 1970s produced the flow drift tube (FDT) that was incorporated into the FA system [18.7]. This was a key feature of the new technique because it enabled the relative ion-neutral kinetic energy in the reaction region to be increased from thermal conditions to several electron-volts (eV) kinetic energy and allowed for precise control of the conditions within the chamber [18.6].

At the time of the experiments leading to the introduction of the FDT, concurrent studies by *Adams* and *Smith* at the University of Birmingham, UK made use of a quadrupole mass filter that was coupled to a FA system, being placed between the low pressure ion source and the FA region to allow single reagent ions to be preselected and injected via an aspirator into the flow tube [18.8]. In particular, the technique allowed these selected species – primarily either H_3O^+, O_2^+ or NO^+ – to be injected into the flow tube at sufficiently low energy to reduce fragmentation from ion collisions. Based on this feature of reagent ion preselection, *Adams* and *Smith* named their technique the selected ion flow tube (SIFT) and its initial exploits were in the investigation and characterization of ion-neutral reactions in the terrestrial upper atmosphere and in interstellar clouds [18.9].

This notion of selecting reagent ions prior to introduction into the flow tube reaction chamber was adopted in the FDT system of the NOAA group to create a selected ion flow drift tube (SIFDT) [18.10]. This combined system offered the advantage of providing a pure reagent ion beam, which was injected into the reaction chamber via a Venturi inlet aperture, with the versatility of the drift tube that employed an electric field to allow the ions to be accelerated to several eV kinetic energy [18.7, 10]. As with the SIFT-MS instrument, the selected ion flow drift tube mass spectrometer (SIFDT-MS) was operated extensively for studying ion-molecule reactions, rather than being used as an analytical detection system per se.

It was not until the mid-1990s that these CIMS techniques shifted in their use from basic research tools to applied analytical instruments for compound detection. Two decades after the SIFT-MS technique was introduced its use in the detection of the volatile constituents in exhaled breath was demonstrated, a research field that at the time was in its infancy and was certainly a niche discipline for the SIFT-MS device [18.11]; similar studies were performed using the SIFDT-MS instrument [18.12]. Around the same time, Werner Lindinger, Armin Hansel, Alfons Jordan and colleagues at the Institute of Ion Physics at the University of Innsbruck, Austria, replaced the preselection quadrupole mass filter of their SIFDT-MS system with a hollow cathode discharge ion source to generate hydronium – or more correctly, oxonium or hydroxonium – ions, H_3O^+, as reagent ions at a purity of $\geq 99.5\%$ that were channeled via a Venturi inlet into the downstream FDT; the high purity reagent ion production in the hollow cathode obviated the need for a preselection mass filter and its associated bulky vacuum pumping system, thus making for a more compact instrument. Because the chemical ionization within the FDT reaction chamber proceeded exclusively via proton transfer reaction (PTR) from the hydronium reagent ions to the neutral target analytes, the name proton transfer reaction MS (PTR-MS) was adopted for this new system [18.13]. Unlike the SIFT-MS instrument, the PTR-MS system was developed with the specific intention of use as an analytical detection system for VOCs, rather than for ion-molecule reaction kinetics studies. As such, key fields of application for this tool were identified and explored upon its conception [18.13–15].

The early developments of techniques leading to atmospheric pressure ionization (API) took place at a number of independent research laboratories, each with its own apparatus and specific mode of ionization, and proceeded independently from the advancements on the FA and FDT systems. Although experiments that made use of reaction chambers at atmospheric pressure were reported in the 1960s, the acronym API first appeared in the early 1970s in the studies reported by

Horning, *Carroll* and coworkers [18.16, 17]. In their system, a ^{63}Ni radiation source was used to ionize N_2 and reference compounds being injected directly into a pure nitrogen carrier gas flow. This system was deemed to be particularly suited as an ultrasensitive detector for gas or liquid chromatography systems [18.18, 19]. An alternative device with a corona discharge ionization source made use of a curtain gas to promote declustering of ions before they entered the mass spectrometer, which critically allowed humid air to be sampled and ionized directly [18.18–20]. It might be noted that inclusion of the term *chemical* in API, i. e., atmospheric pressure chemical ionization (APCI) was generally adopted to better distinguish between CI systems operating at very high pressures and other high pressure ion sources such as plasma discharges [18.20].

The developments and diverse applications of APCI-MS are reported in a number of comprehensive review articles [18.20–24]. For the purposes of this chapter, however, a critical development in conceptualizing direct sample injection for APCI-MS, as required for on-line analysis, was made in the late 1970s by *Lovett* and coworkers, who interfaced a mouthpiece sampler to a commercial APCI-MS system – the Trace Atmospheric Gas Analyzer (TAGA (Sciex Inc., Toronto, Ontario, Canada)) – for the real-time analysis of volatiles in exhaled breath [18.25]. Their breath analysis dilution inlet system comprised of a mouthpiece through which breath gas was exhaled and mixed via a tapered glass pipette into a carrier gas flow from a pressurised zero-grade air source for subsequent introduction into the TAGA instrument. Using this system it was possible to perform direct analyses of breath ammonia, as well as other metabolites and endogenous compounds such as acetone, within seconds, which was a major breakthrough compared to the several hours required in conventional breath analysis involving sample workup, derivatization, column separation, etc. Further developments of this direct sample injection system were reported by *Benoit* and colleagues, who constructed a breath inlet system that consisted of filters, valves and a chamber to mix breath gas with the carrier gas before sample introduction into the ion chamber of the atmospheric pressure ionization mass spectrometer (API-MS) [18.26]. The major progress introduced by this development was that the interface eliminated the high ammonia and its associated interferences that had been observed in the earlier system devised by Lovett and colleagues, yet achieved sufficient sensitivity to detect other species such as methanol and ethanol in exhaled breath.

Alternative samplers using either a membrane [18.27] or fiber [18.28] to improve the interface for breath analysis applications of APCI-MS – specifically for food-flavor applications – were also reported, both showing promising results for novel real-time assessment of aroma compounds during food mastication. A further critical development in APCI-MS and its application in the detection of odorants occurred in the mid-1990s when *Robert Linforth* and *Andrew Taylor* at the University of Nottingham, UK constructed a new sampling interface for the direct analysis of breath, which they named MS-Nose, that overcame the shortcomings of previous attempts by offering a minimal dead volume and fast linear flow rates for sampling [18.29, 30]. Their system comprised of a short length of deactivated fused silica capillary sheathed in a tube carrying a flow of nitrogen such that breath gas was transferred via a Venturi into the corona discharge ionization source. The advantage of this system was that it enabled continuous distal sampling of breath at a constant rate and in real-time, without the need to regulate the exhaled breath flow, as was demonstrated by in vivo analyses of the release of volatile flavor compounds in food applications [18.30]. Subsequently, alternative interfaces for direct injection of APCI-MS for optimized sampling have emerged, similarly showing excellent functionality for food-flavor release studies [18.31].

It is perhaps worthy of note that all three techniques emerged as on-line tools for the dedicated application of detecting volatiles in gas samples (rather than their use to study ion-molecule reaction kinetics) at approximately the same time, partly owing to the great degree of interaction and exchange of ideas between the pioneers of these techniques.

18.1.2 Sampling and Measurement

On-line CIMS techniques are unique in their ability to sensitively detect a wide range of compound classes over concentrations spanning several orders of magnitude and down to ultratrace levels in real-time. The general aspects of sampling and measurement that are specific to on-line CIMS techniques are briefly discussed here.

Chemical Ionization

The International Union of Pure and Applied Chemistry (IUPAC) defines chemical ionization as *the process whereby new ionized species are formed when gaseous molecules interact with ions* and *may involve transfer of an electron, proton or other charged species between the reactants* [18.32]. Although chemical ionization (CI) can proceed via both positive and negative ionization, in general the term CI refers to positive-ion CI, whereas negative-ion CI is specifically stated as such, i. e., NICI. This chapter considers only appli-

cations of positive-ion CI, as applied in the three main on-line CIMS techniques discussed herein. (It might be noted that NICI is also routinely employed in APCI-MS, albeit not extensively in on-line APCI-MS for odorant-related applications.)

In CI, rather than ionizing neutral target molecules via the bombardment of energetic electrons as in EI, by photons as in photoionization, or through a high-potential electrode as in field ionization, the process of ion-molecule reactions are utilized, which take place at lower energies and thereby cause less fragmentation of the target molecules upon charge acquisition [18.18]. The ionization energies encountered in CI generally range from thermal energies to only a few eV; by comparison, the conventional EI in commercial GC-MS systems typically occurs at energies of $\approx 70\,\mathrm{eV}$. As such, CI is referred to as a *soft ionization* method to indicate the less energetic nature of ionization. A direct outcome of this soft ionization process, as opposed to the wide range of molecular fragments known to be produced by EI, is that it frequently produces the molecular – or quasi-molecular – ion in quantifiable abundance and, as indicated above, the characteristic mass spectra of target analytes obtained by CI are generally stable, with limited interferences and high reproducibility.

Ionization of neutral molecules by CI can occur in one of four modes:

1. Proton transfer
2. Charge exchange (or transfer)
3. Anion abstraction, and
4. Electrophilic addition [18.18, 33].

The three main on-line CIMS techniques discussed in this chapter make use of proton transfer and charge exchange reactions, therefore types 3 and 4 will not be dealt with further here.

Reagent Ions and Proton Affinity

A common feature of all of the three on-line CIMS systems discussed here is their predominant use of proton transfer reactions for the ionization of neutral target molecules, more specifically their use of H_3O^+ as the reagent ion species. It is therefore pertinent here to briefly introduce and discuss the concept of proton affinity (PA). The IUPAC defines proton affinity (for a species M) to be *the negative of the enthalpy change in the gas-phase reaction … between a proton (more appropriately hydron) and the chemical species concerned …* [18.32], i. e., in the reaction $M + H^+ \rightarrow [M + H]^+$ *at a specified temperature, usually* 298 K [18.34]. Alternatively, it can be considered as the energy required to separate a proton, H^+, from a species, M, in the reaction $MH^+ \rightarrow M + H^+$. Broadly speaking, if a neutral molecule, M, encounters a protonated reagent, RH^+, the proton will be transferred to the neutral, i. e., $RH^+ + M \rightarrow MH^+ + R$ on the condition that the proton affinity of M is greater than that of R. A great benefit of employing proton transfer is that the reactions are exothermic, proceeding rapidly and with similar reaction rates that are close to the collisional rate of several $10^{-9}\,\mathrm{cm^3/s}$ [18.3, 35]. Moreover, these reactions generate singly charged product ions that are stable and rarely undergo further decomposition. By comparison, endothermic reactions are comparatively slow and less suitable for on-line applications.

The use of hydronium as the reagent ion species in proton transfer reactions is particularly propitious for the detection of VOCs in air because the proton affinities of the major constituents of air, i. e., N_2, O_2, CO_2, Ar, etc., are less than that of water and as such these neutral molecules do not undergo ionization upon collision with H_3O^+. Conversely, the proton affinities of most VOCs are greater than the PA of water – as summarized in Fig. 18.1 and compiled in the literature [18.36, 37] – and are thus ionized upon every collision. This fortuitously means that air (or pure N_2) can be used as the sample carrier gas without the need for prior dilution or filtering. Moreover, the high water vapor content in humid gas samples – such as exhaled breath – does not pose a problem in the analysis and can be accounted for by careful control and reduction of the amount of water entering the source [18.30], knowledge on the proton transfer and ligand switching reactions involving hydrated hydronium ions [18.38], or adequate calibration of the instrumentation [18.39, 40].

As indicated above, SIFT-MS, as well as an adapted PTR-MS system employing the selected (switchable) reagent ion (SRI) feature [18.43], make use of reactions from NO^+ or O_2^+ precursor ions. Whereas the reactions involving O_2^+ reagent ions proceed exclusively via charge exchange, several additional reaction processes can occur with NO^+, namely hydride, hydroxide or alkoxide ion transfer, or ion-molecule association reactions [18.44]. Charge transfer reactions from O_2^+ are highly energetic and produce multiple fragments of neutral analytes, making their use less suitable for general VOC analysis. However, the benefit of CI via O_2^+ – particularly with reference to odorant detection – is in their ability to ionize small molecules such as ammonia, NH_3, and carbon disulfide, CS_2, which are otherwise not detectable via PTR using hydronium ions. Additionally, charge exchange reactions from O_2^+ allow for the detection of some alkanes such as methane and pentane [18.44]. Ionization via NO^+ precursor ions results in the generation of unique fragmentation patterns for certain isobaric species, thereby aiding compound iden-

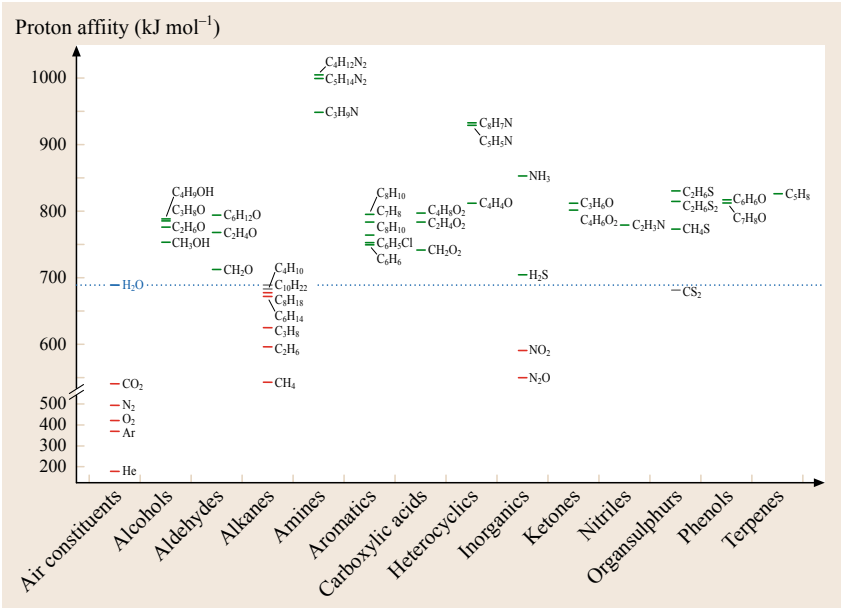

Fig. 18.1 Proton affinity (PA) scale indicating the position of water amongst the major constituents of air and selected (odorant) volatile organic classes (Fraunhofer IVV). Values taken from [18.37], [18.41] (alkanes), [18.42] ($C_6H_{12}O$, hexanal), and [18.38] (C_4H_4O, furan; C_7H_8O, *p*-cresol)

tification when used in combination with CI via H_3O^+ and O_2^+. Further discussion on the ion chemistry of the reactions involving O_2^+ or NO^+ with neutral molecules will not be included here but can be found in the literature [18.9, 43, 44]. Importantly, however, the combined use of these three precursors for the ionization of neutrals extends the analytical range and can improve compound identification, since techniques that use single reagent ions cannot discriminate between isobaric or isomeric analytes. Due to their limited and rather specific use, chemical ionization via other reagent ions such as Kr^+ or Li^+ will not be treated here, but can be found in the corresponding literature [18.45, 46].

Gas Sampling

By definition, on-line CIMS techniques allow for the real-time detection of volatile compounds present at trace levels in a gas matrix such as air. Correspondingly, air or other gases can be sampled directly without the prior need for dehydration or enrichment. This warrants an inlet sampling system that allows for a continuous delivery of the sample gas into the ionization or reaction chamber, which is commonly achieved by use of a small-diameter sampling line capillary – with inner diameter on the order of 0.25–1.5 mm – typically made of an inert material such as Teflon (perfluoroalkoxy (PFA) or polytetrafluoroethylene (PTFE)), polyether ether ketone (PEEK), or passivated stainless steel such as Silcosteel. The inlet line is generally heated to above-ambient conditions, ranging from around 40 to up to 150 °C to avoid condensation or compound adsorption within the sampling tubes. This offers the advantage that thermally labile compounds reach the ionization chamber intact, although compounds with low vapor pressure might adhere to the inner surfaces of the inlet system even at such elevated temperatures; these are typically oxygenated species such as large terpenoids and sesquiterpenes, furans, and carboxylic acids, amongst others. The flow of sample gas through the inlet system is usually variable, ranging from several tens of milliliters to a few liters per minute. The choice of sample gas flow depends on the measurement application: typically, slow flows are used to limit the perturbations of the sample equilibrium at source whereas fast flows might be employed in the assessment of rapid processes to minimize the residence time of a sample within the inlet system and effectively increase the detection response time.

The evaluation of odorant emissions from physical samples is generally achieved by sampling the headspace gas – that is, the gas immediately above and surrounding the sample – and analyzing its constituent odor compounds. In *static headspace* analysis the sample under investigation is placed within a closed container such as a glass vial under defined conditions and, after a period of equilibration, sample gas is withdrawn and analyzed accordingly. Although this mode of analysis provides relevant information by *fingerprinting* constituent odorants and quantifying relative abundances, it offers limited insights into the dynamics of emissions processes. The dynamic profiles of odorants released from specific sample matrices is of interest in many disciplines, for instance in flavor research for the aroma release from food, or in indoor air quality for the

emissions of odorants from building materials, as well as in several other applications described in this chapter. Although direct-injection GC-MS exists in the form of portable instruments, the standard benchtop GC-MS approach for such analyses is to perform intermittent gas sampling of the headspace gas of a solid or liquid sample, for example via purge-and-trap onto adsorbents or into vessels, or by adsorption onto fibers as in solid-phase microextraction (SPME). A further desorption step is subsequently required to release the analytes into the carrier gas and the chromatographic column, thereby creating an inherently discontinuous depiction of the dynamic release. In *dynamic headspace* analysis using on-line CIMS the headspace gas of a sample is measured continuously under defined conditions to generate a more detailed picture of the release kinetics. This uninterrupted sampling and the lack of the chromatographic step thus affords the dynamic analysis of a sample in a highly time-resolved fashion that is not attainable with GC-MS.

Measurement Principles and Modes

The majority of on-line CIMS instruments are equipped with a quadrupole mass spectrometer (QMS) which operates by means of establishing a defined electromagnetic field along the axis of the filter rods that allow specific ions – or rather, ions of specific m/z – to pass through to the detector. Accordingly, only single m/z per setting are transmitted through the mass spectrometer. This imposes a limitation on the detection speed, since the individual setting for each m/z and corresponding product ion must be cycled through sequentially, each for a minimal detection time on the order of a few tens of milliseconds, depending on the system and the abundance of the target compound. This is commonly referred to as the duty cycle of the QMS. These quadrupole-based systems can be operated in one of two modes, namely on an individual ion detection basis referred to as selected ion detection (SID), selected ion monitoring (SIM) or multiple ion detection (MID) mode (subsequently referred to as SID mode), or by a full scan of individual m/z over a predefined m/z range, known as *scan* mode. Each mode of analysis is suited to specific applications.

SID mode is generally used when the compounds are known a priori and allows for targeted dynamic profiling of single or multiple analytes. This analysis mode offers the benefit of a reduced sampling time, since only selected analytes are targeted. In addition, if only a few analytes are selected then the instrumental detection limit can effectively be reduced by extending the integration or dwell time of the MS, without compromising the detection time to any great extent. The integration time is the period for which the quadrupole filter is set to select a specific m/z value: extending this period increases the signal-to-noise ratio by allowing more time for trace analytes to reach the detector and correspondingly reduces the statistical noise of the signal at that particular m/z. Although a longer dwell time can be imposed in measurements made in scan mode, this introduces an extended analysis period due to the accumulation of the individual dwell times over the entire scan period.

Scan mode is typically employed when analyzing gas samples with unknown constituents. In this mode, the entire mass spectrum within a defined m/z range is measured, usually repeatedly, to provide clues to constituent compounds. Scans can be made continuously for monitoring purposes, albeit with a slower time resolution of measurements compared to SID mode, depending on the dwell time and m/z range selected. Generally, mass scan measurements are useful for full mass spectral fingerprinting of samples and are often accompanied by the subsequent application of data-mining tools to identify distinctive MS patterns that specifically relate to the sample properties of interest. This application is discussed in more detail in Sect. 18.2.1.

Replacement of the QMS with a time-of-flight (TOF) mass spectrometer has been tested for APCI-MS [18.30] and is available as a standard configuration in PTR-MS, i. e., PTR-TOF-MS [18.47]. TOF mass spectrometers rely on injecting an ion swarm into an electromagnetic field-free region under high vacuum. The ions enter the flight chamber with equal initial kinetic energy but become separated in space and time as they traverse the chamber due to their different velocities. This phenomenon allows the ions to be counted at the detector on the basis of their arrival time, that is, by their time of flight. The inherent difference between TOF-MS and QMS is that in TOF-MS only the *scan* mode of measurement exists per se, in that there is generally no filter for preselecting individual ions. Instead, all ions are pulsed into the flight chamber and it is a matter of the settings of the pulse-counting electronics as to which defined m/z range is included in the data acquisition and subsequent processing. This offers the distinct advantage that all product ion signals within the measurement range are registered without the need for their selection a priori, as is required in QMS. The rapid pulsing and flight times result in scan times of fractions of a second, thus entire mass spectra are gathered much more rapidly than in QMS [18.48]. Furthermore, the highly accurate registration of flight times afford a greater mass-resolving power that enables many isobaric compounds – that is, compounds with the same nominal mass but different chemical composition – to be separated [18.49, 50].

A third, alternative option to the use of QMS or TOF-MS is an ion trap MS. IT-MS-based systems offer distinct advantages over QMS, primarily related to the extended analytical range of several hundred m/z almost simultaneously, as well as the possibility to initiate collision-induced dissociation (CID) reactions that enhance the resolution by generating characteristic fragmentation patterns and thereby allow for isobaric compound separation in many cases. The details of IT-MS will not be discussed here and the reader is referred to several review papers on the topic [18.51–54], but it should be noted that IT-MS has been employed in some of the on-line CIMS techniques discussed here, from prototype to commercial instrumentation, notably APCI-ITMS [18.31] and PTR-ITMS [18.55–57].

18.1.3 On-line CIMS Techniques

Chemical ionization is exploited in a diverse number of on-line analytical instruments that utilize mass spectrometry, but in terms of prevalence three systems in particular have been at the forefront of scientific research over the past two decades, namely SIFT-MS, PTR-MS and APCI-MS, and are thus at the focus of this chapter. Schematic representations of each instrument are presented in Fig. 18.2.

Although these three techniques have found use in most of the branches of odor detection outlined in this chapter, historically each can be considered to dominate a specific field: SIFT-MS in breath gas analysis for medical purposes, PTR-MS in the environmental sciences and varied niche applications, and APCI-MS for food and flavor-related studies. This will become apparent in the weighting of citations to related studies throughout this chapter. Nevertheless, in recent years each of these CIMS techniques has diversified and proliferated in these major sectors, as well as other niche areas, as is evident in the ensuing discussion. Since the focus of this chapter is on the application of on-line CIMS to odorant detection, technical details of their operation is kept to a minimal here and the reader is referred to the cited literature for more detailed information.

A comparative review of the performances of APCI, SIFT, PTR-MS for the rapid monitoring and quantitation of biogenic VOCs of interest in food, medical and environmental sciences has been published [18.58], summarizing the key technological differences and providing examples of applications to these fields. Table 18.1, adapted from the above, provides a useful overview of the main technological features and differences between the three on-line CIMS techniques reviewed in this chapter.

Selected Ion Flow Tube Mass Spectrometry, SIFT-MS

Selected ion flow tube MS, SIFT-MS, combines the use of proton transfer reactions from H_3O^+ and charge exchange reactions from NO^+ and O_2^+ for the detection of gas-phase organic compounds in diverse applications. Unlike proton transfer reactions, charge transfer involved in the reactions of neutrals with NO^+ or O_2^+ are more energetic and generally incur a greater degree of fragmentation of the target molecule. However, this is a desirable effect that is exploited in SIFT-MS to separate isomeric species and improve compound identification in the mass spectra of unknown gas mixtures based on known fragmentation pathways. Generation of the three main precursor ions is achieved in an external ion source – typically a microwave resonator – from which the reagent ions are selectively transferred into a flow tube via a preselection QMS (Fig. 18.2a) [18.11]. At the exit of this mass filter these primary ions are injected into a fast-flowing inert carrier gas – usually pure He at ≈ 100 Pa (or ≈ 1 Torr) – via a Venturi-type orifice and travel as a thermalised ion swarm along the length of the flow tube. Sample gas containing the neutral target compounds is added to the downstream flow and these constituents interact and react with the reagent ions to form positive products that allow for subsequent mass filtering and detection via a channeltron multiplier/pulse counting system [18.38]. The early use of SIFT-MS to study the ion chemistry of ion-neutral reactions has generated a database containing kinetic data on thousands of ion-molecule reactions, which is of great value in understanding the mass spectra produced when detecting trace gas constituents and offers a means for broad quantitation of these compounds from a theoretical approach, without the imperative need for calibration.

The literature contains a wealth of information on the operational aspects and ion-neutral kinetics taking place in SIFT-MS and the interested reader is referred to these for more detailed reading material [18.11, 38, 44].

Proton Transfer Reaction Mass Spectrometry, PTR-MS

Proton transfer reaction MS, PTR-MS, traditionally makes use of proton transfer reactions from hydronium reagent ions to neutral target molecules [18.13], although the recent introduction of the selective (or switchable) reagent ion (SRI) feature of the commercial instruments has broadened the ionization regime to alternatively utilize charge exchange reactions via NO^+ or O_2^+ (as in SIFT-MS) and other reagents such as Kr^+ [18.43, 45, 61]. The PTR-MS instrument comprises a separate front-end ion source to gener-

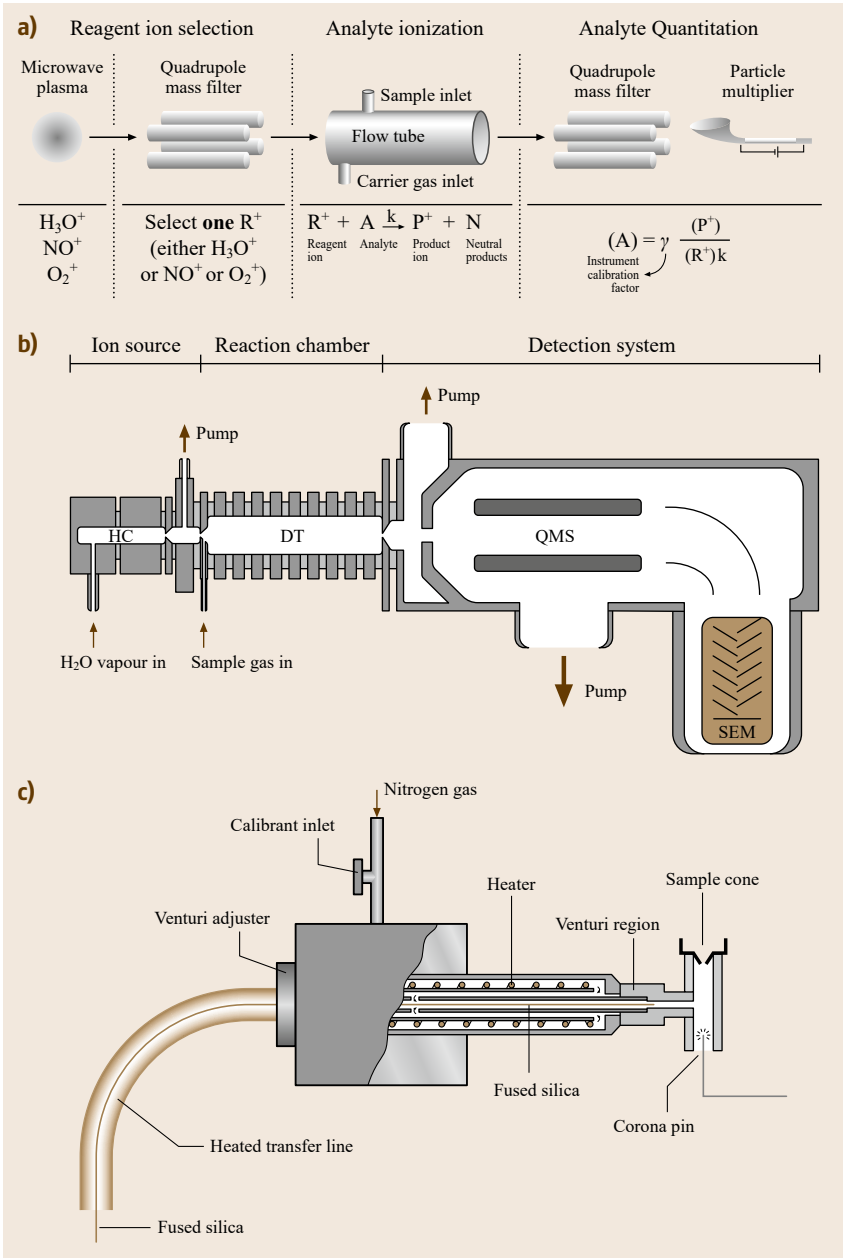

Fig. 18.2a–c Schematic representations of the on-line CIMS techniques of **(a)** SIFT-MS, **(b)** PTR-MS (HC = hollow cathode, DT = drift tube, QMS = quadrupole mass spectrometer, SEM = secondary electron multiplier), and **(c)** APCI-MS. Reproduced with kind permission from **(a)** Syft Technologies Ltd., New Zealand, **(b)** Fraunhofer IVV, Germany, and **(c)** Waters Corporation, UK and Robert Linforth, University of Nottingham, UK

ate the necessary reagent ions that are subsequently transferred to a flow drift tube (FDT) region wherein they encounter the neutral analytes of the sample gas (Fig. 18.2b). Reagent ion generation is typically achieved to a high degree of purity ($\geq$ 99.5%) using a hollow cathode discharge ion source [18.13], although direct current discharge [18.59] and ^{241}Am radioactive ion sources also exist [18.60].

Sample gas containing the volatile targets at trace concentrations is added to and flows through the FDT of the PTR system whereupon it encounters the reagent ions emerging from the ion source that drift along the tube by means of the applied electric field, typically at 40–60 V/cm. Proton transfer reactions occur upon every collision of a hydronium ion and neutral analyte within the sample gas and the ionized target molecule is subsequently subjected to the electric field and drifts with the precursor ions axially along the reaction chamber. Ions reaching the end of the drift tube are channeled by means of ion transfer lenses into the

Table 18.1 Comparative overview of the main features of SIFT-MS, PTR-MS and APCI-MS (after [18.58])

Feature	SIFT-MS	PTR-MS		APCI-MS
		PTR-QMS	PTR-TOF-MS	
Ion source	Corona discharge	Hollow cathode discharge[a]		Microwave discharge
Reagent ion[b]	H_3O^+, NO^+, O_2^+	H_3O^+ (NO^+, O_2^+)[c]		$(H_2O)_nH^+$
Buffer gas	He	Air (sample matrix)		N_2 (dry)
Sample dilution	Yes	No		Yes
Reaction region	Fast flow tube	Flow drift tube		None
Mass analyzer	Quadrupole	Quadrupole	Time of flight	Quadrupole, QqQ
Detector[d]	SEM	SEM, MCP[e]	MCP	SEM

[a] Direct current discharge [18.59] or radioactive α-particles [18.60] have also been reported.
[b] Refers only to positive ionization mode: negative ionization is available in both SIFT-MS and APCI-MS.
[c] NO^+ and O_2^+ modes are available through use of a selectable reagent ion (SRI) interface [18.43].
[d] SEM, secondary electron multiplier; MCP, microchannel plate; QqQ, triple-quadrupole
[e] Most PTR-QMS instruments use an SEM, but some are equipped with an MCP.

mass spectrometer, where they are separated according to their m/z and transferred to a detection system. The conventional PTR-MS systems operate a quadrupole mass filter – and are thus sometimes also referred to as PTR-QMS – but recent adaptations have included coupling the PTR reaction chamber with a time-of-flight (TOF) mass spectrometer to create a PTR-TOF-MS [18.47, 62]. This development offers the distinct advantages of TOF mass spectrometry to the PTR technique, namely a high mass resolving power – at least three orders of magnitude greater than the conventional PTR-QMS system – and the rapid (instantaneous) analysis of a complete mass spectrum. The detection of ions passing through the mass filter is achieved via a secondary electron multiplier (SEM), in PTR-QMS or a microchannel plate (MCP) in PTR-TOF-MS. It is also worthy of note that the combination of PTR with ion trap mass spectrometers has been investigated, but since no broad application to odorant detection has been made it will not be further discussed here: the interested reader is referred to the literature reports on these developments [18.55–57, 63, 64].

The use of a drift tube, i. e., the application of an electric field to the flow tube, allows the ionization energy to be controlled to some degree, with typical ion kinetic energies of 1−2 eV. The reaction rates for proton transfer reactions are generally well known from literature reports or from theoretical calculations based on the polarizability and permanent dipole moment of the neutral targets. This enables quantitation of the neutral analytes via calculation, although the accuracy can be greatly improved to between ±15−30% by calibration of the system [18.35, 65]. In this context, it should be noted that the primary gas composition of the sample being analyzed can affect the ion energetics within the FDT reaction chamber due to altered ion mobility. Unlike SIFT-MS or APCI-MS, which employ high flows of separate buffer gas comprising He or N_2, respectively, in PTR-MS the sample gas itself acts as the buffer gas. As such, the choice of sample gas composition is of importance. To give an example, exhaled breath or the exhaust gas from a fermentation tank contain elevated levels of carbon dioxide and water vapor, typically several per cent, both of which are known to affect the ionization processes and lead to clustering within the drift tube [18.66]. Although this is a critical issue, such changes can easily be accounted and corrected for by appropriate normalization of the signal response at the detector [18.67, 68] or by calibration of the instrument using the same primary gas matrix composition [18.39].

Further details on the operating principles of PTR-MS and the varied applications of this technique can be found in review articles [18.35, 69] and in a recently published comprehensive textbook on PTR-MS [18.70].

Atmospheric Pressure Chemical Ionization Mass Spectrometry, APCI-MS

Atmospheric pressure chemical ionization MS, APCI-MS, in the context of this chapter, is a technique that employs proton transfer reactions from protonated water reagents for the chemical ionization and subsequent detection of VOCs. In APCI the ions undergo thermalization by soft collisions that effectively offer a channel drain for the excess energy in the exothermal protonation reaction [18.33]. As such, APCI is typically a softer ionization process than in conventional CI and is especially sensitive for the detection of basic compounds (referring to gas-phase basicity), or those with low ionization energies; conversely, APCI is insensitive to many nonbasic compounds and these are often not detectable [18.3]. Although different manifestations of APCI-MS have found widespread use as detectors in liquid and gas chromatography (LC and

GC, respectively), the on-line APCI-MS technique that is discussed here is a system that enables the direct, continuous and real-time analysis of gas-phase volatiles.

Unlike SIFT-MS and PTR-MS, APCI-MS does not include a separate ion source but rather the process of precursor ion formation, charge exchange and clustering proceeds in one small region [18.71]. Sample gas is introduced into the reaction chamber via a Venturi nitrogen flow that ensures adequate dilution and control of humidity in the sample gas (Fig. 18.2c). The mixing ratio of this flow is approximately 5–50 ml/min sample gas into 10 l/min nitrogen flow [18.30]. The reagent ion plasma within the reaction region is generated and maintained by a corona discharge between a corona pin and the chamber that serves as the counter electrode, which is typically held at 4 kV [18.33]. Precursor ions are formed in this discharge region by the ionization of the sample air constituents to predominantly form hydronium ion water clusters, $H_3O^+(H_2O)_n$ [18.72], which subsequently protonate neutral organic target analytes present in the sample gas according to the conditions imposed by their proton affinities, as discussed earlier. Reagent and product ions are then transferred into the detection system via a mass filter, usually a QMS, although tests with TOF-MS and IT-MS have also been performed [18.30, 73]. Improvements in sensitivity for the detection of compounds in humid gas samples – specifically in relation to food aroma compounds measured in exhaled breath (i. e., nosespace analysis; Sect. 18.2.1) – have been achieved by humidifying both the sheath and auxiliary gases (relative humidity 88–98%) [18.74]; compared to dry conditions, where strong fragmentation of the target molecules is observed, humidification results in the production of the protonated parent ion in the majority of compounds tested.

Technical details of the construction of on-line APCI-MS and the processes of ionization and detection, as well as its applications, can be found in the literature [18.30, 31] and in patent documents [18.29].

Other On-line CIMS Techniques

Ion molecule reaction MS, IMR-MS, is an on-line CIMS technique that closely reflects the operating principles of SIFT and PTR. Unlike those methods, however, IMR-MS utilities primary ions generated from inert gas – either Hg, Xe or Kr – via EI for subsequent ion-molecule reactions with neutral analytes [18.75, 76]. A preselection mass filter downstream of the ion source, similar to SIFT-MS, is used to channel a high-purity ion beam into the IMR chamber. The technique was developed at the University of Innsbruck, Austria and, like PTR-MS, was derived from the SIFDT-MS studies on ion-molecule reactions undertaken in the Institute of Ion Physics laboratories in the early 1990s. IMR-MS has achieved good commercial success as a trace gas analyzer, particularly in the automotive engineering industry for exhaust gas emissions measurements and in the beverage industry as a rapid quality-control tool for monitoring impurities in recycled bottles, to name but a few. The direct application of IMR-MS in the detection of odorous compounds per se is limited to date, thus this CIMS-based method will not be discussed further in this chapter.

Ion mobility spectrometry, IMS, is a method for characterizing chemical substances according to their gas-phase ion mobility [18.77]. Chemical ionization is induced by collisions of the analyte with ionized carrier gas molecules (N_2 or air), mainly produced by radioactive β-radiation sources. Strictly speaking, IMS is not a mass spectrometric system in the classical sense in that it does not involve the discrete separation of charged molecules by a mass filter, but rather utilizes the combined properties of the mobility and m/z of analyte ions. The analysis is achieved by subjecting the analyte ions – generated in a front-end ion source – to a drift tube of fixed length and defined electric field strength at ambient pressure and determining their drift time, which is proportional to the inverse of their mobility. An ion swarm drifting under such conditions experiences a separation process that is based, amongst other instrumental parameters, on the dimension, structure and ion mobility constant of the individual ions, dictating their drift velocities. The ions are then detected upon impaction on a terminating Faraday plate. Because of the structural dependencies it is possible to separate isomeric (but not enantiomeric) ions, thus the resulting ion mobility spectrum contains information on the nature of the different trace compounds present in the sample gas. Due to the occurrence of ion-molecule reactions and the relatively poor resolution of the species formed, IMS is generally not used to identify unknown compounds, but it is being increasingly applied to cases where the volatiles investigated are known. It has been used extensively in the detection of various species, including odorants, in diverse fields of application, as is indicated in the relevant sections of this chapter, and is in widespread use for safety and security applications [18.78]. For more details on the IMS technique the interested reader is referred to the review articles available in the literature [18.79, 80].

Ion attachment mass spectrometry, IAMS, is a less prevalent CIMS technique that utilizes lithium ion (Li^+) attachment to produce mass spectra consisting solely of quasi-molecular ions [18.46, 81]. The method was originally developed by adapting a commercial APCI-MS system to include a lithium ion source to produce Li^+ for subsequent use in the chemical ioniza-

tion reaction chamber [18.81, 82]. Li^+ IAMS has been successfully demonstrated to detect organic compounds at trace concentrations in air, including the detection of aroma compounds from strawberries [18.83], as is briefly discussed in Sect. 18.2.1.

Hybrid Techniques

A major drawback of the on-line CIMS techniques is the inherent difficulty in compound identification within the mass spectrum of complex gas matrices such as food headspace, exhaled breath or environmental odor plumes, i. e., gas mixtures in which the constituent volatiles are not known a priori. This is chiefly due to the limited amount of chemical-structural information inferable from the m/z values of quasi-molecular ions or their isotopic distribution patterns, but is also a consequence of the low (nominal) mass resolution of the QMS fitted in most CIMS instruments, causing a certain degree of signal overlap from individual isobaric compounds or fragments. Furthermore, the lack of a chromatographic separation step prevents the elucidation of stereoisomeric odor-active compounds, since enantiomeric VOCs cannot be resolved by CIMS.

Although compound identification can be aided by the use of different precursor ions, for example as in SIFT-MS [18.84] and in PTR-MS operating with the SRI feature for the discrimination of isomeric compounds [18.60], or by the implementation of high resolution MS systems as in PTR-TOF-MS for the separation of isobars [18.47], the unequivocal identification of many (or most) compounds within complex VOC mixtures is seldom achievable by CIMS alone. The developments of hybrid techniques that interface GC systems to CIMS instruments have addressed this issue, although this is invariably achieved at the cost of the fast time resolution and real-time detection. A brief report on these developments is given, but will not be discussed at length in view of their limited reported use in odorant detection to date.

Gas chromatography (GC) is the analytical gold standard for odorant detection. The technique is discussed in detail in another chapter of this book (Chap. 17), but its key features are briefly recapitulated here. In GC, constituent gas-phase molecules in a sample become separated as they are purged through a chromatographic column. This flow contains intermittently eluting compounds that are continuously transferred to an MS in which individual compounds undergo EI (or CI, depending on the application and analytical intention) to produce characteristic mass spectra that are matched with well-defined elution times. The combined knowledge of this time, referred to as the *retention time* – or in combination with reference compounds, the *retention index* – and the fragmentation pattern allows for compound identification to a high degree of reliability. The characteristic EI mass spectra of thousands of VOCs are well known and available in diverse libraries, as are the retention indices of numerous compounds within defined chromatographic columns. An additional feature for odorant detection is GC-olfactometry (GC-O) in which the flow exiting the column is split in parallel to the detector (an MS or a flame ionization detector (FID)) and to an odor detection port that allows olfactometric detection of the eluting odorant by the human nose, thereby providing an additional dimension in characterizing each compound in terms of the odor impression it elicits.

The advantages offered by GC-MS make this technique an obvious choice for coupling with on-line CIMS techniques. This combination has been made for SIFT-MS [18.85], PTR-MS [18.86–88] and APCI-MS [18.89] with good success, but the downside of these systems in the past was the inherent loss of the real-time detection feature. An alternative hyphenated system that was recently developed coupled a short GC column to a PTR-TOF-MS instrument and allowed for inline switching between direct and GC analysis, with chromatographic separation possible within 90 s due to the resistive heating capability of the column at a rate of 30 °C/s [18.90]. This *fastGC* approach offers a promising compromise between obtaining real-time data and achieving compound identification. In the aforementioned study, the system was used to analyze volatiles present in the headspace of wine, with GC separation indicating that several groups of isomeric (aroma) compounds were present in the wine headspace. Furthermore, channeling the sample via the fastGC interface offered the additional advantage of 'removing' ethanol – which commonly causes a problem in PTR-MS analyses of alcoholic beverages when present in high amounts due to sequestration of reagent ions [18.91, 92]; see Sect. 18.2.1 – due to its early elution from the column, thus allowing for the subsequent unhindered detection of trace aroma compounds. This fastGC-PTR-TOF-MS system has also been demonstrated to be suitable for the headspace analysis of other beverages, as discussed in a recent review article [18.93]. Another alternative GC-type interface approach is the use of a multicapillary column (MCC), analogous to that used in IMS [18.94]. The MCC is constructed of approximately 1000 single, tightly packed capillaries for parallel separation of volatiles in a gaseous sample. This construction allows sufficient gas flow for directly interfacing with PTR-MS without the need for an additional carrier gas flow and offers a complete analysis every 5–10 min. The coupled MCC-PTR-TOF-MS system has been successfully demonstrated in a number of applications, including the

unambiguous detection of individual aldehydes within a mixture, as well as the separation of ketone isomers [18.94].

18.1.4 Instrumental Performances

The capability and suitability of a given analytical method for its intended task is typically expressed in terms of its performance parameters. For the purpose of this chapter this is defined as the ability of an instrument to detect a substance of interest at a given concentration level and within the dynamic range of measurement. The most important performance parameters for the on-line CIMS techniques reviewed here are defined in the following.

Definitions

Sensitivity is a measure of the intensity of the signal response to a given stimulus. For the on-line CIMS techniques discussed here the stimulus is a VOC at a specified concentration and the signal response relates to the counting unit of the detection system, typically given as a current, for example milliampere (mA), or converted to a response frequency in counts per second (cps) or Hertz (Hz). The sensitivity is then expressed as the response per unit concentration, thus is typically reported as cps ppb_v^{-1} or similar; in this notation the sensitivity represents the signal intensity in cps for the detection of a 1 ppb_v stimulus of the target compound. Sensitivities are compound-dependent and can be determined via calibration of the system using certified gas standards containing the compounds of interest [18.39].

Limits of detection and *quantitation* (LOD and LOQ, respectively) give a measure of the lowest analyte concentration that can be reliably detected and quantified. The LOD of on-line CIMS instruments is generally defined as the minimum gas-phase concentration at which a compound elicits an instrumental signal that can be significantly distinguished from instrumental noise, the latter being the signal in the absence of a stimulus. Typically, this is determined by measuring blank gas matrices containing no VOC analytes, and the resulting instrumental signal is referred to as the noise level; the LOD is generally given as three standard deviations above the noise, i. e., a signal-to-noise ratio of 3. By comparison, the LOQ is defined as the minimum analyte concentration that can be reported with a defined confidence level, commonly 95%. LOQ is typically stated as 3.3 × LOD, or 10 times the standard deviation of the blank. For the on-line CIMS described here, LOD and LOQ are VOC-dependent but are mostly in the ppt_v to ppb_v range.

Dynamic range (of measurement) is the concentration range of a given analyte that is linearly proportional to the instrumental signal response: for the on-line CIMS techniques considered here it spans from the LOD (ppt_v or ppb_v) to a few tens of ppm_v, above which the ion-molecule reactions that underlie these methods might deviate from the prescribed kinetics and yield compromised results.

Mass resolving power is a parameter of the mass spectrometer, indicating the ability to distinguish between ions of different exact mass. The commonly adopted definition of mass resolving power R is based on the full-width at half maximum (FWHM) of the mass spectral peak and is given by

$$R = \frac{m_{\text{nominal}}}{\Delta m_{\text{FWHM}}} \,. \tag{18.1}$$

For two ions with nominal peak centers at m_{nominal}, the term m_{FWHM} represents the minimum separation of peak centers at FWHM that allows the two ions to be clearly discriminated. Note that R is mass-dependent and thus varies with the compound measured. Since the on-line CIMS systems reviewed here are commonly fitted with quadrupole mass filters (with the exception of PTR-TOF-MS, PTR-ITMS and APCI-ITMS), the achieved mass resolving power is limited to unity, that is, only integer m/z are measured and thus nominally isobaric compounds cannot be resolved. An exception is offered by PTR-TOF-MS, whereby the TOF-MS affords a mass resolving power of several thousand (> 5000–10 000, depending on the system [18.95]); thus, provided the separation between the FWHM of two neighboring peaks is sufficiently large, the center of the peaks yields the measured mass with an accuracy sufficient for determining the chemical composition of many volatile (odor) compounds [18.49, 50]. Likewise IT-MS-based systems such as APCI-ITMS allow for greater resolution due to the MS^n capabilities [18.31, 74, 96].

Specificity relates to the ability of a technique to accurately (unambiguously) detect a specific compound. This poses problems in PTR-QMS and APCI-MS due to their employment of a quadrupole mass filter, which imposes limitations in the ability to separate isobaric and isomeric species (cf., *mass resolving power*). Although SIFT-MS also incorporates a QMS, separation of such species is achieved to a reasonable degree of success by the interchangeable use of NO^+ and O_2^+ (together with H_3O^+) reagent ions for charge exchange reactions of analytes, which typically produce varying and distinguishable fragmentation products. PTR-SRI-MS makes similar use of these alternative reagent ions to the same effect. PTR-TOF-MS offers separation of isobaric species due to its high mass resolving power. A combined PTR-SRI-TOF-MS system thereby makes the unambiguous detection of many isomeric and isobaric

Table 18.2 Compound classes of volatiles that are generally detectable by SIFT-MS, PTR-MS and APCI-MS, including example compounds and their descriptive characteristic odor attributes

Compound class	Subgroup	Example compound[a]	Typical odor attribute
Inorganic		Ammonia[b]	Ammonia, pungent
		Hydrogen sulfide	Rotten eggs
Alcohols		Methanol, ethanol, butanol	Ethanolic, alcohol
Aliphatic hydrocarbons[b]	Alkanes	Methane, ethane, propane, butane, pentane, hexane	odorless, gasoline
Amines		Trimethylamine, putrescine, cadaverine	Fishy, putrid
Aromatic hydrocarbons	Single-ring	Benzene, toluene, xylene	Gasoline
	Chlorinated	Chlorobenzene, tricholoroethene	Chlorine, solvent
Carbonyls	Aldehydes	Formaldehyde[b], acetaldehyde, hexanal, octanal, 2-methylbutanal	Green/grassy, soapy/citric, malty
	Ketones	Acetone, 2,3-butanedione	Solvent, butter, sweet
Carboxylic acids		Formic, acetic, butanoic acid	Acidic, vinegar, rancid
Esters		Butyl acetate, butyl formate	Fruity, flowery
Heterocyclic hydrocarbons	Aromatics	Pyridine, indole, skatole	Fecal
Nitrogen oxide[c]		NO_2, N_2O	Pungent, irritating, sweet
Phenols		Phenol, *p*-cresol	Ink, fecal
Organosulfur	Reduced sulfur	Carbon disulfide	Rotten eggs
	Disulfides	Dimethyl sulfide, disulfide, trisulfide	Decaying cabbage, garlic
	Thiols	Methanethiol	Rotten, sulfuric
Terpenes, terpenoids		Isoprene	
	Monoterpenes	α-Pinene, (R)-/(S)-limonene, 3-carene	Woody, citric, sharp-pungent, musty
	Sesquiterpenes	Caryophyllene, farnesene	Carrot, parsley, earthy

[a] Common compound names are given.
[b] Compounds not detectable by proton transfer from H_3O^+ due to their lower/similar proton affinities to water. This includes alkanes of chain length $< C_8$, alkenes, cycloalkanes of chain length $< C_5$, ammonia, formaldehyde, amongst others. Such compounds may be detectable via charge exchange using O_2^+ and/or NO^+ (i. e., in SIFT-MS and PTR-SRI-MS)
[c] Not detectable.

species possible, in principle; an addition of the fastGC system extends this ability further. APCI-ITMS makes use of the MS^n feature of the ion trap for fragmentation under well-defined conditions, with many odorants demonstrating unique fragmentation, thus making their unambiguous detection possible [18.97].

Accuracy reflects the proximity of a reported measured value to the estimated *true* value. This is generally assessed by means of a calibration of the system. Calibrations of on-line CIMS are typically performed by repeated measurements of VOC standards carrying certified purity and concentrations, which are sequentially diluted with a VOC-free carrier gas to produce well-defined concentrations of the analyte [18.39]. The latter procedure is also adopted for establishing the quantitative performance, or sensitivity, of an instrument, i. e., to assess the instrumental response to a given VOC stimulus concentration. For the on-line CIMS techniques included here, the quantitative accuracy of known and unknown analytes ranges from approximately ± 10 to $\pm 50\%$ depending on the technique and whether or not an external calibration is performed by means of certified VOC standards. In this sense, an accuracy of $\pm 10\%$ indicates that the reported concentration measured is within 10% of the true concentration.

Precision is a measure of the agreement among replicate measurements of a reference sample under prescribed and repeatable analytical conditions. Alternatively, it is the degree of deviation from a continuous stimulus at a constant concentration, for example by fluctuations in the stability of the signal. Since VOC measurements by on-line CIMS systems typically do not entail sample workup steps, the precision is mainly affected by the sampling mode, for example dynamic versus static headspace. A precision of, say, 2% indicates that the signal for a given constant stimulus fluctuates by only 2%.

Response time is defined as the time required for the system to respond to or detect a change in the stimulus after this change has physically occurred. This parameter is dominated by the gas flows of the inlet system and the corresponding residence time of sample gas in the inlet before reaching the detection region. For the techniques reviewed here, this is typically in the range of fractions of a second, for example 85 ms reported for PTR-QMS [18.98].

Classes of Odorants Detectable by On-line CIMS

Many important odors – or rather, odorants – can be suitably investigated by using the sensitive, real-time CIMS techniques described here. The soft ionization via diverse precursor ions imparts selectivity and produces relatively simple (*clean*) spectral patterns. Mass spectral interpretation and chemical identification is often possible for experienced users without recourse to dedicated MS software, as is generally required for EI spectra interpretation in MS, and this knowledge of reaction schemes and kinetic parameters allows for absolute quantitation in real-time, often with acceptable accuracy. As with all analytical systems, each technique offers its own advantages and disadvantages over the other. An overview of the classes of volatiles generally detectable by the three on-line CIMS techniques of SIFT-MS, PTR-MS and APCI-MS, with examples of common odorants falling in these classes, is given in Table 18.2.

18.2 Applications

The diverse on-line CIMS techniques discussed in the present chapter have varied fields of application. Their implementation for the detection of nonodorous VOCs inevitably is more extensive than their use for odor-active compounds owing to the fact that they are sensitive to most volatiles regardless of the odor activity of the compound. This section focuses on selected applications of on-line CIMS that are primarily relevant to the detection of odor-active compounds, although some nonodorous applications are included for completeness. Studies include the measurement of atmospheric or biogenic VOCs relevant to the environment and climate, security applications such as the detection of illicit drugs or chemical warfare agents, and medical research by investigation of breath-borne volatiles, amongst many others.

18.2.1 Food and Flavor

The most common, frequent and regular exposure of human beings to odorous molecules is from food-related odors, more appropriately aroma compounds. A western diet traditionally involves three meals per day, which equates to over one thousand regular and distinct annual encounters with a suite of odorants, notwithstanding meals being interspersed with flavorful snacks or beverages. As such, food consumption is one of our primary and most important interactions with odorous molecules, particularly in view of its mostly positive associations. The reader is referred to other chapters in this book for more in-depth accounts of food flavors and their physiological and psychological effects on us as consumers.

Research on food-related aromas has been a prominent area of on-line CIMS research over the last two decades. The real-time detection capabilities of these techniques make them essential tools for investigating dynamic changes associated with aroma release from foods. Unlike GC-MS that requires samples to be pretreated via extraction, enrichment and water removal, which greatly limits the sampling frequency to several minutes at best, on-line CIMS techniques enable samples to be analyzed without preconditioning, resulting in sampling frequencies of several hertz (Hz), as outlined above.

Due to the very nature of the food and flavor research applications of on-line CIMS necessarily detecting odor-active (aroma) compounds, and the growing prevalence of these techniques in this field, it is impossible to provide a comprehensive treatment of all published food-flavor studies in the confines of this subsection, which would be a chapter in itself. As such, an overview will be given here based on representative studies and highlights of recent years.

Food and flavor studies using on-line CIMS can be essentially placed in two categories: in vivo or nosespace analysis, and in vitro or headspace analysis. The former allows for the degree of aroma release during food consumption to be studied, relating the dynamics to the properties of the food matrix, consumption behavior and sensory impressions experienced by the consumer; the latter offers the opportunity to study retention and release of aroma compounds for different formulations, to examine degradative processes such as oxidation and microbial spoilage during storage or, when used in combination with multivariate analysis, allows a screening of products to potentially categorize their type, place of origin, or authenticity. These main investigative aspects will be discussed in the individual subsections here. The rapid measurement capabilities of on-line CIMS are essential for both types of studies. In in vivo studies, the release of aroma molecules from a food matrix is detected during the mastication of the food and thus requires fast detection. Equally, the on-line capabilities allow for flavor release of model systems to be assessed via headspace analysis, for instance during storage under different conditions, or to evaluate the aroma compounds experienced by consumers prior to consumption (e.g., the bouquet of a wine or the aroma of a freshly brewed coffee).

The development of the MS-nose interface for APCI-MS in the mid-1990s had the specific intention of enabling the technique to be used for flavor release studies (Sect. 18.1.1); similarly, one of the key areas of application of the PTR-MS in the same period was food-flavor release. By comparison, SIFT-MS has seen its application to flavor studies only relatively recently, but is equally rapidly becoming a recognized tool for flavor release studies. Nevertheless, APCI-MS and PTR-MS dominate the subdiscipline of real-time flavor release, as is evident from the related studies cited here.

In general, APCI-MS and PTR-MS have been well characterized for their detection of individual aroma compounds. (It might be noted that there are extensive SIFT-MS studies on its detection of VOCs reported in the scientific literature, but these mostly are not specifically on odorants.) There are many papers in the scientific literature that report on characteristic mass spectra (fragmentation patterns) of individual aroma compounds at different instrumental operating conditions, as well as with regard to signal response linearity and limits of detection [18.30, 99–103]. Some researchers have performed direct comparisons between APCI-MS and PTR-MS: one such study indicated similar performances of both techniques for volatile aroma compound detection [18.104], whereas another study claimed APCI-MS to have a 10-fold lower LOD and 10-times broader dynamic range than PTR-MS [18.105]. The author of the latter study attributed the poorer performance of the PTR-MS to a high degree of fragmentation, but noted that the settings could be optimized for aroma compound detection: it might be noted here that the PTR-MS operating settings used in the latter study were indeed not those typically used for food-flavor studies, and this offers a note of caution in that the settings of any of the on-line CIMS techniques discussed in this chapter must be optimized for the specific area of application. Studies have also compared individual on-line CIMS techniques with other tools such as GC-MS, e.g., in relation to the detection of volatiles from meat and meat products using SIFT-MS and SPME-GC-MS [18.106], during dry fermented sausage processing [18.107], and for the aroma analysis of dried red bell peppers [18.108], whereby PTR-MS in the latter study was found to produce data that was more comparable to GC-MS and GC coupled with flame ionization detection (FID) than APCI-MS.

Food Headspace Analysis

A key driver of aroma release from food systems is the hydrophobicity and lipophilicity of the odorant in question. Food matrices – both solid and liquid – have high diversity in terms of their nonvolatile composition and rheology, which dictates the degree of release of specific aroma compounds during production, storage, preparation and consumption. As such, the potential for on-line CIMS to study flavor release in model and real systems, both in vitro and in vivo, is seemingly endless. Indeed such studies reported in the scientific literature already number in the hundreds, thus cannot be covered fully here, but rather just a selection of typical applications is presented.

In vitro analysis of foods by on-line CIMS is used to either monitor individual, targeted compounds over time, for example, in view of aroma development or as quality markers (e.g., to indicate spoilage; see related section on food spoilage), or is applied to analyze the entire suite of volatiles in the headspace of the food, typically with subsequent multivariate data-mining analysis to discriminate samples. These two modes of application will be treated briefly here, after first discussing basic studies on flavor release.

Flavor Composition and Release. In headspace analysis one of the key parameters that determines the detection of aroma compounds is the degree of partitioning from the solid or liquid phase of the food matrix to the gas-phase in the headspace. This phenomenon is driven by many factors that include the physicochemical properties of the volatile compound in question and the formulation of the food matrix from which it is released; for the latter, the phase (liquid, solid, foam, gel), pH, relative composition of nonvolatiles such as sugars, proteins, fats, amongst other factors, is decisive (Chap. 13). Conveniently, the rapid analytical capabilities offered by on-line CIMS allow for partitioning to be purposely studied in relation to these factors. In liquid systems, dedicated analyses using stripping cells in both model [18.109] and real systems (coffee) [18.110] provide invaluable information on Henry's law constants that can support explanations for the mechanisms involved in flavor release and perception. The influence of the food matrix composition on aroma release and the resulting partitioning coefficients have been studied for numerous model formulations, including in gelatine, starch and pectin gels with a correlation to their sugar composition [18.111], textural properties such as strength [18.112] and elasticity [18.113, 114], and mechanical treatment [18.115], for oil-in-water emulsions [18.116, 117], low and high viscosity aqueous solutions with different sugar and bulking agent compositions [18.118, 119], in carbohydrate model systems and the influence of buffer and temperature [18.106], and in hydrating powders, focusing on the impact of protein, lipid and carbohydrate composition [18.120]. The extent of aroma release in relation to the degree of carbonation in liquid systems has also been studied, for

instance in PTR-MS measurements that followed the release of six aroma compounds from an artificial throat model, whereby it was demonstrated that increased carbonation typically promoted aroma release, the extent of which was dominated by the physicochemical properties of the compound [18.121].

Many studies use a model mouth or artificial throat to simulate in vivo conditions and monitor flavor release using on-line CIMS. The model mouth developed for the aforementioned studies on oil-in-water emulsions [18.122] was also used to investigate the role that tongue pressure and oral conditions have on volatile release [18.123]. In particular, the release of several flavor compounds, including 1-butanol, ethyl butanoate and ethyl hexanoate, was monitored under different mastication conditions, with upward and downward tongue mastication assessed for different initial positions at constant tongue pressure and mastication duration. The real-time data provided by PTR-MS revealed an initial flavor *burst* after every tongue stroke (Fig. 18.3). Other model mouth systems have been developed and used to study the influence of mastication rate and saliva on aroma release [18.124–126]. A comprehensive treatment of the use of model mouths to simulate and study flavor release is provided in a review article [18.127] and in another chapter of this book (Chap. 14).

Besides investigating the influence of diverse parameters on the aroma release in model systems, on-line CIMS has been used extensively to characterize the general volatile composition from different foods. The details of these numerous studies will not be discussed here, but rather a selection is listed to give an indication of the diversity of such research. PTR-MS has been used for the headspace volatile analysis, amongst others, of red kidney beans [18.128], juice and custard [18.129], to evaluate different treatments (heat versus pressure) of red orange juice [18.130], diverse cheeses [18.131–133], berry fruits [18.134, 135], apples [18.136], bread [18.137], cereal bars [18.138], coffee [18.139–142] and red bell peppers [18.143]. SIFT-MS has been increasingly used in recent years to perform such analyses, for instance in olive oil [18.144], to establish the kinetics of VOC emissions during yeast fermentations [18.145], in dry fermented sausages [18.146], Atlantic cod [18.147], and Parmesan cheeses [18.148]. Diverse fruits and vegetables have undergone SIFT-MS headspace studies on their constituent volatiles, as also reviewed and reported in the scientific literature [18.149], including the investigation of volatiles formed by lipoxygenase (LOX) activity in strawberries during storage, different cultivars of strawberries or at different levels of ripeness [18.150], sliced carrots during air drying [18.151], tomatoes and tomato puree [18.152, 153], and bell and jalepeño peppers during frozen storage and thawing or the effects of enzyme activity [18.154, 155]. Furthermore, nuts, seeds and grains have been studied using SIFT-MS, with roasting being at the focus of study for some targets, for instance, looking at volatiles of cocoa during roasting or at different pH levels [18.156, 157], or investigation into the effects of roasting sweet almonds, peanuts and pumpkin seeds [18.158–161]. Such applications can be extended to processed foods; one example here is the use of headspace analysis by SIFT-MS to investigate the character-impact odorants associated with basil and pesto, primarily the terpenoids methyl cinnamate, eucalyptol, linalool and estragole using NO^+ as a precursor ion to minimize compound interferences [18.162]. APCI-MS has been used to study many foods as diverse as kiwi fruit [18.163], milk [18.164], tea [18.165] and sausages [18.166], amongst others. Although not in widespread use, Li^+ IAMS has been used – in a demonstration of its practical application – to detect aroma compounds present in the headspace of strawberry [18.83]. Although the identification of

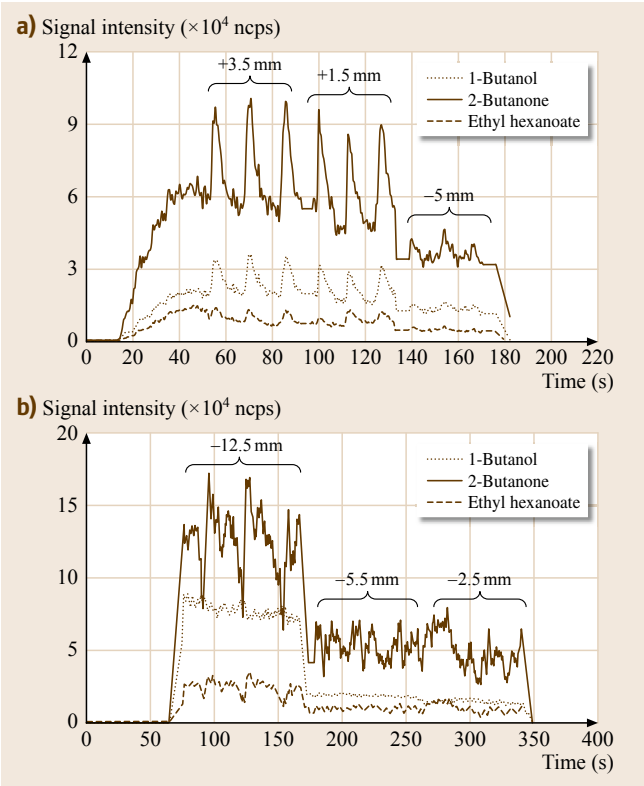

Fig. 18.3a,b Flavor release of 1-butanol, ethyl butanoate and ethyl hexanoate during **(a)** downward and **(b)** upward tongue mastication in a model mouth at constant pressure (25 kPa) and duration (0.4 s). Distance labels (in mm) refer to initial positions of the tongue (plus is above-surface; minus is below-surface) (after [18.123])

the mass spectral peaks was based principally on mass number, thereby carrying a degree of uncertainty, several compounds were detected and tentatively identified, including methanol, acetaldehyde, ethanol, acetone, propanol, methyl propanol, methyl butanol, demonstrating that this technology – like APCI-MS, PTR-MS and SIFT-MS – has potential use for aroma compound assessment from foods.

The headspace analysis of volatile aroma compounds in alcoholic beverages presents a particular challenge for on-line CIMS because of varying ethanol concentrations and interferences from protonated ethanol products. This issue has been addressed in several studies. The ability of APCI-MS to measure such beverages was assessed using an ethanolic model system and by adding ethanol into the source via the sweep gas [18.167]. The system developed was used to measure the partitioning of selected volatiles and was shown to deliver consistent and quantitative ionization provided the ethanol concentration in the source was above a certain threshold, with the ethanolic solutions found to reduce the partition coefficient of most of the aroma compounds tested. Subsequent APCI-MS studies on ethanolic systems under dynamic conditions have explored the influence of ethanol content, temperature and gas flow rate on the release of aroma compounds [18.168, 169]. Similar attempts at utilizing protonated ethanol as reagent ions for subsequent protonation reactions with target volatiles have been made using PTR-MS. In one study the sample headspace was diluted into an ethanol-saturated nitrogen carrier gas flow via a stripping cell to achieve a steady ethanol concentration and to promote conversion of H_3O^+ primary ions into protonated ethanol and ethanol cluster ions, such that subsequent protonation reactions of target volatiles were achieved with ethanol as a primary ion [18.92]. The technique was used successfully for mass spectral fingerprinting of different wine varieties but suffered from complex mass spectra, making individual compound identification difficult. To circumvent this problem, an alternative approach was proposed, whereby the sample headspace was diluted by a factor of 1:40 with nitrogen, and target aromas were ionized in the standard manner via H_3O^+ [18.91]. Here it was demonstrated that the ethanol concentration in the headspace had no influence on the wine headspace composition and the method was deemed better suited for routine applications than the previous approach.

Mass Spectral Fingerprinting. Volatile headspace profiling, or mass spectral fingerprinting, by on-line CIMS has been used successfully to discriminate between food products. In such studies, the entire spectrum of volatiles in the headspace of the samples are rapidly recorded and the resulting data are subjected to multivariate analysis such as partial least squares discriminant analysis (PLS-DA), principal components analysis (PCA), or analysis of variance (ANOVA), amongst others. These powerful statistical tools allow for correlations between large datasets, for instance providing indications of the degree of similarities or differences. By these means, it has been possible to categorize samples based on their headspace volatile profiles for diverse goals, including distinguishing different geographic origins or products with protected designation of origin (PDO) status, predicting sensory impressions, or distinguishing between varieties or

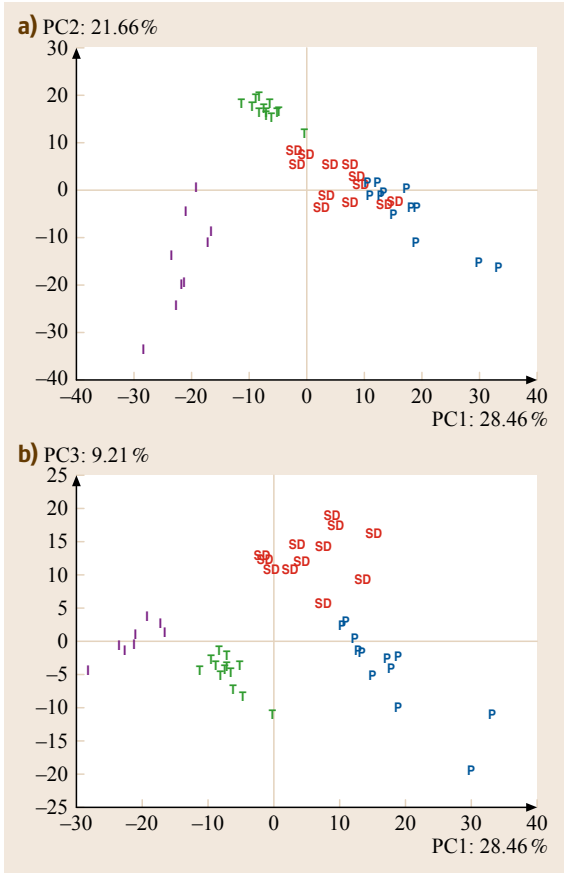

Fig. 18.4a,b Principal component analysis (PCA) score plots based on PTR-TOF-MS mass spectra of four types of ham, namely Iberian (I), Parma (P), San Daniele (SD) and Toscana (T), representing **(a)** the first vs. second and **(b)** first vs. third principal components. The headspace analyses of three replicates of 46 ham samples yielded more than 700 mass spectral peaks; PCA could discriminate between samples with a good degree of success (after [18.170])

types of particular foodstuffs. Examples of each of these applications are given here.

The practice of discriminating products according to their geographic origin is based on the premise that the volatile profiles of these foods will be affected to a lesser or greater extent by many factors during their growth and rearing or production. For plant-based foods these include soil conditions and growth climate, plant or fruit genotype, and ripening conditions. For animal products other factors can additionally play a role, including breed, feeding regime (which also depends on the feed and the factors affecting its production), and rearing procedures. The volatile profiles of both plant-based and animal-based foods are further affected by subsequent processing, thus clearly many factors come into play. On-line CIMS techniques have proven that mass spectral profiling with chemometric data processing can be successfully applied to foods to discriminate between different geographic origin, which has potential use as a rapid, nondestructive tool for food authenticity assessment relating to PDO status or fraudulent production. Such analyses have been demonstrated, for example, for cashew nuts [18.171], honey [18.172, 173] Iberian ham [18.174] (SIFT-MS), olive oil [18.175], dry cured ham [18.170], coffee [18.140, 176], wine [18.91] (PTR-MS), Stilton and cheddar cheeses [18.177, 178] and apples [18.179] (APCI-MS), to name but a few. As an example, the aforementioned study on dry cured ham involved performing rapid PTR-TOF-MS headspace analysis on 138 samples comprising triplicates of 46 hams sourced from four types (Iberian, Parma, San Daniele, and Toscana) [18.170]. The spectral fingerprints comprised over 700 peaks, and these were subjected to PCA, as well as random forest and discriminant PLS, with the resulting score plots indicating reasonably good separation of the samples (Fig. 18.4). Such studies demonstrate the utility of mass spectral fingerprinting to potentially screen foods for PDO status.

The use of mass spectral fingerprinting to predict sensory profiles is a powerful technique and a key area of potential application of on-line CIMS, since it potentially allows manufacturers to quickly assess product quality based on its volatile (aroma) headspace profile. Such assessments are traditionally performed by highly trained sensory assessors, but the use of on-line analytical instrumentation is desired by the industry to monitor sensory quality during production (e.g., during coffee roasting), or as a screening tool for rapid sample throughput to flag problematic samples along the production line (e.g., compromised flavor due to oxidation). It might also be used in more basic studies to ascertain general aroma compositions of related products.

Sensory profiling by on-line CIMS headspace analysis has been reported in several cases. In one study on mozzarella cheese, multivariate statistical data analysis indicated that both the CIMS method and the trained sensory panel delivered comparable sample descriptions [18.133]. Sensory profiles of espresso coffee were predicted by PTR-MS headspace data and were shown to reflect the flavors reported by the skilled sensory panel to a high degree [18.139]. APCI-MS has been used for similar purposes on beer, with a discrimination of samples based on key aroma compounds [18.180], or in Swiss cheeses, distinguishing between cheeses based their VOC profiles and related odor activity values (OAVs) [18.181]. SIFT-MS headspace analysis has been similarly applied, for example, to investigate the flavor interactions for sodium-reduced cheese sauce in wholegrain macaroni, whereby the volatiles detected by SIFT-MS were compared to taste attributes for the pasta with and without sauce [18.182].

In terms of screening approaches, SIFT-MS has been used to discriminate honeys produced from different flowers [18.183]. PTR-MS has been investigated as a potential tool to rapidly assess the oxidative alteration of olive oil via headspace analysis of the volatiles, whereby multivariate analysis of the mass spectral data generated models that could reliably classify oils as extra virgin or defective, with many peaks in the mass spectra – predominantly aldehydes – correlating with the peroxide values of the oils, as were determined independently, thereby suggesting that this was an appropriate nondestructive tool for peroxide determination [18.184]. The ripening of Swiss cheese was similarly studied based on volatiles – primarily sulfur compounds, but also carboxylic acids – released into the headspace, whereby it was found that the formation of propionic acid during curing of the cheese coincided with the production of key flavor impact sulfur compounds such as dimethyl sulfide and methyl mercaptan [18.185].

Food Spoilage. The unpleasant odor that food develops when it spoils is often the first indicator to consumers that the food is no longer fit for consumption. These odorants are by-products of the breakdown and metabolism of food substrates from microbial, enzymatic, oxidative or other degradative activity. Like other applications, on-line CIMS techniques are particularly suited to investigating food spoilage as they allow for continuous, nondestructive analysis, thus enabling the development of such volatile odorous spoilage metabolites to be monitored over time. Despite its suitability, however, there are only few studies that report on the use of on-line CIMS for meat spoilage measurements, and these only involve either PTR-MS or SIFT-MS; no reports of the use of APCI-MS for the detection of volatile spoilage metabolites could be found in the sci-

entific literature. The primary focus of such research is to identify prospective volatile spoilage markers that could be used for quick assessment of food quality and ultimately lead to the development of intelligent food freshness indicators (FFIs) based on emissions from specific compounds that signal when a food has reached its end of shelf-life and should no longer be consumed.

Investigations on the spoilage of meat by PTR-MS appeared soon after the development of the technique, with a series of studies on different meats in relation to packaging conditions and in correlation with bacterial species and colony growth. The first PTR-MS publication on this topic involved measuring the VOC emission profile of beef, pork and poultry under aerobic or anaerobic (vacuum-packed) conditions over a 13-day storage period [18.186]. The headspace of individual samples was analyzed on successive days to create VOC profiles of the different meats and storage conditions. The paper only names a handful of compounds – although apparently more were detected than reported – including 2-butenal and C4-esters, all showing an increase with storage, as would be expected. Differences in the generation of individual compounds were also observed between air-packed and vacuum-packed samples. A follow-up study further looked at the specific microbial species growing on the meat during spoilage [18.187]. Again, a number of VOCs were found to be generated and increase in concentration during spoilage, corresponding to an increase in bacterial numbers. Odorous compounds detected included the sulfurous compounds methanethiol, dimethyl sulfide, thioacetic acid methyl ester, dimethyl disulfide, and 2,3-dimethyl trisulfide. Of these compounds, dimethyl sulfide was found to have the highest correlation with bacterial spoilage. In a complementary study, the same researchers investigated whether ozone treatment of pork meat affects microbial growth, with dimethyl sulfide used as a proxy to determine the latter [18.188]. Although this compound was observed to be at much lower concentrations in the headspace of pork that had been exposed to ozone (compared to oxygen or untreated samples), bacterial numbers were high regardless of treatment. Another study associated with food spoilage looked at the volatile emissions from different bacterial cultures that degrade meat, namely *Escherichia coli*, *Shigella flexneri*, *Salmonella enterica* and *Candida tropicalis* [18.189]. The study made use of PTR-MS to monitor each of the four samples in repeated succession via an on-line sampling setup. The overall mass spectral profiles, as well as the temporal dynamics of individual VOCs were found to vary greatly according to microbial species; another finding, however, was that bacterial numbers generally did not correlate with VOC abundance, principally owing to the highly variable concentrations over time and making a case for the need for real-time as opposed to offline measurements in any such studies.

Milk spoilage has been investigated by PTR-MS from diverse angles. In one study, the microbially induced spoilage of fresh cow milk was followed over a 17-day period, comparing samples treated with sodium azide (to inhibit bacterial growth) with untreated samples [18.190]. Based on the suite of VOCs that increased in concentration in the headspace of the milk samples over the storage period in comparison with bacterial numbers, it was reported that the milk showed signs of spoilage only after a certain threshold abundance of microbial activity was achieved, which has potential utility for the development of FFIs. In another study, photooxidative spoilage of milk was monitored on-line by PTR-MS, with several compounds showing a distinct increase during light exposure of the milk compared to unexposed samples [18.191]. The latter pilot study makes a case for using PTR-MS (or any of the on-line CIMS techniques) to investigate the kinetics of such reactions in far more detail than achievable with offline analytical methods. PTR-TOF-MS has similarly been applied to dynamic headspace analysis of milk, albeit not for spoilage but to study lactic acid fermentation [18.192]. The headspace measurements were made by discontinuous sampling (i. e., not constant monitoring), but nevertheless provided semi-dynamic data with a time resolution of ≈ 20 min. Many key flavor or off-flavor compounds were observed to develop during the fermentation, including diacetyl, methanethiol, dimethyl sulfide and furfural.

Recent studies have demonstrated the applicability of SIFT-MS for food spoilage monitoring based on the strengths of its on-line sampling capabilities. Beef packaged under modified atmosphere was the focus of one such trial, whereby SIFT-MS was used to monitor VOCs produced during lipid oxidation [18.193]. The notable outcome of these trials was that SIFT-MS was able to detect differences in meat samples at an earlier stage than complementary SPME-GC-MS analyses, highlighting its potential suitability for lipid oxidation monitoring. In a different study on the lactic acid bacterial spoilage of sweet bell peppers, SIFT-MS was used to monitor the VOC composition in the headspace of samples packed under different atmospheres [18.194]. The clear advantage of using SIFT-MS over other conventional methods for such analyses was the convenience of not needing to open packaging for the analysis – which might alter the concentrations of volatiles – but additionally that the analysis simulated the sensory impressions experienced by consumers upon opening the packaging.

The microbiological spoilage of fish and crustaceans has been studied using SIFT-MS, notably iridescent shark fillets [18.195], cod fillets [18.196], and brown shrimp [18.197]. In the former, a SIFT-MS method was developed to monitor the growth and metabolic production of bacteria on packaged fillets, either under air, vacuum-packed, or modified atmosphere packaging (MAP) with complementary VOC analyses performed with SPME-GC-MS [18.195]. Several volatiles – including odor-active candidates – were observed to increase during spoilage, notably ethanol, 2,3-butanediol, diacetyl, acetoin, ethyl acetate, acetic acid and sulfur compounds. In the latter aforementioned study on brown shrimp, a selection of 17 volatiles, including alcohols, acids, ketones, amines and sulfur compounds in the headspace of samples inoculated with specific bacterial strains were monitored by SIFT-MS on a daily basis [18.197], whereby VOC concentrations and colony forming units of the bacteria were found to show similar increases. SIFT-MS has also been explored as a potential tool to monitor and establish the oxidative status in oils in comparison with sensory rancidity [18.198], or to determine antioxidants [18.199].

Food Nosespace Analysis

When food is consumed, constituent aroma compounds are released from the food matrix during mastication and swallowing. Their subsequent velopharyngeal transfer through the nasal cavity during exhalation elicits a response in the olfactory epithelium, when the latter is exposed to these odor-active volatile compounds. This simplified explanation does not consider the intricacies of this complex process, such as the physicochemical properties of the individual aroma compounds [18.200], variabilities in mastication processes, the composition of saliva, the anatomy of the oral and nasal cavities [18.127], or potential biotransformation of compounds en route through the nose [18.201]; these phenomena are discussed in detail elsewhere in this handbook. For the purposes of the present chapter, in flavor research it is assumed that the exhaled nasal breath during food consumption generally reflects the composition and quantities of volatile aroma compounds that reach the olfactory cleft and elicit a flavor impression in the consumer. A concept that was made possible by on-line CIMS tools is that of *nosespace* analysis, whereby nosespace refers to the gas directly exiting the nostrils and is assumed to be representative of retronasal aroma perception. This method has a large caveat in that concentrations of odorants detected in the nosespace do not necessarily reflect odor intensity impressions, which is dictated by odor thresholds and potencies of the odorants in combination with the mostly non-linear or

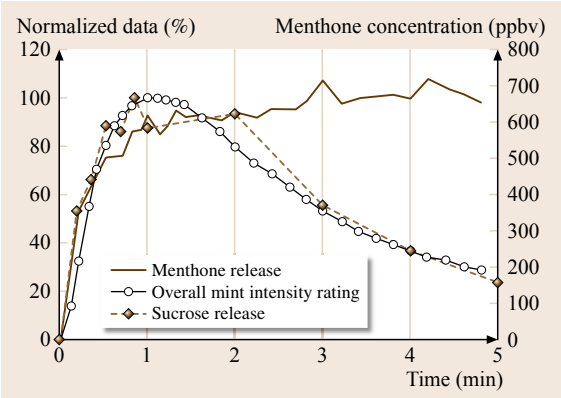

Fig. 18.5 APCI-MS nosespace analysis of menthone release during mastication of a chewing gum, whereby perceived mint intensity followed the sucrose release curve, despite menthone intensity remaining high (after [18.202])

cumulative integration of multiple sensory stimuli by the brain; indeed it was the application of APCI-MS for nosespace analysis that provided unequivocal evidence of the perceptual interaction between nonvolatile and volatile flavor compounds whereby the flavor perception of a mint-flavored chewing gum was shown to be dictated by the temporal profile of sucrose levels in the saliva and not the concentration of menthone in exhaled nasal breath, which remained steady whilst sucrose and flavor perception levels dropped concordantly (Fig. 18.5) [18.202]. Similar studies have been performed on other food matrices, for instance strawberry-flavored yogurt of different formulations, whereby the aroma release of the fruity compounds ethyl butanoate, (*Z*)-hex-3-enol and ethyl 3-methylbutanoate – as measured by PTR-MS – was found to be suppressed by sweeteners [18.203].

Several independent developments to APCI-MS have made it particularly suited to measuring retronasal flavor release. The early construction of an interface for direct injection of breath gas into the APCI-MS ionization chamber that was discussed earlier [18.25, 26] (Sect. 18.1.1) paved the way for later developments by *Taylor*, *Linforth* and colleagues [18.30] to allow for the measurement of flavor release via nosespace analysis. Other APCI-MS developments for optimized flavor release characterization via breath have also been made, including the use of IT-MS with a novel sample gas injection system [18.31], and the development of a mathematical model for characterizing release based on APCI-MS time-resolved data [18.96].

The persistence of several volatile aroma compounds in the breath from a six-person panel after ingestion of aqueous solutions was investigated using the aforementioned APCI-MS system [18.204]. The

extent of persistence was found to be similar for all panelists, whereby hydrophobicity and vapor pressure were key factors influencing the in vivo behavior of aroma compounds. The degree of interindividual variability was also investigated by conducting nosespace analysis of menthone released from a mint sweet using APCI-MS [18.205]. The release of menthone in 68 subjects that were tested according to a strict protocol for mouth movements was dominated by swallowing actions and the degree of degradation of the sweet in the mouth, which was suggested to relate to proteins, particularly the content of enzymes, in the saliva; by comparison, tongue and jaw movements were found to not have an influence on flavor release. Other attempts at modeling the kinetics of flavor release using APCI-MS nosespace analysis have also been made [18.206], whereby the interindividual variability was found to be very high, which was attributed mainly to differing swallowing and breathing patterns between subjects, thereby requiring nosespace data to be corrected for airflow rate in order be comparable. The study concluded that flavor molecules detected in the nosespace were primarily from liquid left in the throat after swallowing. Similar studies using APCI-MS to characterize flavor release similarly concluded that the main flavor impression resulted from a swallow breath from the throat, rather than from the mouth, with flavor-enriched air being delivered to the nose via exhalation immediately after the swallowing event [18.207]. APCI-MS has been used to investigate taste-aroma interactions in citrus-flavored model beverages by following aroma release profiles in combination with sensory analyses by a trained panel on models with altered acid and sugar types [18.208]. The study highlighted a difference in flavor perception due to sugar type, rather than physical factors, suggesting different receptors or receptor mechanisms involved in the perception process.

The interindividual variability and effects of food matrix composition on retronasal aroma release was investigated using APCI-MS [18.209]. The results of the study, which was performed using a panel of 30 subjects consuming nine different food products, suggested that the degree of retronasal aroma release is specific to an individual and relatively independent of the type of food consumed, with subjects who exhibited a high retronasal aroma release profile displaying these regardless of whether the food was semiliquid or solid. It was suggested, however, that foods could nevertheless be tailored to increase retronasal aroma stimulation with a view to potentially enhancing satiety and thereby reducing intake, which is of relevance in the current epidemic of obesity in the western world [18.210].

PTR-MS has been used extensively to investigate in-mouth aroma release and retronasal perception. Two such studies assessed how texture (of flavored whey protein gels) affected aroma release and perception [18.211, 212]. Another study compared trigeminal, taste and aroma perceptions by way of example on mint-flavored carbonated beverages, whereby the addition of CO_2 to the beverage was found to induce physicochemical modifications and sensory interactions with taste and aroma perceptions [18.213, 214].

Model custards that were flavored with strawberry aroma were used to study aroma release in relation to the interactions of oral processing and food texture via nosespace analysis using PTR-MS [18.215]. In particular, the consumption behavior of the panelists undergoing a free-chewing protocol could be separated into groups according to swallowing time, whereby subjects that swallowed relatively quickly displayed higher aroma release from the firmer custard than from the softer custard, whereas the latter matrix exhibited higher flavor release in the group that swallowed later, further highlighting interindividual variability. Similarly, the breath-by-breath aroma release from banana was investigated using PTR-MS, comparing ripe with unripe bananas [18.216]. In particular, two characteristic flavor impact compounds of ripe banana, namely isopentyl and isobutyl acetate, and two character impact compounds of unripe banana, 2-E-hexenal and hexanal, were monitored and compared.

The popularity and rich aroma complexity of coffee has made this beverage a key foodstuff for investigation by on-line CIMS, both in vivo and in vitro. A study that combined in vivo nosespace analysis by PTR-TOF-MS with the measure temporal dominance of sensation (TDS) investigated the roasting degree and sugar addition on aroma release and perception in espresso coffee [18.217]. The degree of roasting was found to have a higher impact on both aroma release and perception, whereas the addition of sugar had no effect on the former, despite influencing the perceived flavor of the coffee, thus highlighting sensory congruency effects. Similar comparisons of nosespace aroma release measurement data with sensory data were made in a PTR-MS study comparing PTR-MS data with time intensity perceptual measurements of flavor release using a strict breathing and consumption protocol [18.218].

Despite the only relatively recent deployment of SIFT-MS to food and flavor release monitoring, the technique has been shown to be suitable for nosespace analysis, such as for the case of aroma release from tomatillo and different varieties of tomato (and in comparison to headspace aroma release) [18.219] or strawberries after processing [18.150].

18.2.2 Environmental Odors

Odors have a range of physiological and psychological impacts on humans, spanning the whole spectrum from pleasantness and relaxation to symptoms of irritation and negative health effects. It is generally recognized that prolonged exposure to negatively rated environmental odors can cause reactions ranging from emotional stress such as states of anxiety, unease or depression, to physical symptoms, such as headache, eye and airways irritation, respiratory problems, or nausea [18.220]. Environmental malodors can diminish quality of life of those affected, raise intolerance and trigger complaints from workers and residents, thus requiring mitigating actions from facilities managements and regulatory acts from the local authorities [18.221, 222] (Chap. 25). Yet odor mitigating actions are hampered by difficulties in assessing the characteristics of community and individual odor exposures, and the variable odor perception thresholds of the odorants. It has been reported that the psychophysiological association between odor annoyance and air contaminants – whether these are odorants or not – is complex, involving several factors including sex, age, health status, social, geographic and environmental context [18.223]. Negative health effects often cannot be reconciled with the chemical constitution of environmental odorants, their chemical composition or concentration [18.224], since a multifaceted relationship exists between odor properties, exposure, sensitivity and individual responses such as annoyance or sensory irritation [18.225]. In general, a dose-response gradient is found to relate reported annoyance with the concentration of air pollutants [18.226], but such relationships are complex and critically dependent upon the olfactory quality assessed and the hedonic tone such as liking or disliking [18.227], while frequency and intensity of episodes contribute to the overall odor impact assessment. Since odors can be smelt at much lower concentrations than those eliciting upper respiratory tract irritation, in exposed workers or communities there is confusion between odor perception and irritation: this factor, coupled with individual variability in odor sensitivity and response, complicates the evaluation of adverse effects or annoyance [18.225].

An environmental odor can be composed of many tens of nonodoriferous substances and only a few compounds with low odor threshold, while in both categories their concentrations are typically at trace levels (i. e., gas volume mixing ratios in the range from ppm_v down to ppt_v or lower). Outdoor and indoor chemoanalytical odor monitoring is commonly conducted by discontinuous active or passive sampling, followed by offline analysis in the laboratory. This is typically achieved with techniques such as thermal desorption (TD) coupled to GC separation and MS detection (TD-GC-MS) in combination with olfactory detection ports as in GC-MS/O (Chap. 17). For this purpose, discrete ambient air samples bearing odors are collected onto VOC adsorbent materials, either actively – by means of a portable pump purging sample gas through tubes packed with adsorbent resin such as Tenax TA or activated carbon (Carbotrap/Carbopack) [18.228] – or passively onto porous materials or fibers, such as in the case of a Radiello diffusive sampler [18.229] or headspace SPME fibers [18.230]. These methods provide a time-averaged quantitation of the odorant VOC over a period of several minutes to several days; however, it is often desirable to instantaneously capture the gas sample at times of short-term or acute malodor events. A common alternative for instantaneous odor sampling consists of capturing, or *grabbing*, defined volumes of sample gas into an evacuated inert gas canister (e.g., SUMMA or Silcocan canisters with a passivated inner-surface coating), or into polymer material bags commonly made of polyvinyl fluoride (Tedlar bags) or polyterephthalic ester copolymer (Nalophan NA). Useful comparisons on the relative efficiencies of these sampling methods with regards to odorant recovery, sample stability and artifact issues have been reported [18.231–233]. The most widely used VOC sample collection and offline analysis protocols are those of the United States Environmental Protection Agency (EPA) Compendium of Methods for the Determination of Toxic Organic Compounds in Ambient Air (TO methods), commonly known as the US EPA TO suite of methods [18.234]. These custom analysis protocols have been central to the understanding of environmental and indoor odors, owing to the power of GC-MS techniques to separate and chemically identify and quantify complex mixtures, despite being somewhat limited in the suite of target VOC classes validated for each TO method [18.235]. It has become evident, however, that these methodologies are prone to significant sampling artifacts and analytical interferences and that many of the more active odorants (especially acids and carbonyl compounds) might not be stable enough to endure sampling, handling and thermal desorption without undergoing chemical transformation before they reach the analyzer. Generally, losses during storage and stability issues limit VOC recoveries in all of the aforementioned sampling methods [18.236]. Moreover, being rather work-intensive and discontinuous in nature, these methods are not suitable for long-term, highly time-resolved and directional monitoring of VOC sources in the field or indoors, as is generally desirable to address issues of

odor complaints. The shortcomings of the discontinuous headspace, purge-and-trap or sample-grab methods have been partly overcome by the development of rapid and field-deployable devices such as handheld GC-MS systems [18.237]. Nevertheless, the application of this tool to complex environmental odor issues is somewhat restricted, being less sensitive than laboratory GC-MS and constrained in the suite of VOCs that can be monitored at trace levels, owing to the limitations of the built-in GC column. Some form of sample preconcentration is required for increasing the sensitivity, while chemical identification and accurate quantitation are limited to the compounds for which reference standards are run.

In perspective, the discontinuous sampling and offline analysis methods outlined in the US EPA TO suite of methods do not offer sufficient time resolutions to allow the capture of the real-time fluctuations of odorant compounds in the ambient air and are subject to analytical artifacts. Although these shortcomings have been partly overcome by field-portable GC-MS and open path-Fourier transform infrared (OP-FTIR) spectroscopy, optimum performance is only achieved when the odor nuisance compound is known a priori and the instruments can thereby be used as source-monitoring tools. On-line CIMS techniques have the advantage of giving instantaneous snapshots of the ambient air composition, regardless of whether or not the compounds of interest are known a priori. Given the lack of sample handling required, the high time resolution and enhanced sensitivity, and the wide range of nonspecific VOCs detectable, on-line CIMS techniques offer a highly time-resolved record of the unidentified compound mixture in the sample air at times of complaints and can thus be used as fast screening tools. The VOC concentration datasets generated can then be post-analyzed focusing on selecting specific ions for VOC fingerprinting. Nevertheless, the reviewed techniques are principally tools for fast monitoring and for gas-phase fingerprinting, rather than for chemical-structural elucidation of unknown compounds. Although these can provide molecular mass information on the target analytes, the capability of separating isobars and isomers (or specific odorant stereoisomers) and to unambiguously ascertain the chemical identity of unknown analytes remains a strongpoint of the slower and specific GC-MS techniques.

In this section the status of on-line CIMS as research tools for the instantaneous monitoring of complex air pollutant mixtures – both indoor and outdoor – is discussed. The scope here is to describe the applications of CIMS to environmental odors arising from VOCs, or for which VOCs are markers, therefore the measurement of inorganic odorants – commonly conducted by means of highly specific monitors or sensors – will be omitted. A treatise on environmental odor detection would, however, not be complete without mentioning the relative standing of CIMS techniques to those of the electronic noses (e-noses), which are semispecific gas sensor arrays that are sensitive to a wide range of odorants and employ complex algorithms for pattern recognition; the operating principles and performances of this technology are outlined at length in another chapter of this book (Chap. 21).

While on-line CIMS instruments stand as research tools capable of revealing molecular and structural chemical information on the sampled air irrespective of the odor attribute of the constituent VOCs, e-noses are fast and semispecific screening tools aimed at the detection of selected VOCs whose odor characteristics are known from the outset [18.238]. The on-line CIMS techniques discussed in this chapter are unspecific, thus are ideal analytical tools for complex mixtures of unknown odorants. Moreover, the underlying soft ionization processes produce little to no fragmentation of the parent ions, making it easier to deconvolute the resulting mass spectra and to extract the chemical information. The spectra produced by these techniques represent essentially the gas-phase fingerprints of the target molecules or mixture, irrespective of its odor activity. For completeness it has to be noted that, when a specific odorant or a limited range of VOCs is known a priori to be emitted in ambient air in certain areas but their source apportionments are unknown, the closest approach to on-line and continuous trace level monitoring is achieved by applying OP-FTIR. This optical spectroscopic technique has recently been successfully applied to monitor VOCs continuously along different optical paths for identifying multiple odor sources by targeting a limited suite of odorants or a nonodorous plume tracer such as methane [18.239–241].

Indoor Odors

Odors within the indoor environment can generate sensory irritation and annoyance that can result in psychological effects, including a distraction from work and a consequential loss in productivity [18.242]. Indoor odor is discussed in depth in another chapter of this book (Chap. 26) and will not be treated in detail here, but rather the application of on-line CIMS techniques to monitor such odors.

People assess the quality of the air indoors primarily on the basis of its odor and on their perception of associated health risk. The major contributors to indoor odorants are human occupant odors (i. e., body odor), environmental tobacco smoke, volatile building materials, bio-odorants (particularly mold, human and animal-derived materials), air fresheners, perfumes and

residues of cleaning products. These are mostly present as complex mixtures, thereby complicating the assessment of the total odorant problem [18.243].

It is important to note that many of the VOCs that are primarily emitted from indoor sources such as building materials, air conditioners, furniture, flooring and carpeting materials, cleaning products, printers, etc. are not odor-active [18.244]. Thus, although they might be the cause of general discomfort, often it is the perceived odor that is presumed to be the culprit. Furthermore, even if the concentrations of certain odor-active VOCs inside buildings are far below their reported odor thresholds, compounded effects at sub-detection threshold levels might cause the exacerbation of symptoms that lead to a complaint [18.245, 246]. Moreover, chemical reactions between VOCs and oxidants, such as ozone, nitrogen oxide and electromagnetic radiation, can produce oxygenated VOCs [18.247] and secondary organic aerosols (SOA) [18.248] that might be responsible for the reported symptoms, while the overall impact of toxic compounds adsorbed onto particles or forming SOA has not been clarified to date [18.249].

Keeping in mind the aforementioned remarks, indoor environmental odor or associated irritant VOCs have been investigated by on-line CIMS in a number of recent studies. The CIMS techniques can provide a snapshot of the air composition at the time of complaint, but for the unambiguous identification and characterization of active odorants these must be used in combination with offline techniques, typically TD-GC-MS/O, for detailed chemical identification and quantitation of the compounds measured within the mass spectrum, and to resolve isomeric or isobaric compounds that are indistinguishable by the on-line CIMS system.

Among odor-active compounds as specific markers of indoor air pollution, formaldehyde is a well-documented indoor irritant [18.250] with a characteristic pungent odor [18.251]. Formaldehyde can be measured by all of the described CIMS techniques, although in some cases its detection is hindered by interferences, humidity effects and low sensitivity when using hydronium for proton transfer reactions due to the similar proton affinities of the reagent and analyte (see Fig. 18.1 and Table 18.2). This is the case for PTR-MS [18.252], although an optimized mode of operation can significantly enhance its performance for on-line formaldehyde detection at low absolute humidity [18.253].

Common terpenes such as limonene, α-pinene, citronellal, geraniol and linalool are the main odorous ingredients of virtually any cleaning agent or ambient air fresheners. They readily react with ozone, and the resulting oxidation products have a high irritation potential [18.250, 254], partly due to the formation of formaldehyde, and can equally be odor-active [18.255]. Ozone-initiated terpene reaction products may therefore be of concern in ozone-rich environments ($\geq 0.1\,\mathrm{mg/m^3}$) with elevated limonene concentrations, such as office environments [18.256]. Gas-phase oxidation products of several terpenes (monoterpenes, sesquiterpenes and oxygenated terpenes) arising from the reaction with ozone have been measured by PTR-MS in environmental simulation chambers such as Teflon chambers and fluoroplastic bags [18.257, 258]. The temporal evolution of the concentrations of products such as formaldehyde, acetaldehyde, formic acid, acetone, acetic acid, and nopinone (tentatively identified by their m/z) were followed although no discussion on the potentially odorous and irritant characteristics of these volatiles was presented, since the rationale of these studies was on the kinetics of outdoor and indoor atmospheric chemistry and aerosol formation. Similarly, APCI-MS was used in a chamber study to follow the kinetics of terpene oxidation processes at different ozone ratios [18.259] with focus on the kinetics of formation of gas-phase tracers relevant to aerosol formation and subsequent modeling. A background study on the ion-molecule reaction of H_3O^+, NO^+ and O_2^+ with various monoterpenes by SIFT-MS has been reported [18.260], investigating the feasibility of using these ions as primary reagents for the detection of monoterpenes by SIFT-MS. The reaction of individual monoterpenes with each reagent ion was found to proceed at the collisional rate, although in a mixture of the studied monoterpenes the concentration of each constituent could only be approximated from the SIFT-MS spectra by assuming averaged rate constant and product distribution.

Many building materials are persistently perceived as odorous, although the concentrations of the detected organic compounds are close to or below their reported odor thresholds [18.261]. Any of the on-line CIMS techniques outlined in this chapter can be effective tools for establishing VOC emission signatures of building materials, although PTR-MS is a prominent technique in this field of application [18.262]. A direct measurement of building material emissions has been reported by PTR-MS [18.263], with the results correlating well with measures of odor acceptability by humans that allow for fingerprinting and chemometric analysis on the material release patterns. A series of emissions tests on building products and characterizations of the release of paint additives was successfully carried out using both PTR-QMS and PTR-TOF-MS [18.264]. Owing to its enhanced sensitivity and specificity compared to PTR-QMS, the

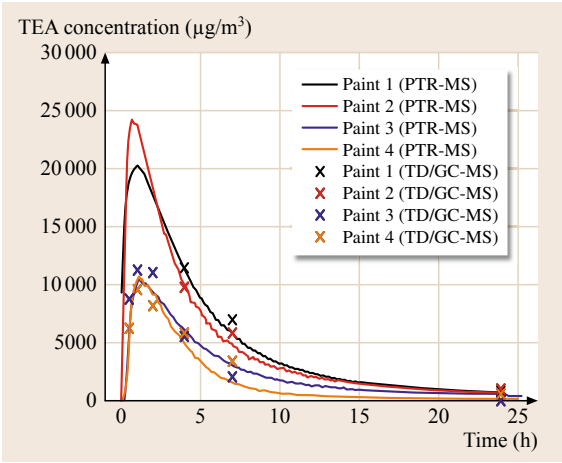

Fig. 18.6 Comparison of PTR-MS and TD-GC-MS analysis of the release of trimethylamine (TEA) from four freshly applied paints (after [18.264])

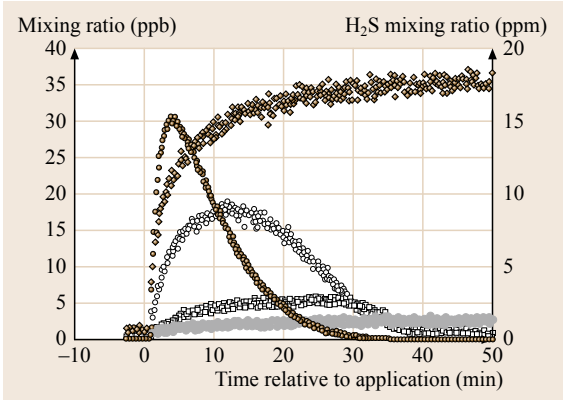

Fig. 18.7 Presence of hydrogen sulfide (*closed black circles*), methaniethiole (*open circles*), dimethyl sulfide (*open squares*), 4-methylphenol (*open diamonds*) and 3-ethylphenol (*closed grey circles*) within a static emission chamber after application of a pig manure, as measured by PTR-MS (after [18.267])

PTR-TOF-MS instrument was deemed a more suitable tool for the task of generating VOC datasets that can undergo multivariate analysis to disentangle the complex patterns of indoor VOCs, or for predicting parameters that cannot be directly measured. Further, the real-time detection offered by PTR-MS was demonstrated to reveal the kinetics of release in far greater detail than TD-GC-MS, as shown in the emission of trimethylamine from freshly applied paints [18.264] (Fig. 18.6).

Another selected, but not exhaustive, example of applications in this field is the assessment of emission characteristics of odorous VOCs from incense and scented candle burning by PTR-MS [18.265], in which the concentration profiles from the onset of burning and the emission rates of tens of VOCs were revealed along with other indoor air pollutants such as NO_x and particulates (the latter measured by dedicated techniques). The on-line detection capabilities of APCI-MS have also been put to use in detecting VOCs in cigarette smoke via use of a smoking machine, whereby puff-by-puff analyses of VOCs including acrylonitrile, crotonaldehyde, benzene and toluene demonstrated the applicability of this on-line method [18.266].

Generally, research in these fields using on-line CIMS techniques has revealed the presence of compounds previously overlooked in surveys of indoor pollutants, reflecting the limitations of the analytical methods routinely used for such monitoring purposes. Moreover, the application of uni- and multivariate statistical modeling to the complex chemical data matrix obtained by on-line CIMS allows for the prediction of parameters that cannot be directly measured, such as odor impact in relation to VOC concentration.

Outdoor Odors

The topic of environmental odor monitoring is the subject of another chapter of this book (Chap. 25), thus only an overview of its main challenges and of the potential of on-line CIMS techniques to contribute analytical solutions is given here. When monitoring outdoor environmental odors, a particular analytical challenge lies in the highly transient nature of the odorant abundances, which is affected by meteorology and local dispersion conditions. Furthermore, it is necessary to discern the odorant signal from the background ambient air, which is often made of a complex combination from mixed sources such as urban, agricultural, landfill or industrial [18.268]. Odor complaints are associated with a wide variety of industries and operations including agriculture and livestock, sewage treatment plants, paints, plastics, resins and chemical manufacturers, refining operations, rendering plants, pulp mills and landfills, to name the most prevalent. A range of inorganic and organic compounds are responsible for industrial and environmental odors, depending on the source. Commonly encountered inorganic substances with powerful odorants properties are ammonia (NH_3) from agriculture and animal farming, inorganic amines (e.g., chloramines, NH_2Cl) from chlorine disinfection in drinking water and swimming pools, inorganic sulfur compounds including hydrogen sulfide (H_2S) from petrochemical, paper milling and photographic industries, and sulfur dioxide (SO_2) and carbon disulfide (CS_2) emitted by sewage pipes and aerobic sewage water treatments [18.269]. Nevertheless, organic volatiles are by far the most commonly encountered causes of environmental odor complaints.

Table 18.3 Chemical groups of odorous compounds associated with industrial processes

Industrial process	Chemical group
Petroleum refining	Carboxylic acids, aldehydes, thiols, organic sulfides, phenols
Vehicles (incomplete fuel combustion)	Aliphatic and aromatic hydrocarbons, nitrogen oxides
Pulp/paper processing	Alcohols, aldehydes, ketones, thiols, reduced sulfur compounds, organic sulfides, terpenes and sesquiterpenes
Pharmaceutical production	Organic amines, reduced sulfur compounds
Agriculture (fertilizers, pesticides, animal rearing)	Alcohols, aldehydes, amines, carboxylic acids, chlorinated compounds, esters, ketones, thiols, phenols, reduced sulfur compounds
Chemical manufacture (paints, solvents, plastics, rubbers)	Carboxylic acids, alcohols, aldehydes, amines, chlorinated aromatics, esters, ketones, thiols, phenols
Metallurgy	Carboxylic acids, aldehydes, aromatic and aliphatic hydrocarbons
Sewage/municipal wastewater treatment	Carboxylic acids, aldehydes, ammonia, aliphatic and aromatic hydrocarbons, hydrogen sulfide, organic sulfides, terpenes
Bio-waste composting	Carboxylic acids, alcohols, aldehydes, esters, furans, ketones, organic sulfides, terpenes
Waste management (landfill, incineration, composting, sorting)	Carboxylic acids, esters, ketones, terpenes
Rendering plants	Aldehydes, esters, ketones, aromatic hydrocarbons, halogenated hydrocarbons, organic sulfides

The VOCs commonly investigated in studies on environmental odor complaints include the classes listed in Table 18.3.

An example amongst the processes listed in Table 18.3 is the application of on-line CIMS to monitor odorous emissions from animal production facilities, in particular swine rearing operations, which has been recently reviewed [18.270] (and references therein). Pig farms are a source of a complex mixture of VOCs over a wide range of molecular weights and with diverse physicochemical properties, with the VOCs emitted being preferentially bound to smaller-size dust particles inside and outside swine barns. The major VOC sources investigated include air inside barns, in the headspace of manure storages or composts, or in the open atmosphere above wastewater and surrounding swine farms. Some studies have also included liquid swine manure due to its use as a fertilizer in agricultural land. The most abundant VOCs that were quantified in at least two independent studies included acetic, butanoic, isovaleric, valeric and propionic acid, dimethyl sulfide and disulfide, p-cresol, skatole and trimethylamine in air, although the majority of the studies used a range of discontinuous sampling, preparation and analysis methods based on GC and LC with different detectors. Only two studies were reported on continuous and real-time odorous VOC monitoring in air samples, both deploying PTR-MS. More recently, PTR-MS has been applied for studies on animal agriculture, mainly for swine [18.267, 271–274] (Fig. 18.7), but also for monitoring emissions from cattle [18.275, 276]. Additionally, an application of on-line CIMS to measure the headspace above pig feces and urine in a laboratory setting was conducted by SIFT-MS [18.277].

The coupling of on-line CIMS with olfactometric assessment tools is highly desirable for the purpose of producing accurate yet real-time determination of ambient odor concentration. This approach has been tested, for instance, by measuring odor emissions and abatement efficiency of biofilters in composting plants [18.278, 279], a common source of odor nuisance complaints. In that study, the approach of correlating PTR-MS instrumental signal response to olfactometric assessment by means of multivariate calibration yielded a good estimate of odor concentration. Another application of on-line CIMS in ambient air monitoring is to measure instances of odor nuisance and determine its source by comparison of volatile profiles with library data, for instance, by the unique volatile emissions profiles from pig or chicken livestock [18.280, 281].

The application of on-line CIMS has led to unprecedented advancements in both atmospheric environmental studies and in plant physiology, by allowing the detailed measurement of localized or diffuse atmospheric and biogenic VOC emissions. Besides being inherently odorous to the human nose, these compounds are relevant to the environment and climate. Pivotal studies on the emissions of biogenic VOCs such as isoprene, terpenes and terpenoids (amongst which are the odorous α-pinene and limonene, among tens of others) from vegetation have been conducted by PTR-MS [18.282] and by SIFT-MS [18.283]. Another potential area of application for on-line CIMS in environmental gas monitoring is in the detection of hydrocarbon seeps from oil or gas extraction, whereby SIFT-MS has been demonstrated to meet the conditions for detecting the small linear hydrocarbons with high precision [18.284].

In conclusion, the chemical composition of a gas mixture – either indoor or outdoor air – does not represent the odor perception, which is a subjective impression in human olfaction. Nonetheless, on-line CIMS monitoring of small organic compounds is extremely useful for the purpose of investigating indoor and outdoor air quality and associated odor issues, both in the field or under simulated conditions such as in gas and smog chamber studies. It is, however, crucial to apply these techniques with a deep knowledge of their limitations, interferences, and of the uncertainties associated with the measured data. The chemical composition and concentration information obtained by CIMS has then to be critically linked with the characteristics of community and individual odor exposures, whilst accounting for the subjective odor perception thresholds of odorants. With the caveat that complementary human sensory assessments is indispensable to identify the odor impression, indoor and outdoor VOC monitoring by on-line CIMS is generating unprecedented data with high temporal resolution and chemical information. Materials emission factors and flux measurements can thus be measured, which undoubtedly represents the future of research in this area.

18.2.3 Human Odor Emissions

The human body is a continuous source of odor to its immediate surroundings. Odor emanations from the body are primarily from breath, typically in the form of oral malodor, and sweat (Chaps. 48 and 49), or more specifically from the bacterial breakdown of food particles in the mouth or sweat constituents after secretion. Further, odorants are present in most other bodily fluids, even if these do not contribute to the exterior *odor-print* of an individual. Blood, urine, feces, human milk, semen and vaginal secretions all contain distinct constituent volatile molecules (including odorants) that can be affected by health state and diet, amongst other things; the totality of the volatile constituents of these bodily fluids – comprising at least 1840 VOCs – has been termed the *human volatilome* [18.285, 286].

The detection of odorants from bodily media by on-line CIMS has been limited to date, primarily because of the lack of a need for rapid detection of samples that are intrinsically slow or infrequent to acquire. The physiological and psychological role of such odorants has been treated elsewhere in this handbook. This section details the role that on-line CIMS has played in detecting such emanations.

Breath Analysis and Oral Malodor

Breath gas analysis has seen high activity in recent years with the emergence of new technologies that enable fast and comprehensive analysis of breath gas constituents. Primarily, breath analysis focuses on the search for volatile or nonvolatile constituents of exhaled breath gas, aerosols or condensate that represent unique biomarkers as indicators of state of health or specific diseases. The major advantages of analyzing breath for disease biomarkers over other bodily fluids such as blood or urine is that breath is inexhaustible, allows for noninvasive sampling, and can provide point-of-care results. In the context of this chapter, the emphasis on odorous molecules in breath restricts the discussion to predominantly oral malodor, whereas the majority of breath gas studies focus on end-tidal breath, i. e., gas from the deep lungs. The interested reader is referred to review articles that deal with breath analysis and biomarkers in general and not selectively odor-active compounds [18.287, 288]. Further, a recent comprehensive compendium of VOCs reported to be found in diverse bodily fluids highlights the complexity of the human volatilome and provides further indications of human endogenous odorous molecules [18.285, 286].

Of the on-line CIMS techniques outlined in this chapter, two stand out as the prevailing technologies in the analysis of exhaled breath volatiles in terms of their cumulative implementation in breath analysis, namely SIFT-MS and PTR-MS [18.44]. As is evident from Sect. 18.2.1, APCI-MS has been used extensively in the analysis of aroma compounds exhaled during food consumption, i. e., in nosespace analysis, to characterize in vivo aroma release. By comparison, its implementation in the analysis of endogenous breath constituents or oral malodor has been very limited, despite this technique being the first of the on-line CIMS tools to be used for analyzing breath. Indeed, as reported in the introduction, the development of a direct breath sampling interface for an APCI-MS in the late 1970s and early 1980s was a catalyst for establishing methods for the direct injection CIMS techniques of today [18.25, 26]. These initial studies reported on the detection of the most predominant volatiles in breath, namely ammonia, acetone, methanol and ethanol. More recent breath studies using APCI-MS include the analysis of fatty acid vapors in breath [18.289], and the detection of volatiles – including pyridine, isoprene and hydrogen sulfide – in a simulated breath atmosphere in view of its use to monitor exposure or disease biomarkers [18.290, 291]. Medically related breath analysis by APCI-MS, however, remains a niche area of application.

As indicated above, SIFT-MS and PTR-MS have been used more extensively in breath research, and both techniques were identified early on in their development as tools that are ideally suited to detecting VOCs in breath. Like APCI-MS, they fulfill the key requirements for the favorable aspects of breath analysis, i. e., rapid

and direct detection with immediate results. Moreover, unlike offline analytical methods such as GC-MS that are typically selective for specific compound classes, the on-line CIMS techniques offer nonselective detection of VOCs, thus the targeted molecules must not necessarily be known a priori. Further, the high humidity of exhaled breath is not a hindrance to the analysis when appropriately accounted for, thus samples must not undergo dehydration prior to their analysis [18.44].

There is a wealth of studies that have investigated the volatiles present in exhaled breath in view of discovering disease-specific biomarkers. As mentioned earlier, these typically do not focus on odorous compounds, but a brief summary is given here as a matter of course. The majority of studies on potential disease biomarkers have used a *discovery* approach, detecting a suite of VOCs in breath and performing statistical analyses on the data to find significant correlations. Alternatively, *targeted* studies investigate known VOCs and examine their correlations with specific diseases. In terms of general breath gas analysis, SIFT-MS has been employed for studies on endogenous volatiles such as methanol [18.292], isoprene [18.293], ethanol and acetaldehyde [18.294], C3-C10 aldehydes [18.295] and carbon dioxide [18.296], for disease-specific biomarker discovery, such as pentane as a marker for bowel disease [18.297], or for exposure monitoring, for instance in a study on xylene and trimethylbenzene levels in breath and blood after exposure [18.298] or for pollution exposure monitoring in breath [18.299]. The use of PTR-MS in breath analysis is similarly widespread, ranging from studies on the kinetics of endogenous compounds such as isoprene and acetone during exercise or sleep [18.300–303], detection of specific compounds in smokers' breath [18.304–306], the pharmacokinetics of compounds such as 1,8-cineole after ingestion of a pharmaceutical preparation [18.307], or investigating medical conditions such as renal transplantation [18.308], liver cirrhosis [18.309, 310], coeliac disease [18.311], or gestational diabetes mellitus [18.312], to name but a few. Other breath-related studies using on-line CIMS have investigated procedures in breath sampling and storage, and how these can influence constituent volatiles detected [18.233, 313–317], or a comparison between exhaled breath volatiles and those present in exhaled breath condensate (EBC) [18.318]. An overview of further breath-related studies using SIFT-MS and PTR-MS are available in review papers [18.44] or in recent journal special issues dedicated to these techniques [18.319, 320].

Let us now return to the focus of this chapter, namely odorant detection, in relation to breath. Halitosis (or bad breath) is caused by the presence of a range of odoriferous VOCs in exhaled breath. The offending odors are principally generated directly in the oral cavity, but in some cases halitosis is associated with episodes of eructation of food-related volatiles present in the gut or is related to the pulmonary excretion of systemic volatiles, such as in the case of ketosis or in conditions such as trimethylaminuria. The key candidate volatiles that cause oral malodor are volatile sulfur compounds (VSCs), typically hydrogen sulfide, methanethiol, dimethyl sulfide and other sulfides, but also short-chain fatty acids such as propionic, butanoic, pentanoic and 4-methylpentanoic acids, polyamines such as cadaverine and putrescine, phenolic compounds such as indole, skatole and pyridine, and nitrogen-containing compounds such as urea and ammonia can all contribute, depending on the specific medical condition [18.321]. Unfortunately for halitosis sufferers, these compounds are not only characterized by their unpleasant odor, but they typically have very low odor thresholds, such that they exhibit a potent odor even at ultratrace concentrations. For deeper insights into halitosis and its organoleptic assessment, the reader is referred to review articles on the topic [18.321, 322].

The very low detection limits offered by the on-line CIMS techniques, in combination with the direct sampling capabilities that reduce the potential losses of these labile substances, make these ideal tools to investigate oral malodor. Despite this, studies on oral malodor using on-line CIMS techniques are far sparser than general breath analysis studies; indeed to date only SIFT-MS has been used for oral malodor-related studies. A case study on oral malodor used SIFT-MS to characterize the ion-molecule reactions occurring with different reagent ions on the malodorous compounds indole and 3-methylindole (skatole), with results demonstrating a higher concentration of these compounds in oral air of subjects with clinically significant oral malodors compared to healthy controls [18.323]. SIFT-MS has also been demonstrated to detect the polyamines cadaverine and putrescine, which can be present in oral air [18.324]. Another study used an in vitro matrix biofilm perfusion model to test the efficacy of treatment with putative antimicrobial or anti-malodor actives based on the concentration of H_2S and other VSCs detected by SIFT-MS, as well as using SIFT-MS to validate microbial fuel cell-based H_2S sensing electrodes [18.325]. The study revealed higher levels of VOCs in the post-treatment placebo and negative treatment samples, and reduced levels after treatment with the active agents.

Ammonia in breath has been the subject of analysis by SIFT-MS, whereby it has been shown that this compound is primarily generated in the oral cavity, rather than arising from the systemic circulation, which of-

fers a note of caution on general breath analysis that contributions of volatiles from the oral cavity can be significant [18.326] and present a potential source of confounders. Sulfur compounds have been investigated in a similar fashion using SIFT-MS, comparing concentrations in mouth- and nose-exhaled breath and in the oral cavity. It was found that the concentrations of hydrogen sulfide, methanethiol, dimethyl sulfide and dimethyl disulfide were higher in the closed mouth than in nose-exhaled breath, suggesting that they are largely produced in the oral cavity [18.327].

In a diet-related malodor study, SIFT-MS has been employed to investigate the effect of drinking milk on suppressing or eliminating breath malodor in relation to garlic consumption. It was demonstrated that milk was effective in reducing the major odor compounds of garlic in breath, namely diallyl disulfide, allyl methyl disulfide, allyl mercaptan, allyl methyl sulfide and methyl mercaptan, with whole milk producing a greater reduction effect than fat-free milk [18.328], although other treatments were investigated in a follow-up [18.329]. PTR-MS has similarly been employed to investigate the compounds arising after garlic consumption. In the study, the VSC constituents of garlic were monitored in exhaled breath for up to 30 h after ingestion of raw garlic [18.330]. Allyl methyl sulfide and disulfide, diallyl sulfide, disulfide and trisulfide, and dimethyl sulfide were all detected in exhaled breath, whereby most of the compounds followed a transient increase and decrease over a 2–4 h period with the exception of dimethyl sulfide, which was still elevated in exhaled breath 30 h after ingestion of the garlic.

Body Odor

Human body odor is primarily related to the microbial breakdown of sweat excretions, which generates and releases odor-active compounds. Diet can influence the overall odor emanations from the body, whereby aroma compounds consumed with food can be excreted through the sweat glands, either in unaltered form or after being metabolized and converted into derivative forms. The use of on-line CIMS to investigate body odor or skin emanations has been limited to date. In terms of direct analyses with human subjects, one PTR-MS study investigated the presence of carboxylic acids in the axilla of volunteers, comparing use of a deodorant with use of an antiperspirant for one week [18.331]. The measurements, which utilized a specially designed sampling unit that cupped the axilla of the volunteer for direct measurement of odorants at the armpit, found acetic, propanoic, butanoic, pentanoic and hexanoic acids at high concentrations. Although the data represented only a single subject, an inverse effect of antiperspirant use and concentrations of selected fatty acids was evident, which was confirmed by the subjective impressions of a sensory assessor. This study has potentially high commercial value, as it allows sensory assessments of axillary odor following use of underarm deodorant or antiperspirant to be validated from a chemo-analytical approach.

An alternative method to investigate axillary odor is to use worn clothing as a surrogate, as was performed in a PTR-MS study that aimed to differentiate between different types of fabrics [18.332]. Short-chain carboxylic acids were at the focus of the investigation, where it was found that worn polyester fabric had significantly higher levels of odorous compounds compared to wool or cotton, as was confirmed in sensory assessments. The degree of adsorption of body odor compounds on three different textiles was similarly studied by PTR-MS, whereby specific textiles were found to adsorb compounds less or more efficiently, with decanal and cyclohexanone adsorbing more to wool, and ethylbenzene and butanoic acid methyl ester more to polyester, with all three textiles, including cotton, showing high adsorption of phenol [18.333]. Another study that used worn clothing as a proxy for skin emanations investigated the effects of ozone exposure on skin at ozone concentrations relevant to typical altitudes of air travel. Participants were asked to wear t-shirts overnight and subsequently place these in a simulated aircraft cabin, which was then flushed with ozone and the cabin air monitored by PTR-MS [18.65]. The presence of ozone led to a production in saturated and unsaturated aldehydes, and carboxylic acids, which reflected anecdotal observations of a higher odor in the cabin after ozone exposure compared to before. The theme of ozone-initiated lipid oxidation was followed further by direct PTR-MS measurements of skin emanations after ozone exposure [18.334]. In particular, the investigation revealed oxidation products such as dicarbonyls that may be respiratory irritants. Volatile lipid peroxidation products were similarly investigated in one study that exposed skin to ultraviolet (UV) light. PTR-MS was used to monitor reaction products during and after exposure, whereby compounds such as acetaldehyde and propanal, as well as some unidentified product ions, showed dramatic increases in concentrations above the skin during UV exposure [18.335].

One example of a SIFT-MS application loosely related to body odor is experiments performed on skin emanations after ingestion of glucose in the fasting state, with corresponding analyses of exhaled breath [18.336]. The results did not produce directly correlative responses in breath and skin in terms of their temporal developments, but demonstrated that most

compounds were present in both, with ammonia flagged by the authors to be of particular value in future applications, for instance to monitor the efficacy of haemodialysis treatment. Acetone emissions showed the highest correlation with blood glucose in breath and skin emanations, equally holding promise for potential future clinical use. APCI-MS has been used to investigate fatty acids from skin by use of secondary electrospray ionization (SESI) and subsequent detection via APCI-MS, whereby lactic acid and a complete series of saturated and singly unsaturated fatty acids were observed, as well as other metabolites such as ketomonocarboxylic and hydroxymonocarboxylic acids [18.337].

Bodily Fluids

In addition to bad breath and body odor discussed above, on-line CIMS techniques have been employed to characterize the volatiles present in other bodily fluids such as blood, urine and feces. As with breath analysis, most on-line CIMS studies of these bodily media focus on characterizing the overall volatile emissions, rather than purposely looking at the odorants, with the aim of identifying disease-specific volatile biomarkers. However, since odor-active volatiles are inevitably measured in these fluids, a brief review of research in this area is included here for completeness.

On-line CIMS analysis of the constituent volatiles in blood or urine is typically performed via direct headspace analysis, i. e., by sampling the gas immediately above the liquid sample. SIFT-MS has been used, for instance, to assess the correlation between breath and blood concentrations of ethanol after alcohol consumption. A comparison between breath ethanol levels – analyzed both directly and via sampling bags – with the ethanol levels in the headspace of blood measured by SIFT-MS, as well as data from a routine clinical determination of ethanol levels in blood, showed a high correlation and revealed that ethanol in the exhaled breath is in equilibrium with the ethanol in the blood, which is the main premise for alcohol *breathalyzers* used in law enforcement for drunk driving [18.298]. Other studies have similarly compared the concentrations of VOCs, primarily endogenous compounds, in breath and blood. PTR-MS, for instance, was used to investigate the potential of exhaled breath to be used as a surrogate for blood in detecting such compounds, although in those studies only the breath and not the blood was analyzed using PTR-MS [18.338, 339].

Urine headspace has been analyzed by SIFT-MS for several compounds, including acetonitrile [18.340], acetone, butanone, pentanone, hexanone and heptanone [18.341], and 3-hydroxybutanoic acid and other ketones [18.342], or to investigate potential volatile metabolites for profiling gastro-esophageal cancer [18.343]. Acetonitrile in urine was similarly investigated by PTR-MS in a study to correlate urinary levels with smoking habits wherein significantly higher concentrations were found in the urine of heavy smokers compared to nonsmoking controls [18.344]. The headspace of urine was also analyzed recently to ascertain changes in its composition after strenuous walking, whereby acetic acid, amongst other metabolites, was identified as a significant marker for exercise effects [18.345].

Decomposition Odor and Entrapment Odor

Postmortem decomposition odor and entrapped human odor are of interest in forensic science and search and rescue endeavors. Most studies investigating human odor emission are conducted offline by use of hyphenated GC techniques [18.346] and GC-MS has indisputably been pivotal in acquiring a good understanding of human odor, ranging from breath gas, blood, and urine to tissue decomposition [18.346–352].

Law enforcement and rescue agencies have long made use of the highly sensitive canine sense of smell to locate human remains or survivors after natural or manmade disasters [18.353]. Despite the exceptional abilities of trained dogs in this task, their usage has certain shortcomings such as a high cost for training, a rapid onset of fatigue leading to limited working hours (typically only 20 min before they require several hours of rest to recuperate) and only 6–8 years average working lifetimes. The development of field-portable analytical instruments that mimic the canine ability of locating live humans or remains is thus highly desirable.

The on-line CIMS techniques covered in this chapter have a range of potential applications in the field: the equipment can operate continuously and reproducibly with sufficiently low LODs that are necessary for the detection of human odor emanations, and their fast time resolution could allow for monitoring the intermittent nature of human odor emissions in real-time at the point of interest. Although these techniques cannot yet achieve the sensitivity of the canine nose, these could be used as a complementary tool in the field, for instance for the purpose of preliminary screening of large sites.

To increase the recovery rate of surface and subsurface human survivors or cadavers, the artificial decomposition (or bodily fluid) odor surrogates used for canine training and instrumental calibration should closely mimic the composition and time-intensity release curves encountered under realistic scenarios. Perhaps the greatest barrier to wider applications of on-line CIMS applications in these fields is the lack of complete characterization of the odor signature (chemical fingerprint) of human decomposition odors and the

large spectrum of VOCs emitted by bodily fluids – i. e., the human volatilome [18.286] – which consequently limits the availability of comprehensive synthetic odor surrogates, for example those used as canine training aids [18.354, 355]. Such synthetic odorant mixtures should also be used to calibrate the on-line CIMS signal responses and to improve spectral identification, while their accuracy and appropriateness as training and instrumental calibration aids should be thoroughly evaluated by applying procedures similar to those used for assessing food flavoring compositions and odorant delivery systems described elsewhere in this chapter.

Despite the great potential for successful applications in the field of forensics and search-and-rescue, the technical literature is currently devoid of reports on the implementation of on-line CIMS. Near-real-time applications have been reported only for multicapillary column ion mobility spectrometry (MCC-IMS). Specifically, these relate to the fingerprinting of urine odorants [18.356] that can be used for purposes of subject location under ruins, VOCs emitted from humans entrapped in confined spaces [18.357] and general fingerprinting of skin odorants [18.358]. Recent advancements in the sensitive and quantitative detection of skin-borne volatile biomarkers of entrapped humans have been achieved by PTR-SRI-TOF-MS [18.359].

Despite the fact that the chemical identification capabilities of on-line CIMS do not yet reach those of GC-MS, and although complex patterns of odor mixtures are not immediately discernible as with e-noses or sensors arrays (the latter have nonetheless limited applications in these fields [18.360]), the techniques reviewed in this chapter have distinct advantages that could contribute to in situ applications for survivor or cadaver localization. They offer highly sensitive measurements of specific target VOCs and require no sample separation, thereby enabling fast screening purposes based on molecular masses, in addition to being deployable in the field and offering a relatively low cost and high throughput for sample analysis when compared to multiple steps and handling required for the offline GC methods. As such, once the chemical fingerprinting of body fluids and decomposition odors has been narrowed down to a core set of tracer VOCs, it is anticipated that the application of CIMS to these types of investigation will proliferate in the near future.

18.2.4 Olfaction

The field of olfactology, i. e., the study of the sense of smell, has been discussed extensively in several chapters of this book. On-line CIMS techniques are of utility in certain aspects of this field of research, but have been far from exploited to date. As with in vivo aroma release or exhaled breath odor studies, specific olfactology-related investigations can greatly benefit from the real-time analytical capabilities of on-line CIMS. Applications of on-line CIMS in olfactology-related studies can be broadly categorized into two branches: validation of tools used in olfactology, and the study of human physiology and olfaction. These are treated briefly below.

Validating Tools Used in Studies on Olfaction

The assessment of olfaction in terms of the sensitivity and function (or dysfunction) of the human sense of smell is typically made by presenting the subject with a specific odorant or series of individual odorants at defined concentrations. In terms of olfactory ability, this might involve identifying or naming a specific smell within a selection of different smells. For sensitivity, the odor and perception thresholds can be determined by ascertaining the presented odor concentration below which the person no longer detects or perceives it. Similar methods are employed for dysfunction in relation to varying forms of anosmia, whereby odors must be named or their perception must be indicated during presentation of specific odorants at varying concentrations.

Most olfaction-related tools used by otorhinolaryngologists in determining olfactory ability have undergone rigorous evaluation from a clinical perspective, but few have been the subject of chemo-analytical validation. In other words, they have been demonstrated to be reliable tests for their intended purposes, but – especially for olfactory sensitivity testing, whereby the presented odorant concentration is a key feature of the test – the odorants in use in such tests are rarely accurately quantified analytically, but rather are presumed based on their methods of preparation.

There are diverse tools that are in routine use for assessing olfaction, the most prominent being the University of Pennsylvania Smell Identification Test (UPSIT) [18.361], the Connecticut Chemosensory Clinical Research Center Test (CCCRC) [18.362], and the combined odor identification, discrimination and threshold assessment using Sniffin' Sticks [18.363]. Human olfactory function assessment methods are treated in more detail elsewhere in this book (Chap. 23). As with the other applications outlined in this chapter, the benefits of using on-line CIMS for such investigations are the wide range of detectable compounds, the broad dynamic range of measurable concentrations, and the real-time capabilities.

Sniffin' Sticks have found widespread use in testing olfactory sensitivity and acuity [18.363], and the threshold test comprises a set of odorant pens covering a range of concentrations of *n*-butanol that are used to determine the threshold of human subjects to this

particular odorant, and by inference approximate the keenness of their sense of smell. This test battery has been validated from a clinical approach [18.364, 365], but previously not to any great extent from a chemo-analytical perspective, with only one study reporting on the release of *n*-butanol from the tips of individual pens, as measured by GC-MS [18.366]. PTR-MS was recently used to provide a comprehensive validation assessment of the gas-phase concentrations of *n*-butanol released from the tips of the Sniffin' Sticks olfactory threshold test set in terms of concentration linearity and stability over time [18.367]. Pens were presented to the PTR-MS via a custom-made analytical setup according to the same protocol used for testing human subjects. By continuously monitoring the *n*-butanol signal, the gas-phase concentration linearity across the pens could be determined; for these specific investigations it could be shown that gas-phase *n*-butanol released from the tips of the pens was linear over the full concentration range, as had been previously only assumed. Furthermore, assessments of older pens indicated good odorant (concentration) stability provided the pens were stored correctly. This one example highlights the power of on-line CIMS for validating such tools in a quick and reliable fashion.

An air-dilution olfactometer is a device that generates odorant pulses at predefined concentrations and for predetermined pulse durations [18.368, 369]. It can be used for odorant delivery with simultaneous measurement of olfactory event-related potentials via electroencephalography (EEG) in assessments of neurological activity in response to odorant exposure. As with the tools outlined above, the odorant concentrations presented to the subjects undergoing examination are typically only calculated. In this case, calculations are made based on the specific conditions of the system, i. e., the set mixing ratios of the (presumed) saturated odorant gas and the air dilution flow. Past attempts at validation have used sensors such as a photoionization detection (PID) system to measure the temporal resolution of odorant pulses, but typically rely on GC-MS analyses for quantitation [18.369]. On-line CIMS techniques offer both a high temporal resolution and accurate quantitation and are thus highly appropriate tools for validating such tools. PTR-MS was recently used to characterize the odorant pulses generated by one such air-dilution olfactometer [18.370]. Focusing on the odorous compounds hydrogen sulfide, ethyl butanoate, ethyl hexanoate, and 2,3-butanedione, the PTR-MS analyses revealed a dependence of the delivered odorant concentration on the pulse duration. In particular, the briefest pulse of 50 ms produced a H_2S concentration that was less than half that of the longest pulse of 3.2 s. Furthermore, the relative odorant concentrations set at the olfactometer were not necessarily reflected at its delivery port and depletion in concentration was observed for odorants that were delivered by the olfactometer from aqueous solutions. These results indicated that such gas-dilution olfactometer systems should not be operated with the assumption that the set concentrations are accurately delivered by the system, but nevertheless the device was deemed suitable for the settings typically encountered in its clinical use [18.370]. Two commercial field olfactomters were also recently validated by SIFT-MS with respect to their ability to generate accurate dilutions, specifically for the compounds acetic acid, propanoic acid, *n*-butanol, dimethyl sulfide and dimethyl disulfide [18.371]. One of the two systems showed a good performance, albeit with a breakthrough of the compounds through the activated carbon filter at the highest concentrations; the other system was found to produce linear concentrations, although these were higher than expected theoretically.

APCI-MS (MS-Nose) was used to measure the in vivo flavor release of the compound ethyl butanoate from milk (whole and skim milk samples) and to replicate this via defined odorant pulses from a gas-dilution olfactometer [18.372]. The latter were then verified using the same APCI-MS, where it was shown that the areas under the flavor release curves were consistent in most cases. This study further demonstrates how odorant delivery tools such as the gas-dilution olfactometer can be calibrated using on-line CIMS techniques. APCI-MS was also recently used to assess the variability in odor delivery of several simple systems commonly used for human sensory testing, namely from squeezable wash bottles, hinged-lidded plastic sniff pots, cosmetic cotton buds placed in a tube, and a dynamic system of purging air through the odor solution via a fine sinter [18.373]. An odor mixture containing eight compounds of different physicochemical properties in water was used to study odor delivery from these systems during typical procedures of use, for example squeezing the wash bottle for 10 s for sampling, followed by a 30 s pause in a repetitive manner. The assessments via on-line APCI-MS detection demonstrated the rapid decrease in headspace concentrations from the initial values to subsequent squeezes, lid-openings, and purge time for the different systems for most of the compounds, whereby 3-methylbutanol and diacetyl showed the least change for the latter two systems. Following this initial concentration drop, however, the levels stabilized to produce fairly consistent concentrations. Although the systems tested are primarily used for determining odor attributes rather than for threshold assessments, whereby fluctuations in the absolute concentration might not be too critical, this study nevertheless demonstrates the need for validating

such methods to ensure consistency. Furthermore, the authors attributed some losses in compound concentrations from the squeezable wash bottle to interactions of individual compounds with the plastic material of the bottle, further highlighting the importance of choosing appropriate materials when working with volatile odor compounds.

Human Olfaction

The rapid and sensitive detection capabilities of on-line CIMS techniques have long been exploited in food-flavor research for aroma detection in relation to flavor release from foods, in particular in the form of correlative determinations of exhaled *nosespace* aroma compound concentrations with perceived sensory impressions, as reported and discussed in Sect. 18.2.1. Despite the wealth of information gained from such studies, critics have often voiced their concerns that the odor concentrations detected in the exhaled breath exiting the nostrils do not necessarily represent those that reach the nasal epithelium in the olfactory cleft, located at the upper apex of the nasal cavity, and interact with the epithelial cells to elicit an odor impression in the brain. The basis of these arguments is that the physicochemical properties of individual odorants and their affinity to the nasal mucosa will determine the extent to which they can successfully traverse the nasal cavity; odorants released in the oral cavity have a different route to the olfactory cleft (retronasal perception) than through the nares (orthonasal perception). As such, in the field of human olfaction it is desirable to determine odorant concentrations directly at their site of physiological detection, i. e., at the nasal epithelium. Despite this, on-line CIMS has been largely overlooked in this niche area of research, but has recently been used in several investigations for probative assessments of specific odorants at different positions within the nose.

Very few studies in the past have made progress in this direction by conducting in vivo detection of odorants within the nasal cavity. Amongst these, the trigeminal sensation associated with exposure to carbon dioxide (CO_2) by its directed stimulation has been investigated at different locations within the nose by simultaneous intranasal detection of this compound [18.374]. In that study, however, the temporal attribute of the stimulus, which is a critical parameter for perception, was not considered, but rather the intranasal concentrations of CO_2 were determined only once these had stabilized. Another study used an e-nose sensor with a sampling rate of 8.3 Hz to detect and quantify H_2S at the olfactory cleft at two stimulus concentrations (2 and 10 ppm_v) and comparatively for orthonasal and retronasal odor administration routes [18.375]. This investigation successfully demonstrated that odorants can be measured intranasally with a high temporal resolution, with indications that there was no difference in concentration observed at the olfactory cleft from orthonasal or retronasal administration; it should be noted here that other odorants might be expected to perform differently to this specific compound.

Based on the initial success in detecting a single odorant intranasally using a sensor system, trials of multiple-aroma detection in relation to perception and administration routes were conducted using PTR-MS by measuring the concentrations and temporal profiles of stimuli of specific aroma compounds released from model food custards [18.376]. In particular, the chosen aroma compounds were measured at four positions within the nasal cavity, namely in the nostril, in front of the middle turbinate, in the area of the olfactory cleft, and in the nasopharynx, as released via the oral cavity during mastication of two custards of different viscosity; a liquid and a solid custard. The highly time-resolved measurements showed clear differences in release, both between compounds and between custard viscosities for individual compounds. Moreover, the concentration maxima of all compounds were observed to be highest at the nasopharynx and lowest at the olfactory cleft, with the different positions also showing varying latencies of compound arrival.

In a similar study, PTR-MS was used to detect individual odorants exiting the nostrils in relation to modes of presentation, either an orthonasal or a retronasal stimulus of discrete odorant pulses generated by a gas-dilution olfactometer [18.377]. In particular, a comparison was made between ipsilateral and contralateral orthonasal and retronasal stimulus presentations, i. e., between odorant concentrations at the nostril coming from pulses presented either on the same side or on the opposite side of the nasal cavity as the sampling position. Here it was shown that, as intuitively expected, the odorant concentrations exiting the nostril from retronasal stimulus presentation were similar for ipsilateral and contralateral sampling, since the air containing the odorant essentially flows equally between the two chambers of the nose. By comparison, orthonasal stimulus presentation elicited much lower concentrations at the nostril during contralateral (compared to ipsilateral) detection, since the odorant was required to flow in through one nostril and out through the other. An interesting finding of this study was the large discrepancy in the concentration of the odorant detected directly as emitted from the olfactometer compared to its intranasal detection. The authors explained this phenomenon to be a culmination of the distribution of the odorant within the nasal cavity and adsorption of the odorant to the mucosal lining, the degree of which depends on gas flows, the nasal anatomy and the

physicochemical properties of the compounds in question [18.200]. In addition to the evaluation of cortical olfactory processing – detailed below – the visualization of swallowing processes during food consumption provides additional insights into olfaction in this context. The aforementioned study used videofluoroscopy of the oral and pharyngeal processes [18.378] in combination with PTR-MS nosespace analyses to identify consumption behavior responsible for retronasal aroma release. In one example using model food gels of different hardness, videofluoroscopy showed that panelists tended to press the gel against the frontal region of the hard palate using their tongue, without direct chewing action [18.377]. It was found that individual consumption patterns greatly affected flavor release, whereby the velum-tongue border remained closed in nonchewers until the sample was swallowed, which was then followed by a higher intensity swallow breath compared to panelists that chewed their sample.

Subsequent studies have employed on-line CIMS to assess how the mode of sniffing can affect intranasal concentrations of individual odorants. One study investigated how the odorant 2,3-butanedione (diacetyl) was delivered to the region of the olfactory cleft in relation to the sniffing behavior of subjects, with a comparison between normal inspiration, rapid sniffs and a single, forced sniff [18.379]. The normal sniff was observed to elicit the maximum concentrations at the olfactory cleft, which anecdotally reflected the highest intensities subjectively perceived. In a further study the concentrations of *n*-butanol at the olfactory cleft, as detected by PTR-MS, were compared to the odor thresholds of individual subjects that were ascertained by means of odorant pens (Sniffin' Sticks). Comparison of intranasal concentration and odor threshold indicated an overall alignment of *n*-butanol concentrations at the olfactory cleft with the corresponding odor threshold and the reports from subjects that they did or did not perceive the odor (false/correct perception responses) [18.380]. Additionally, concentrations in the pens generally correlated positively with concentrations at the cleft, which provides evidence of the pen performance – as similarly demonstrated in a separate study [18.367] – as well as demonstrating the validity of such intranasal sampling methods to perform corresponding subjective threshold and odorant delivery tests.

Moving to ex vivo studies, a series of measurements using APCI-MS was made to study the dynamics of odorant uptake by odorant-binding protein (OBP) [18.381, 382]. The dynamic biomimetric odorant-binding system (DyBOBS) developed for these studies featured a capillary tube with its inner surface coated in a thin aqueous film of recombinant rate OBP, through which known concentrations of odorants were passed and their uptake and release monitored by on-line APCI-MS. The system showed time-dependent changes and remarkably high reproducibility, offering a promising technique for investigating odorant mass transfer from the gas-phase to the liquid-phase of OBPs. On-line APCI-MS has also been used to validate a system for retronasal olfactory stimulation that combines an automated odorant stimulus delivery system with functional magnetic resonance imaging (fMRI) acquisition of brain activity to complement basic sensory research in terms of cortical olfactory processing [18.383] (see also Chap. 38).

This handful of studies demonstrates the utility of on-line CIMS techniques for investigations in the field of olfaction and highlights the benefits for scientists working in the field of chemosensation to increasingly embrace these tools in years to come.

18.2.5 Miscellaneous Applications

This final applications section reports on niche areas in which on-line CIMS techniques have been deployed, with only loose association to odorant detection. It is included here for completeness to provide the reader with some insights into other areas of use for these on-line CIMS tools. Inevitably there are some further minor areas of application that are not well documented in the scientific literature and are therefore not reported here.

Chemical Warfare Agents and Toxic Industrial Compounds

Chemical warfare agents (CWAs) and highly toxic industrial compounds (TICs) are briefly considered under the umbrella of detecting volatile *odoriferous* compounds using on-line CIMS techniques for scenarios such as real-time, in situ threat monitoring, for example first responders to war or terrorist attacks, or in the protection of workers operating at chemical facilities or on location at environmental contamination sites. TICs are also potentially dispersed as attack or contaminant agents and thus fall in the same category as CWAs. It might be noted that such situations typically involve CWAs or TICs at trace levels that are below human odor perception or detection thresholds.

CWAs are synthetic compounds with low molecular weight that are characterized as fast acting and potentially lethal for humans at very low levels. Typical compounds in this group include the nerve gases sarin (GB) and tabun (GA), or the blister agents mustard gas (HD) and lewisite 1 (L1), to name but a few. A compilation of chemical structures, volatility at 25 °C, and inhalation toxicity data of CWAs is given in the literature [18.384]. Most of these substances exist as stable liquids or solids with low volatility at room tempera-

ture; these sometimes exhibit characteristic odors (e.g., mustard gas smells like garlic, onion or mustard), although the human sense of smell is generally numbed rapidly after exposure, typically after only a few inhalations. Common chemicals that are widely produced, transported, and stored can also be classified as TICs, i. e., having high toxicity and being easily vaporized. These are especially noxious when released at elevated concentrations, and can include compounds such as ammonia, formaldehyde, sulfur dioxide, phosgene, chloroacetone, chloroacetophenone, and many others compiled and ranked into health-hazard lists.

CWAs and TICs are usually released as vapor or aerosols and enter the body mainly via inhalation or dermal exposure, hence the need for detection in the gas and/or particulate phase. Due to their often rapid action and high lethality there is a necessary demand for rapid and reliable methods for the early detection and identification of CWAs and TICs and their degradation products at the point of interest (hence portability is also a requirement for these methods), typically within a few seconds to less than a minute, and a high detection specificity to avoid false alarms [18.385]. Because of the acute toxicity at low concentration levels the required detection limits are in the range of ppb_v down to ppt_v levels. For example, the detection requirements for sarin are gas-phase concentrations of $150\,\mu g/m^3$ (ca. 25 ppb_v) [18.384], which is necessarily well below the toxicity level (LCt50 within 1 min) and often at subodor thresholds for humans. Interfering environmental signals from emissions, for example by exhaust gases or smoke resulting from burning materials at the site of an attack or accident, as well as the influence of variable sample gas humidity, pose significant issues for the reliable detection and quantitation of these agents.

The recommendation for analytical field inspections of CWAs under the Chemical Weapons Convention include GC-MS and IMS for on-site screening [18.386]. Application requirements are fast analysis times (< 30 s), high sensitivity, high selectivity towards the specific agents, and good precision with a wide dynamic range and suitable accuracy. Although commercially available portable GC and GC-MS can provide highly sensitive and specific detection of CWAs in air, often the required real-time detection is not achievable. IMS has found widespread use for screening CWAs [18.387–391], yet the technique is affected by a low sensitivity and specificity (the latter leading to frequent false alarms) and by relatively long response and recovery times due to strong adsorption of CWAs on the internal surfaces of the device [18.384, 392]. Auxiliary reagent gases (e.g., ammonia) affect ionization processes and are quite common in IMS-based CWA detectors for suppressing fragmentation effectively and thereby allow for the implementation of algorithms for automated agent identification in field applications.

APCI-MS has long been reported as a detector capable of real-time detection of CWAs down to ppt_v-levels, both in positive and negative ionization modes [18.393–395]. Lately, an improved method for sensitively and selectively detecting CWAs was developed by using counterflow introduction APCI-MS. This latest technique relies on corona discharge to form reactant ions that are then driven by an electric field in an opposite direction to the airflow, thus eliminating the interfering neutral molecules ozone and nitrogen oxide and resulting in a more efficient ionization of the target compounds, particularly in the negative ionization mode [18.396].

SIFT-MS has also been trialled for CWA detection, with a paper reporting on the rate coefficients and branching ratios of the reaction of fifteen CWA precursors and surrogates with hydronium, NO^+ and O_2^+ reagent ions, thus enabling quantitative detection [18.397]. For most of the investigated compounds the real-time LODs were in the ppb_v to ppt_v range at a detection time of 10 s. Despite its promise, no further work on SIFT-MS applications for CWA detection has emerged in the literature since the aforementioned paper.

PTR-MS shows promise as a suitable technique for the sensitive, selective and fast detection of CWAs and TICs, particularly with the higher accuracy spectral resolution afforded by the PTR-TOF-MS instrument configurations [18.398], but also in the simpler and compact PTR-QMS model. Both systems have been trialled using hydronium as the main reagent to detect CWAs and TICs [18.399]. Additionally, ammonia has been used as a reagent ion in combination with a reduced drift voltage to effectively suppress the fragmentation of product ions of the nerve agents sarin, soman, cyclosarin and tabun [18.400], which is necessary in order to generate and implement algorithms for automated agent identification in field applications. On the other hand, appearance of particular fragments might deliver additional information. Making use of these effects allows switching between fragment and molecular ion peak spectra; thus targeted fragmentation can be used to confirm identification based on molecular peak detection.

In conclusion, all of the on-line CIMS techniques deliver the necessary requirements for both immediate detection and continuous on-site monitoring of CWAs and TICs. Currently, limits of detection for commercial and research grade on-line CIMS instruments are estimated in the ppt_v to ppq_v levels, and the range of detectable CWAs and TICs agents in on-line CIMS

is being constantly expanded by advancements in theoretical studies and measurements of ion chemistry properties [18.401].

Process Analysis

The processing industry as a whole, including chemical, pharmaceutical, biochemical, cosmetic and food processing sectors, strive for simultaneous analysis and continuous process control. The information for the task is provided by integrating a variety of analyzers encompassing near-infrared spectroscopy, electrochemical probes and multiarray gas sensors to generate thousands of different real-time signals from chemical or biological processes. Providing on-line/in-line/at-line and in situ measurements has to meet specific requirements, for example warrant aseptic conditions and detect a high number of analytes simultaneously and without disrupting the process. On-line mass spectrometry equipped with carefully designed inlet interfaces, in some instances CIMS techniques, are well established in several fields of industrial and bioindustrial processing [18.402] where their capability for real-time sample analysis and high analytical throughput yields insights into the dynamics of chemical or biochemical reactions and kinetics, also for labile intermediates or metabolites [18.403]. Although such VOCs do not strictly belong to the odorants category, a selection of on-line CIMS applications in the field of industrial process monitoring is provided in the following for completeness.

PTR-MS has been deployed in the biopharmaceutical industry to support the understanding and control of production processes and to monitor these in view of increasing the product yield, an application demonstrated by the measurement of the headspace gas from within a bioreactor [18.404]. Constituent VOCs in the headspace of the reactor used for fermentation of *Escherichia coli* were monitored in real-time and compared with biomass dry matter weight and recombinant protein production rate. Twenty VOCs that correlated with these bioprocess parameters were identified and proposed to be of potential use as indicators for on-line monitoring of fermentation progress. Moreover, advanced on-line monitoring of cell culture off-gas has also been achieved by deploying PTR-MS [18.405, 406]. PTR-MS has also found application in biogas-producing facilities to monitor odorous trace impurities such as H_2S, which presents an operational and environmental challenge, along with monitoring of marker VOCs as quality indicators of the biogas product [18.407]. On the chemical processing side, PTR-MS was used for the analysis of syngas in an industrial Fischer-Tropsch process to detect a variety of volatile organic and inorganic compounds, such as HCN, H_2S, RSH, carbonyls, acids, alcohols and others, potentially odorous VOCs [18.407].

18.3 Conclusion and Outlook

On-line chemical ionization mass spectrometry, CIMS, has developed over the past two decades to become a powerful tool in the real-time detection of VOCs, including odor compounds. The use of such systems in the detection of odorants has evolved from a handful of niche and classical areas of study to a wide range of fields of application that cover food aromas, environmental and indoor odors, studies related to olfaction, detection of chemical warfare agents, forensics and many more. This chapter has introduced and discussed the three current major on-line CIMS techniques of selected ion flow tube MS (SIFT-MS), proton transfer reaction MS (PTR-MS) and atmospheric pressure chemical ionization MS (APCI-MS), and their implementation for the detection of odorous compounds in diverse disciplines. The less common techniques such as ion-molecule reaction MS (IMR-MS), ion mobility spectrometry (IMS) and ion attachment MS (IAMS), are also introduced, with references to specific odor-related applications given, where appropriate. The chapter provides an up-to-date compilation of the state of research using these powerful CIMS tools.

The on-line CIMS techniques reviewed in this chapter have their primary strength in providing very fast and highly sensitive analysis of VOCs, including odorous compounds, whilst practically eliminating analytical artifacts and sample losses arising from pretreatment, sample storage and thermal desorption steps that are mandatory for routine analytical methods. The use of fast, on-line CIMS techniques overcomes the need of discontinuous purge-and-trap or preseparation or preconcentration steps customarily required by the GC-MS methods, thus allowing for real-time detection of odorous VOCs down to ultratrace levels in diverse areas of application. As such, the CIMS techniques greatly increase the number of samples analyzed on-site, thus significantly reducing data uncertainty and improving time resolution for determining fast odorant emission or release processes. Furthermore, the method of analyzing solid or liquid samples is nondestructive since measurements proceed only in the

gas-phase from the sample headspace, thus samples can be analyzed repeatedly, as appropriate, as is the case for monitoring food spoilage or aroma development.

Despite the applicability of the on-line CIMS techniques in the fields addressed in this chapter, it is crucial to apply them with knowledge of their strengths and limitations, bearing in mind that these are principally tools for real-time measurement, gas-phase fingerprinting, and nontargeted screening of VOCs, rather than for exhaustive chemical-structural elucidation of known and unknown analytes. Although on-line CIMS can provide some chemical-structural information on the target analytes, the capability to thoroughly separate and identify isobars, isomers and, in many cases, specific odorant stereoisomers, remains a strongpoint of the slower and specific GC-MS techniques. For certain applications, accurate concentration assessment of selected odorants can only be achieved by the GC-MS techniques, especially by the hyphenated (GC-GC) systems, for which customary quantitation procedures (such as internal standard additions or stable isotope dilution assays) provide unsurpassed accuracy and precision in quantitation, although there is evidence indicating that on-line CIMS techniques perform better for some applications [18.58, 408, 409]. Further, in terms of quantitation, a particular strength of on-line CIMS techniques is the opportunity to make relative comparisons of dynamic changes, which is made possible by the mostly linear response to analytes over a wider range of gas-phase concentrations.

The generation of large, chemically detailed and highly time-resolved concentration datasets afforded by on-line CIMS opens the path to the application of powerful multivariate statistical methods, encompassing chemometrics, data-mining and statistical modeling techniques. These can be applied to solve problems of fingerprinting, classification or calibration across virtually all the fields of applications described in this chapter. Successful examples have been described for applications with SIFT-MS [18.148], APCI-MS [18.179], PTR-MS [18.131] and PTR-TOF-MS [18.282]. The continuing endeavors to increase detection sensitivities, reduce the limits of detection, and expand the range of detectable compounds by improved compound discrimination are welcome developments that will broaden the appeal of on-line CIMS techniques for odorant detection. As the mass spectrometric detectors (QMS, TOF-MS) and the CI method described in this chapter have reached a steady state of development, the latest technological progress heads toward better collimation and preselection of the analyte ions before these proceed to the detector. Some of these developments have already been implemented in research or commercial instruments and have been demonstrated to further increase the detection sensitivity, expand the detection range and to increase mass resolution and enhance selectivity. In the case of APCI-ITMS, for instance, the ion trap enhances the detection sensitivity of polychlorinated biphenyls and allows for the monitoring in ambient air [18.410].

A dual-purpose drift tube/ion funnel can be coupled to various types of mass spectrometers to increase the detection sensitivity, which has been reported in terms of a radio frequency ion funnel in PTR-MS [18.411]. Alternatively, in PTR-QiTOF-MS a quadrupole ion guide (Qi) preselects the ions entering the TOF-MS area, to enhance the mass resolution [18.95]. The implementation of a fastGC add-on to PTR-MS instruments offers fast gas chromatographic performances by separating the neutral analyte molecules before these undergo proton attachment, which in turn enhances discrimination and allows identification of isomers. This has readily found application in food and flavor research, for example in wine aroma analysis or in medical application such as breath gas research [18.90, 94], as well as in biogenic emissions [18.412]. The recent launch of a commercial SIFT-MS instrument with negative reagent ions, namely O^-, O_2^-, OH^- and NO_2^-, extends the analytical range of detectable compound classes – albeit not focused on odorants – to include acidic gases (HCl, HF, SO_2), greenhouse gases (CO_2, CH_3, NO_2, H_2O), photochemical smog precursors (NO_x, ozone, PAN), and tracer gases (e.g., sulfur hexafluoride SF_6). Pending developments in this field also include the planned launch of a commercial APCI-MS tailored for the sensitive, on-line detection of VOCs.

Although on-line CIMS have an analytical range beyond only odorous substances, their implementation for odorant detection is highly valid, as is evident from the diverse range of applications reviewed in this chapter. To date their main dedicated use to detect odorous molecules has been in aroma research, but many niche fields can profit from the analytical capabilities of the on-line CIMS techniques of SIFT-MS, PTR-MS, and APCI-MS, most notably due to their real-time detection capabilities, wide dynamic range, and low limits of detection. Certainly this technology will be increasingly deployed in such fields once its potential is fully realized.

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19. Enantioselective Gas Chromatography with Cyclodextrin in Odorant Analysis

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This chapter concerns enantioselective gas-chromatography (Es-GC) with cyclodextrin derivatives as chiral stationary phases for chiral recognition of volatile odorants in the flavor and fragrance field. The text is divided into two main parts. The first one is more general and deals with enantiomers and odor and need for chiral recognition, evolution of chiral stationary phases for Es-GC since its beginning, followed by the history of cyclodextrins and their applications to enantioselective GC. It also includes some theoretical aspects of enantiomer separation with cyclodextrin derivatives and their influence on routine chiral recognition.

The second part concerns the strategy of chiral recognition in routine analysis with cyclodextrin derivatives as chiral stationary phases illustrated by examples with real natural product samples. This part describes enantiomer automatic identification or their excess or ratio determination in complex mixtures by enantioselective GC combined with mass spectrometry; in particular it deals with the potential of multidimensional techniques and of fast GC in chiral recognition and the role played by mass spectrometry. The last paragraph concerns the use of total analysis systems in chiral recognition.

Metabolite formation in a biological matrix (plant or animal) is almost always stereoguided, and the resulting components are very often chiral and present an enantiomeric excess. Enantiomer recognition and enantiomeric excess (ee) and/or ratio (er) determinations of a chiral compound are therefore important parameters to characterize the matrix and its biological activity, in particular in the fields of food, flavor, and fragrance. Enantiomeric recognition is therefore very important:

1. To correlate chemical composition and organoleptic properties
2. To determine the biosynthetic pathway of a compound
3. To classify a sample
4. To determine the geographic origin of a *natural* sample
5. To implement quality control and detect frauds or adulteration of *natural* samples: enantiomeric composition can reveal the addition to *natural* products of cheap synthetic materials or volatiles from other sources, mainly to reduce costs.

The interaction of a molecule with olfactory receptors is usually stereoselective, meaning that the

Table 19.1 Examples of different odor characteristics of enantiomers of chiral molecules [19.1]

Compound	Enantiomer	Characteristics
Nootkatone	(5*R*, 6*S*, 8*R*)-(+)	Grapefruit
	(5*S*, 6*R*, 8*S*)-(−)	Woody-spicy
2-Methylbutanoic acid	(*R*)-(−)	Penetrating, reminiscent of cheese and sweet
	(*S*)-(+)	Pleasant, sweet, elegant, fruity note
Ethyl 2-methylbutanoate	(*R*)-(−)	First medical, phenolic note, later sweet, fruity
	(*S*)-(+)	Ether-like, sweet, after dilution pleasant apple note
2-Ethylhexanoic acid	(*R*)-(−)	Herbaceus, earthy
	(*S*)-(+)	Sweet, herbaceus, faint musty
1-Octen-3-ol	(*R*)-(−)	Intensive mushroom note, fruity, soft
	(*S*)-(+)	Herbaceus, green, musty
Linalool	(*S*)-(+)	Flower-fresh, reminiscent of lily of the valley
	(*S*)-(+)	Differs slightly in odor
(*E*)-Nerolidol	(*R*)-(−)	Pleasant, woody, warm, musty
	(*S*)-(+)	Slightly sweet, mild, soft, flowery different to (*Z*), less intensive
(*Z*)-Nerolidol	(*R*)-(−)	Intensive, flowery, sweet, fresh
	(*S*)-(+)	Woody, green, fresh bark
Limonene	(*R*)-(+)	Fresh, pleasant, orange-like
	(*S*)-(−)	Faint mint note, turpentine note
α-Ionone	(*R*)-(+)	Fine violet-like, fruity, flowery, raspberry-like
	(*S*)-(−)	Strong woody aspects, raspberry-like
α-Terpineol	(*R*)-(+)	Strong, flowery sweet, lilac
	(*S*)-(−)	Tarry, reminiscent of colp pipe
Carvone	(*R*)-(−)	Herbaceus odor, reminiscent of dill seeds
	(*S*)-(+)	Herbaceus odor, reminiscent of spearmint
α-Phellandrene	(*R*)-(−)	Citrus odor, slight peppery note
	(*S*)-(+)	Weed-like, dill-like
Menthol	(1*R*, 3*R*, 4*S*)-(−)	Refreshing, mint note, cool
	(1*S*, 3*S*, 4*R*)-(+)	Mint, phenolic note, medical note, camphor-like, not refreshing
Whiskey lactone	(3*R*, 4*S*)-(−)	Strong coconut note, reminiscent of celery
	(3*S*, 4*R*)-(+)	Piquant celery note, faint coconut note, green walnut note
	(3*R*, 4*R*)(+)	Sweet woody, bright fresh coconut note
	(3*S*, 4*S*)-(−)	Faint coconut note, faint musty, earthy, reminiscent of hay
δ-Decalactone	(*R*)-(+)	Sweet, fruity, milk note
	(*S*)-(−)	Sweet, fruity, peach note, fatty, butter-like
Theaspirane	(2*R*, 5*S*)-(−)	Highly attractive intense fresh-fruity
	(2*S*, 5*R*)-(+)	Naphtalene-like
	(2*R*, 5*R*)-(+)	Weak camphoraceus note
	(2*S*, 5*S*)-(−)	Fresh camphoraceus note
2-Methyl-4-propyl-1,3-oxathiane	(2*R*, 4*S*)-(−)	Sulfurous, herbaceous-green, roasty, linseed oil-like, onion
	(2*S*, 4*R*)-(+)	Sulfurous, fatty, fruity-green- tropical fruit, grapefruits
	(2*R*, 4*R*)-(−)	Green grass soot, earthy, red radish note
	(2*S*, 4*S*)-(+)	Sulfurous, slight bloomy-sweet
γ-Pentalactone	(*R*)	Faint, sweet
	(*S*)	Nearly odorless
γ-Hexalactone	(*R*)	Faint, sweet coconut with a fatty-herbaceous hay note
	(*S*)	Sweet, creamy coconut with some woody aspects
γ-Heptalactone	(*R*)	Sweet, spicy, herbaceous hay note, reminiscent of coumarin
	(*S*)	Fatty, coconut note with fruity-sweet aspects, less intense than the opposite enantiomer
γ-Octalactone	(*R*)	Spicy-green, coconut note, with almond notes
	(*S*)	Fatty, coconut note, less intense than the opposite enantiomer
γ-Nonalactone	(*R*)	Strong, sweet, soft coconut with fatty-milky aspects
	(*S*)	Fatty, mouldy, weak coconut less intense than the opposite enantiomer
γ-Decalactone	(*R*)	Strong, fatty-sweet fruity note, some reminiscence to coconut, caramel
	(*S*)	Soft, sweet coconut note with fruity-fatty aspects

Table 19.1 (continued)

Compound	Enantiomer	Characteristics
γ-Undecalactone	(*R*)	Strong, fatty-sweet, reminiscent of peach, with some bloomy aspects
	(*S*)	Fatty-sweet aldehyde note, less intense than the opposite enantiomer
γ-Dodecalactone	(*R*)	Strong, fatty-sweet, bloomy note with aldehyde and woody aspects
	(*S*)	Fatty-fruity, milky notes, less intense than the opposite enantiomer
2-Pentanol	(*R*)	Light, seedy, sharp
	(*S*)	Heavy, wild berry, ripe, dusty, astringent
2-Hexanol	(*R*)	Mushroom, dusty, oily
	(*S*)	Mushroom, green, ripe, berry, astringent, metallic
2-Heptanol	(*R*)	Fruity, sweet, oily, fatty
	(*S*)	Mushroom, oily, fatty, blue cheese, mouldy
2-Octanol	(*R*)	Creamy, cucumber, fatty, sour
	(*S*)	Mushroom, oily, fatty, creamy, grape
2-Pentyl acetate	(*R*)	Fruity, muscat, green, metallic, chemical
	(*S*)	Fruity, apple, plum, metallic
2-Hexyl acetate	(*R*)	Sour, fruity, cherry, plum, strawberry
	(*S*)	Sweaty, sour, fruity, plum, nectarine
2-Heptyl acetate	(*R*)	Green, fatty, banana, methyl ketone
	(*S*)	Mushroom, earthy, wild berry
2-Octyl acetate	(*R*)	Fatty burnt, boiled vegetable
	(*S*)	Fruity, plum, dusty

organoleptic properties of the enantiomers of a chiral molecule can be different and may elicit different odor sensations or intensities. The earliest evidence of this different perception of chiral odorants was found by *Rienacker* and *Ohloff* [19.2]; it concerned β-citronellol, whose (+)-β-enantiomer was described as having a typical citronella odor, while that of the (−)-β-enantiomer was rated as a geranium type smell. Since then the different odor responses of a very large number of enantiomeric chiral odorants have been investigated [19.1, 3]; Table 19.1 reports some examples of chiral compounds whose enantiomers present different odors.

19.1 Chiral Recognition and Enantioselective Gas-Chromatography (Es-GC)

Gas chromatography (GC), in particular, in combination with mass spectrometry (MS), is the most widely used and powerful technique to define the composition of the volatile fraction of natural product samples. However, GC with conventional stationary phases fails with er because separation is based on physicochemical properties, whose values are the same for both enantiomers of the chiral molecule. Stationary phases with a chiral selector in their structure that interacts differently with each enantiomer of the chiral compound(s) must therefore be used to achieve enantiomer separation. The first stationary phase for chiral separation of enantiomers by GC was introduced by *Gil-Av* et al. of the Weizmann Institute of Science, Israel [19.4–6]. They separated racemic amino acid alkyl esters on glass capillary columns coated with N-trifluoroacetyl-L-isoleucine lauryl ester.

Several chiral selectors have since been proposed, which operate on different principles. Three of these, distinguishable for the mode of selector–selectand interaction, have been successfully applied to routine analysis [19.7–9]:

- Separation of enantiomers on chiral amino acids via hydrogen bonding [19.4–6, 10–14]
- Separation of enantiomers on chiral metal coordination compounds via complexation [19.15, 16]
- Separation of enantiomers on cyclodextrin derivatives via inclusion (inter alia) [19.17, 18].

Cyclodextrin (CD) derivatives are the chiral stationary phases (CSP) most widely used in the flavor and fragrance field today, because of their wide range of application and high enantioselectivity, which offer the possibility to separate the underivatized enantiomers of a large number of chiral molecules with different structures and organic functions. The development of stationary phases based on different separation approaches has, however, made available appropriate CSPs for almost all enantiomer separations.

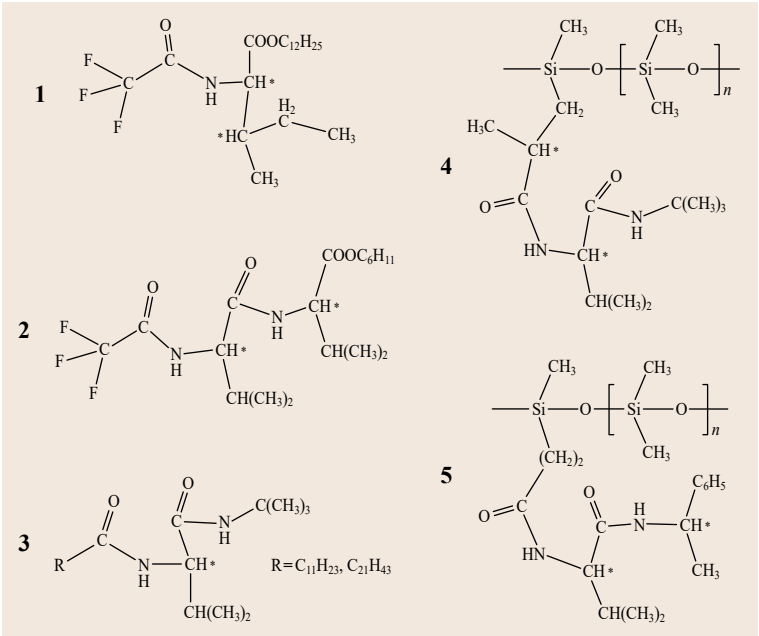


Fig. 19.1 Hydrogen-bonding type CSPs (1) *N*-trifluoroacetyl-L-isoleucine lauryl ester; (2) *N*-trifluoroacetyl-L-valyl-L-valine cyclohexyl ester; (3) *N*-Lauroyl-L-valyl-tert-butylamide; (4) L-valine-*t*-butylamide coupled to a copolymer of dimethylsiloxane; (5) L-valine-*t*-butylamide grafted on to modified polycyanopropylmethyl phenylmethyl silicone (OV-225)

The next paragraphs will briefly discuss the characteristics and chiral separation mechanisms of CSPs operating via hydrogen bonding, complexation with chiral metal coordination compounds, and CD, this last in greater detail because of the importance CDs now have in the flavor and fragrance field.

19.1.1 Chiral Stationary Phases Based on Hydrogen Bonding

The first CSP available were those based on hydrogen bonding. They are usually enantiomerically pure amino acid derived selectors, mostly applied to separate racemic amino acid derivatives, although they can also be used to separate other chiral molecules.

As already mentioned, separation of the enantiomers of *N*-trifluoroacetyl amino acid alkyl esters was first achieved in 1966, on *N*-trifluoroacetyl-L-isoleucine lauryl ester (**1**) (Fig. 19.1) using a glass capillary column [19.6] and then, in 1967, on *N*-trifluoroacetyl-L-valyl-L-valine cyclohexyl ester (**2**) (Fig. 19.1) using a packed column [19.5], in both cases by *Gil-Av*'s group. It was later shown that an additional amide function was fundamental to provide additional hydrogen bondings. The C-terminal amino acid was therefore replaced by an amine derived from valine, yielding the diamide (**3**) [19.19], and then coupled to a copolymer of dimethylsiloxane via the amino function, to yield Chirasil-Val (**4**) in both enantiomeric forms [19.11, 12]. Epimeric CSPs (L,*R* and L,*S*) (**5**) [19.20], containing two chiral centers, were also introduced, although they can either enhance (matched-case) or compensate for (mismatched-case) enantioselectivity. *Koppenhoefer* et al. modified the chiral backbone in Chirasil-Val (**4**) by varying loading and polarity, and by introducing rigid spacers [19.21–23]. Calixarene derivatives (Chirasil-Calix) [19.24] were also tested, but failed to markedly improve enantioselectivity.

Enantiomer separation by hydrogen-bonding CSPs usually requires the analyte (generally an amino acid) to be derivatized, to increase its volatility, and/or to introduce functions suitable for additional hydrogen-bonding association [19.13].

19.1.2 Chiral Stationary Phases Based on Metal Coordination

Complexation GC was introduced by *Schurig*; he applied a chiral metal coordination compound (dicar-

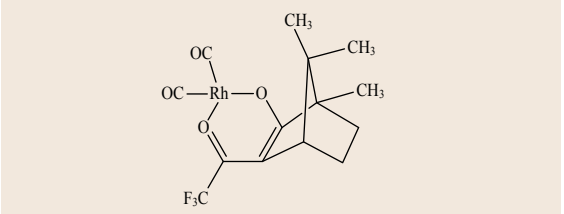


Fig. 19.2 Coordination-type stationary phases: dicarbonyl rhodium(I)-3-trifluoroacetyl-(*1R*)-camphorate

bonyl rhodium(I)-3-trifluoroacetyl-(1*R*)-camphorate) (Fig. 19.2) dissolved in squalane to separate the enantiomers of the chiral olefin 3-methylcyclopentene [19.25].

The chiral recognition effectiveness of these CSPs was confirmed by the enantiomer peak inversion when both enantiomeric selectors obtained from (*1R*)- and (*1S*)-camphor were applied, and by peak coalescence with the racemic selector [19.26]. Complexation GC was later extended to chiral oxygen-, nitrogen-, and sulfur-containing compounds, using various chiral 1,3-diketonate bis chelates of manganese(II), cobalt(II) and nickel(II) derived from perfluoroacylated terpene-ketones. The main limit of these CSPs was the low operative temperature range (25–120 °C); their thermostability was increased when the immobilised polymeric CSPs (*Chirasil-Nichel*) were introduced [19.27]. Before the advent of modified CD, complexation GC was successfully applied to chiral analysis of volatile nonhydrogen-bonding compounds such as pheromones [19.28], flavors and fragrances [19.28, 29], and products of enzymatic reactions, such as oxiranes [19.30]. Enantioselective complexation GC has also been used extensively to study the principles of chiral recognition.

19.1.3 Chiral Stationary Phases Based on (Inter Alia) Inclusion

The separation of enantiomers based on inclusion, using CD derivatives as chiral selector, is probably now the most widely adopted approach for Es-GC analysis, in particular in the flavor and fragrance field.

Cyclodextrin Derivatives as Chiral Selectors

The first reference to a substance that later proved to be a CD was published by *Villiers*; he isolated a crystalline substance from starch digested from *Bacillus amylobacter* [19.31, 32]. Twelve years later, *Schardinger* found that small amounts of two different crystalline products were formed when starch was digested by that microorganism [19.32]. Then, in the 1930s, *Pringsheim* and his group described the inclusion properties of crystalline dextrins and their acetates [19.33] and discovered their ability to form complexes with organic compounds having different structures. An important contribution to the elucidation of the crystalline structure of Schardinger's dextrin was given by *Freudenberg* and coworkers who, later in that decade, hypothesized that CD consisted of maltose units linked through α-1,4-glycosidic linkages. In 1936, they postulated the cyclic structure [19.34]. In the 1950s several groups, in particular those of *French* [19.35] and *Cramer* [19.36], studied in depth the chemical and physical properties of CDs, at the same time clarifying their enzymatic production. By the late 1960s, these studies led to the full elucidation of CDs' structures and physical properties, and their ability to form inclusion complexes was demonstrated; methods for their laboratory-scale preparation were also now developed. The three major CDs are crystalline, homogeneous, nonhygroscopic compounds consisting of torus-like macro-rings built up of 6–8 glucopyranose units (Fig. 19.3), the α-CDs, β-CDs, and γ-CDs, respectively. The CD ring is a conical cylinder in which the primary hydroxyl groups (position 6) are situated on one of the two edges of the ring and the secondary groups (positions 2 and 3) are placed on the other edge. For a general overview of CD chemistry refer to [19.37].

Several thousands of publications have discussed possible applications of CDs: a large proportion (nearly 25%) is in the pharmaceutical field, while about 20% concern applications to analytical chemistry and diagnostic preparations. The analytical applications of CDs mainly refer to their use in GC, high performance liquid chromatography (HPLC), capillary zone electrophoresis, and, to a lesser extent, thin layer chromatography (TLC), and to enhance of ultraviolet–visible (UV–Vis) absorption and luminescence/phosphorescence.

Chiral Stationary Phases Based on Cyclodextrin Derivatives for Es-GC. The first chiral separation in GC with CD as stationary phases was due to *Koscielski* and *Sibilska* [19.38]; they separated the enantiomers of α- and β-pinene, Δ-3-carene, and hydrogenated derivatives, on columns packed with a mixture of native α-cyclodextrin in formamide. Despite their high separation factor α, these columns had a limited lifetime and low efficiency. CDs were applied to capillary GC almost contemporarily, by *Juvancz* et al. [19.39] and *Schurig* et al. [19.16]. In other pioneering studies, *Alexander* et al. [19.40] and, shortly afterward, *Venema* and *Tolsma* [19.41], demonstrated that undiluted permethylated β-cyclodextrin could be employed in capillary columns for high-resolution separation of enantiomers at high temperatures. Subsequently, two different approaches were introduced to overcome the problems associated with the high melting point of permethylated CD:

(a) *Konig* and coworkers proposed using per-*n*-pentylated CD, which are liquid at room temperature. These CD derivatives can thus be used as such to separate enantiomers belonging to several classes of compounds [19.42–45].

(b) At about the same time, *Schurig* and *Nowotny* introduced permethylated-β-cyclodextrin dissolved in

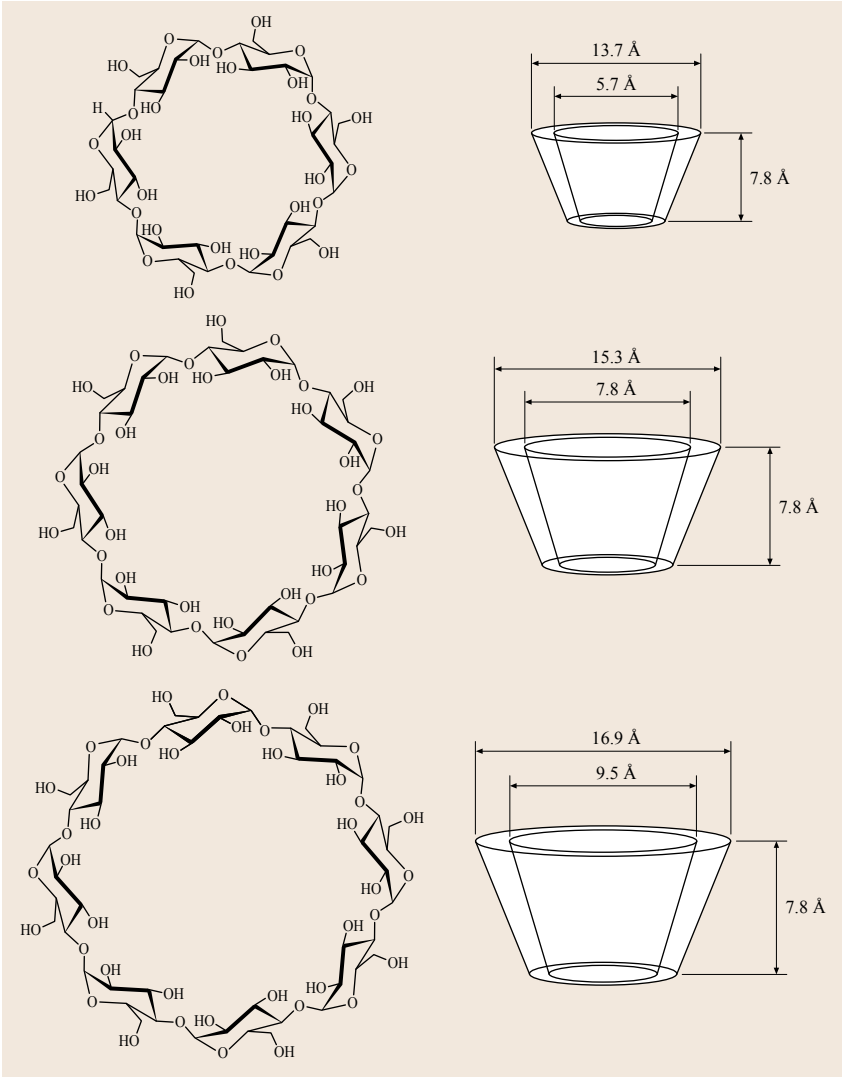


Fig. 19.3 Structure of α-, β-, and γ-cyclodextrins

moderately polar polysiloxanes (OV-1701) [19.16, 46]. This approach combined the high enantioselectivity of the modified CD with the very good gas chromatographic characteristics of polysiloxanes.

Dilution in polysiloxane is now almost the only approach in routine use. A number of in-depth investigations [19.47, 48] have, in fact, made it possible to obtain chiral columns with chromatographic properties, efficiency, and reliability comparable to those of conventional columns, which can operate successfully over an extended range of temperatures (0–240 °C).

Chemistry of Cyclodextrin Derivatives for Es-GC. The presence of three hydroxyl groups that can be regioselectively alkylated and acylated offers an enormous number of possible α-, β-, γ-cyclodextrin derivatives. Numerous derivatives have been described, mainly based on β-cyclodextrin derivatives, although no universally applicable derivative has yet been found. A fundamental improvement was the introduction of bulky substituents, the *tert*-butyldimethylsilyl (TBDMS) or *tert*-hexyldimethylsilyl- (THDMS) groups [19.49, 50] at the primary C6-hydroxy groups, first introduced by *Blum* and *Aichholz* [19.49, 51], but mainly developed by *Mosandl*'s group [19.51]. The bulky substituent conditions the CD conformation and inhibits entrance to the cavity at the smaller rim, thus orienting the analyte/CD interaction toward the wider rim, with the substituents at the C2- and C3-secondary hydroxy groups responsible for enantioselectivity.

The development of new CD derivatives is still necessary to extend the operative range and to embrace new applications such as multidimensional or fast Es-GC, as well as to increase enantioselectivity in comparison to existing ones. Unfortunately, the number of new CD derivatives with higher, wider, or *new* enantioselectivity that have been introduced over the last 10 years is very small. The authors have quite recently introduced *fully asymmetrically substituted derivatives* [19.52]. The already existing CD derivatives present the same *small* substituents (mainly acetyl, methyl, or ethyl groups) in the C2- and C3- secondary hydroxy groups, and bulky substituents (*t*-butyldimethylsilyl-, TBDMS) in the C6-primary hydroxyl groups, and at the same time provide good enantioselectivity and columns with good chromatographic properties. However, their enantioselectivities are very often complementary; therefore CD derivatives with a *mixed* substitution pattern in positions 2 and 3 of the wide rim may provide the desired synergy in enantioselectivity (the term *asymmetric* is here used to indicate the different nature of the substituents in positions 2, 3, and 6). The asymmetrically substituted methyl–ethyl derivatives (6^{I-VII}-*O*-TBDMS-3^{I-VII}-*O*-ethyl-2^{I-VII}-*O*-methyl/3^{I-VII}-*O*-methyl-2^{I-VII}-*O*-ethyl-β-cyclodextrin) were the most successful, since they were found to be more effective, in terms of both the number of chiral compounds separated into their enantiomers, and the resolution values, than the corresponding symmetrical CDs (6^{I-VII}-*O*-TBDMS-3^{I-VII}-*O*-methyl-2^{I-VII}-*O*-methyl/3^{I-VII}-*O*-ethyl-2^{I-VII}-*O*-ethyl-β-cyclodextrin), proving to be very useful in routine analysis for control of samples containing several chiral markers in a single run.

Several other approaches to improve enantioselectivity and chromatographic results have been attempted: (a) applying two or more chiral selectors in a single column, for example, (1) mixing two CDs [19.53–56] although the synergy in terms of enantioselectivity was not as high as expected; similar unsatisfactory results were obtained when two CD units were condensed; and (2) mixing two structurally different chiral selectors, amino acid derivatives, and CD derivatives; these were grafted simultaneously [19.57], linked to polysiloxane [19.58] or covalently linked to amino acid derivatives/CDs [19.59, 60]. These binary phases retained the enantioselective properties of each component, thus extending the range of enantioseparation achievable. (b) CD selector(s) chemically and permanently linked to a polysiloxane backbone [19.61–63] (Chirasil-Dex) as a way of advancing the dilution approach.

However, in the authors' opinion great efforts must yet be made to develop a further generation of CD derivatives with extended enantioselectivity and better chromatographic properties.

Mechanism of Chiral Recognition. It is difficult to rationalize chiral recognition involving modified CDs, since almost all classes of chiral compounds, ranging from apolar to highly polar, are susceptible to being separated into their enantiomers on the appropriate CD-derived CSP; there is often no logical dependence on molecular shape, size, or functionalities of either selectand or selector. This is because the recognition process is multimodal and may, inter alia, involve inclusion, hydrogen-bonding, dispersion forces, dipole–dipole interactions, electrostatic interactions, and hydrophobic interactions [19.9, 64–66]. The separation of enantiomers is due to the differences between the diastereomeric CD selector–selectand (enantiomer) association equilibria; although this is a low-energy interaction, separation is achieved, partly thanks to the high efficiency of capillary GC columns [19.13]. *Lipkowitz* et al. [19.67–69] made an important contribution to the molecular modeling of enantiomer separation with CDs, demonstrating the dichotomy that exists between the location of the preferred binding site of a selectand within the cavity, and that of the optimum chiral discrimination domain, which may, a priori, be different; they also discussed the importance of short-range dispersion forces as intermolecular forces. Another significant contribution, from Schurig's group, showed that inclusion of a molecule within the CD cavity may not be a prerequisite for a successful chiral recognition; they reported enantiomer separations obtained with per-*n*-pentylated amylose [19.70] and with modified linear dextrins [19.71, 72].

All these considerations combine to demonstrate that a strong molecular association is not always a prerequisite to efficient chiral discrimination [19.64] and that a weak selectand–selector interaction can often lead to successful chiral recognition. These results also explain possible changes of elution order of enantiomers from members of homologous series [19.73] and the unreliability of correlating absolute configuration with elution order.

Thermodynamics of Enantioseparation by Gas Chromatography. *Schurig* and coworkers introduced a thermodynamic model to describe enantiomer separation with CD derivatives [19.18, 74]. The model is based on two equilibria that condition the elution of a volatile solute, B eluting from a column containing a solution of a CD derivative, A in an achiral solvent, S, as stationary phase; K_L^0 is the partition coefficient of

B between the gas (g) and the liquid (l) phases, and K is the thermodynamic stability constant of the complex AB in the achiral solvent:

1. $B_{(g)} \overset{K_L^0}{\longleftrightarrow} B_{(l)}$
2. $B_{(l)} + A \overset{K}{\longleftrightarrow} AB.$

K_L^0 in the two equilibria can be defined through the activities (a) of the reagents in the solvent, that is,

$$K_L^0 = \frac{a_{B_{(l)}}}{a_{B_{(g)}}} \text{ for equilibrium (1)} \tag{19.1}$$

and

$$K_L^0 = \frac{a_{AB}}{a_A a_{B_{(l)}}} \text{ for equilibrium (2)} . \tag{19.2}$$

Since the total amount of solute B in the liquid phase is $a_{B_{(l)}} + a_{AB}$, the apparent partition coefficient K_L is

$$K_L = \frac{a_{B_{(l)}} + a_{AB}}{a_{B_{(g)}}}, \tag{19.3}$$

that, from (19.1) and (19.2), can be rewritten as [19.75, 76]

$$K_L = K_L^0(1 + Ka_A) . \tag{19.4}$$

On the basis of the fundamental equation of chromatography

$$t' = \frac{K_L}{\beta} t'_m, \tag{19.5}$$

where t'_m is the dead time, t' the net retention time, K_L the partition coefficient between mobile and stationary phase, and β the phase ratio; an analogous equation can be obtained for the net retention time

$$t' = t'_0(1 + Ka_A) , \tag{19.6}$$

where t_0 is the net retention time on an identical reference column, containing only the solvent S as stationary phase.

Equation (19.6) affords a definition of the retention increase (or retention increment) R' [19.77] as

$$R' = Ka_A , \tag{19.7}$$

as the quantitative measure of the increase in the retention of B due to the CD derivative, A diluted into the achiral solvent, S.

Equation (19.6) must be rewritten in terms of relative retention data to obtain an equation that is independent of column length, diameter, and film thickness, since experimentally it is impossible to obtain truly identical columns to determine t' and t'_0 [19.77]

$$r' = r_0(1 + R') \tag{19.8a}$$

or

$$R' = \frac{r'}{r_0} - 1 , \tag{19.8b}$$

where r' is the relative retention of solute B calculated versus an inert reference standard B*

$$r = \frac{t'}{t'^*} \quad \text{[chiral column (A in S)]} \tag{19.9}$$

$$r_0 = \frac{t'_0}{t'^*_0} \quad \text{[achiral reference column (only S)]}. \tag{19.10}$$

If solute B is a racemic mixture of the enantiomers B_R and B_S, and R arbitrarily represents the last-eluted enantiomer, since $K^0_{LR} = K^0_{LS}$, enantiomer separation of B_R and B_S must be due to different values of K_R and K_S. The chiral separation factor is therefore given by

$$\alpha = \frac{K_{LR}}{K_{LS}} = \frac{t'_R}{t'_S} = \frac{r_R}{r_S} . \tag{19.11}$$

Equation (19.11) can be modified on the basis of (19.4) and (19.7)

$$\alpha = \frac{K_R a_A + 1}{K_S a_A + 1} = \frac{R'_R + 1}{R'_S + 1} . \tag{19.12}$$

From the thermodynamic relationship of free enthalpy

$$\Delta G = -RT \ln K, \tag{19.13}$$

and the ratio of the thermodynamic stability constants

$$\frac{K_R}{K_S} = \frac{R'_R}{R'_S} = \frac{r_R - r_0}{r_S - r_0} , \tag{19.14}$$

it is possible to determine the difference in the free enthalpies of formation of the diastereomeric associates

$$\begin{aligned} \Delta_{R,S}(\Delta G) &= \Delta G_R - \Delta G_S \\ &= -RT \ln \frac{K_R}{K_S} = -RT \ln \frac{R'_R}{R'_S} , \end{aligned} \tag{19.15}$$

and the enthalpic and entropic contributions to $\Delta_{R,S}(\Delta G)$, from the *Gibbs–Helmholtz* equation

$$\Delta_{R,S}(\Delta G) = \Delta_{R,S}(\Delta H) - T\Delta_{R,S}(\Delta S) . \tag{19.16}$$

This equation also indicates that, for a 1 : 1 molecular association, the quantities $\Delta_{R,S}(\Delta S)$ and $\Delta_{R,S}(\Delta H)$

display an opposite effect $\Delta_{R,S}(\Delta G)$. At the isoenantioselective temperature T_{iso}

$$T_{iso} = \frac{\Delta_{R,S}(\Delta H)}{\Delta_{R,S}(\Delta S)} , \tag{19.17}$$

a peak coalescence occurs ($-\Delta_{R,S}(\Delta G) = 0$, $K_R = K_S$, no separation of enantiomers). Above T_{iso}, the enantioselectivity ($\Delta_{R,S}(\Delta G)$) changes and is governed by $\Delta_{R,S}(\Delta S)$, while below T_{iso}, it depends on $\Delta_{R,S}(\Delta H)$.

The most important impact of these results on routine Es-GC is that, in most cases, even at high temperatures, enantioselectivity is dominated by enthalpy-control, with an increase in the separation factor α with decreasing temperature. As a consequence, the lowest possible temperature for Es-GC separation of enantiomers must be applied, not least because the separation of enantiomers by GC with CD derivatives as chiral selector is based on fast kinetics and is thermodynamically driven [19.78].

19.2 Measurement of the Enantiomeric Distribution

The main aim of the GC separation of enantiomers is to precisely determine the enantiomer distribution of one (or more) marker components in a matrix, in order to characterize it. This aim is further stimulated by the legislation on chiral compounds, which is becoming increasingly stringent, thus requiring that reliable methods for enantiomer composition determination be developed.

Enantiomeric distribution is usually expressed in terms of *enantiomeric excess* (ee), *enantiomeric composition* (ec), or *enantiomeric ratio* (er) [19.79]. *Enantiomeric excess* expresses the superabundance of one enantiomer over the other, and is defined as

$$\text{ee} = \frac{E_1 - E_2}{E_1 + E_2} , \tag{19.18}$$

where E_1 and E_2 are the areas of the enantiomers, E_1 being the major enantiomer; ee ranges from 0 for racemic mixture to 1 for pure E_1. In routine practice, ee is often expressed as a percentage

$$\%\text{ee} = \frac{E_1 - E_2}{E_1 + E_2} \cdot 100 = \%E_1 - \%E_2 . \tag{19.19}$$

Enantiomeric purity has also been used as a synonym for ee.

Enantiomeric composition (ec) is defined as the molar fraction of the major enantiomer x_{E_1} in a mixture

$$\text{ec} = x_{E_1} = \frac{E_1}{E_1 + E_2} . \tag{19.20}$$

In this case too, in routine, ec is in general expressed as a percentage

$$\text{ec}\% = x_{E_1} = \frac{E_1}{E_1 + E_2} \cdot 100 . \tag{19.21}$$

Lastly, the *enantiomeric ratio*, er, is defined as

$$\text{er} = \frac{E_1}{E_2} , \tag{19.22}$$

where E_1 is the major enantiomer; er extends from ee = 1, for a racemic mixture, to ee $= \infty$, for pure E_1. The terms er and ee are correlated as follows

$$\text{ee} = \frac{(\text{er} - 1)}{(\text{er} + 1)} \tag{19.23}$$

and

$$\text{er} = \frac{(1 + \text{ee})}{(1 - \text{ee})} . \tag{19.24}$$

In routine analysis, correct measurement of the above parameters requires that the peaks of the two enantiomers be baseline separated, that their resolution is

$$R_S \geq 1.5 .$$

The separation factor α cannot therefore be used routinely in the flavor and fragrance field, because it does not consider peak widths, and must be determined isothermally.

19.3 Enantioselective GC Analysis with Cyclodextrins in the Flavor and Fragrance Field

Enantiomer separation by Es-GC with columns coated with modified CDs as chiral selector has been successfully applied to several fields of contemporary research, such as checking essential oil authenticity [19.80], flavors and fragrances and alcoholic beverages [19.81, 82], and clinical chemistry [19.82]. Terpenoids [19.83], enzymatic reactions [19.84], organochlorine pesticides [19.85, 86], alkyl nitrates as atmospheric constituents [19.87], and volatile pharmaceutical compounds can also be investigated [19.64].

The second part of this chapter will be devoted entirely to chiral recognition strategies in the flavor and fragrance field by Es-GC, with CD derivatives as stationary phase; it is based on the authors' day-to-day experience in this field.

CD derivatives are the most widely used chiral selectors in Es-GC of chiral components in the flavor and fragrance field, most probably because their physicochemical characteristics (polarity, reactivity, and volatility) are highly *compatible* with the enantiomer discriminative interactions provided by CDs.

As is the case of all stationary phases, CDs have advantages and disadvantages, both absolutely and in comparison to the other CSPs described above. Their main advantages are as follows:

- They can separate underivatized enantiomers, thereby enabling real natural product samples to be directly analyzed without any manipulation.
- They can separate almost all classes of volatile chiral compounds, thanks to the wide range of selectivity covered by the large number of available CD derivatives.
- They possess good chromatographic properties (efficiency and inertness) and a wide range of operative temperatures of columns prepared with CDs, thanks to their dilution in moderately polar polysiloxanes.

However, CDs also present some disadvantages, limiting not only their popularity but also the routine use of Es-GC, because they tend erroneously to be considered complex:

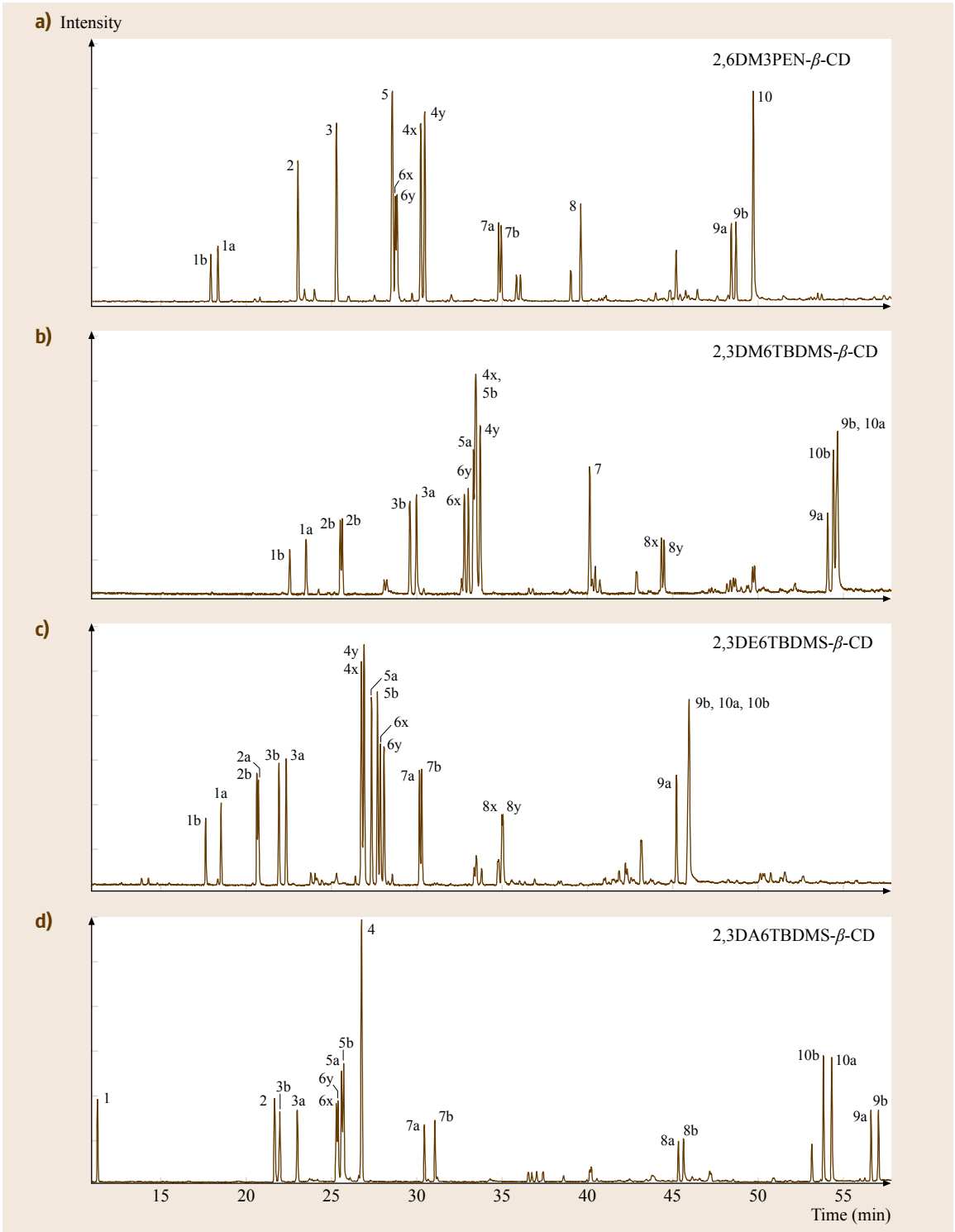
- Absence of a *universal* CD derivative separating most chiral compounds; this means that a laboratory must be provided with a number columns to extend the range of analyzable chiral compounds.
- Difficulty of identifying enantiomers and measuring ee and/or er in real-world complex samples with mono-dimensional Es-GC, because of the increased probability of interferences due to the doubling of the number of chiral analyte peaks; this limit is extremely frequent in the flavor and fragrance field.
- Long analysis time, due to the use of long columns and low temperature rates, because of the CD mechanism of separation.

Partly in view of the above three points, the next paragraphs will examine the optimization of strategies for the chiral recognition of odorous markers by Es-GC and Es-GC-MS, with CD as chiral selectors in complex mixtures in the flavor and fragrance field, based mainly on the authors' experience.

19.4 Testing Column Efficiency and Enantioselectivity

A short paragraph must first be dedicated to a critical and often-neglected point: column efficiency and enantioselectivity. Conventional GC columns in general, but even more so Es-GC columns, must be tested periodically for their efficiency and enantioselectivity, in order to obtain reliable results. This evaluation requires the use of dedicated test mixtures, such as the Grob test, to evaluate chromatographic performance of a column over time, and a chiral test for enantioselectivity. In the authors' laboratory, a test mixture containing racemates with different volatilities, structures, and polarities has been designed to check column enantioselectivity; it is used continually [19.47] (its composition is reported in the caption of Fig. 19.4). Figure 19.4 reports the chiral test profiles carried out on the four columns used to build the chiral library (Sect. 19.5.1).

Fig. 19.4 Total ion chromatogram of chiral test profiles carried out on the four columns used to build the chiral library: **(a)** $6^{I\text{-}VII}$-O-methyl-$3^{I\text{-}VII}$-O-pentyl-$2^{I\text{-}VII}$-O-methyl-β-cyclodextrin, **(b)** $6^{I\text{-}VII}$-O-TBDMS-$3^{I\text{-}VII}$-O-methyl-$2^{I\text{-}VII}$-O-methyl-β-cyclodextrin, **(c)** $6^{I\text{-}VII}$-OTBDMS-$3^{I\text{-}VII}$-O-ethyl-$2^{I\text{-}VII}$-O-ethyl-β-cyclodextrin, **(d)** $6^{I\text{-}VII}$-O-TBDMS-$3^{I\text{-}VII}$-O-acetyl-$2^{I\text{-}VII}$-O-acetyl-β-cyclodextrin. Analysis conditions: Injection mode: split; split ratio: 1 : 50. Inj. temperature: 230 °C, detector: FID, temperature 250 °C; temperature program, 50 °C/2 °C/min/220 °C; peak identification: 1: limonene, 2: 2-octanol, 3: camphor, 4: isobornyl acetate, 5: linalyl acetate, 6: 2-methyl-(3*Z*)-hexenyl butyrate, 7: menthol, 8: hydroxycitronellal, 9: γ-decalactone, 10: δ-decalactone; (a) (*R*) enantiomer, (b) (*S*) enantiomer, x and y: enantiomer configuration not assigned ►


a)
Intensity
2,6DM3PEN-β-CD
1b
1a
2
3
5
4x
4y
6x
6y
7a
7b
8
9a
9b
10
b)
2,3DM6TBDMS-β-CD
1b
1a
2b
2b
3b
3a
6x
5a
6y
4x,
5b
4y
7
8x
8y
9a
10b
9b, 10a
c)
2,3DE6TBDMS-β-CD
1b
1a
2a
2b
3b
3a
4y
4x
5a
5b
6x
6y
7a
7b
8x
8y
9a
9b, 10a, 10b
d)
2,3DA6TBDMS-β-CD
1
2
3b
3a
5b
5a
6y
6x
4
7a
7b
8a
8b
9a
10b
10a
9b
15
20
25
30
35
40
45
50
55
Time (min)

19.5 Analysis of Enantiomers in Complex Samples

Reliable chiral recognition of marker compounds in complex real-world samples generally requires a two-dimensional approach, in order to avoid erroneous identifications due to peak overlapping; this is a particular risk because of the doubling of the number of peaks of chiral analytes when separated by Es-GC. Two complementary but distinct approaches are therefore available (Sect. 19.5.1): the first is to adopt *a second dimension in separation* (by conventional heart-cut GC-GC or comprehensive GC), while the second approach implies using a *second dimension in detection*, by coupling GC with MS. The latter method applies a strategy that is the converse of the conventional one: the enantiomer(s) is(are) located in the chromatogram by their MS spectrum and identified by GC data (Kovats retention indices (I_s) [19.88], or linear retention indices (I_s^T)) [19.89, 90]. This is because, as is known, MS is not a chiral probe so that the mass spectra of two enantiomers cannot be distinguished. This makes it impossible to determine which enantiomer is present in a sample. Conversely, chromatographic data (and in particular linear retention indices) are the most reliable and effective parameters for enantiomer identification, being characteristic for each analyte (enantiomer), since they depend on its chromatographic interaction with the adopted stationary phase; similar considerations can be made with locked retention times, which were introduced by *Giarrocco* et al. and by *Blumberg* and *Klee* [19.91, 92].

19.5.1 Location and Identification of Enantiomers in Complex Samples

The basic concepts of *multidimensional* gas-chromatography are described in Chap. 17 of this book, and will therefore not be discussed here. Two techniques commonly known as multidimensional GC are at present used in chiral recognition:

1. Heart-cut GC–GC is as yet the most widely used approach for ee and er determination of chiral compounds in complex mixtures, in particular for applications in which a small number of chiral components (or fractions) (possibly eluting at relatively different temperatures) are to be submitted to chiral recognition. With this technique, *the selected chiral components* (peaks) eluting from the first, achiral, column (first dimension – ^{1}D) coated with a conventional stationary phase are on-line and automatically transferred to the second, chiral, column (second dimension – ^{2}D), through a dedicated time-programmable interface, for a programmed time fraction of the whole chromatographic

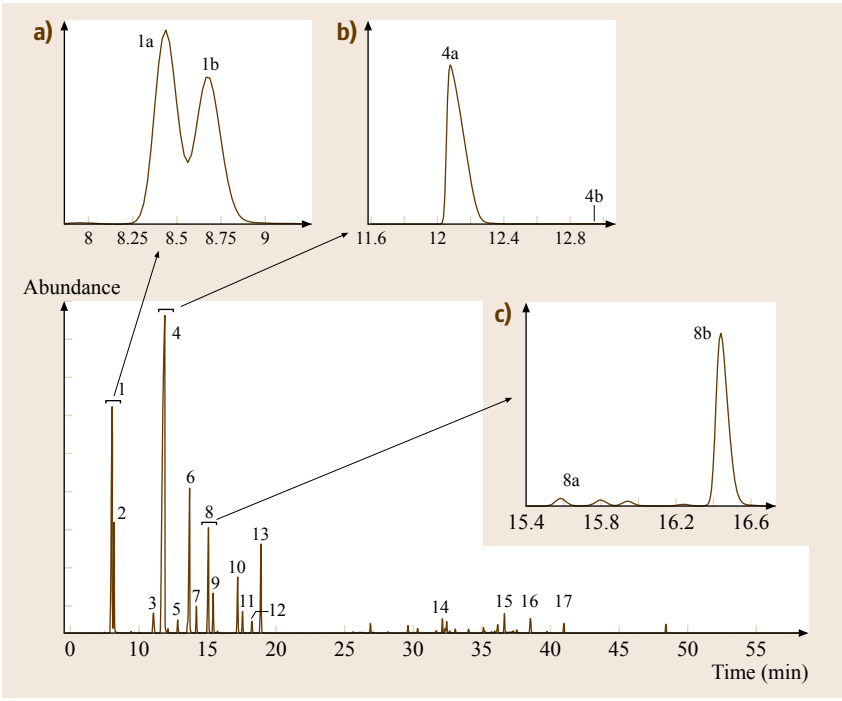


Fig. 19.5 HS-SPME heart-cut GC-GC analysis of the volatile fraction of a juniper (*Juniperus communis* L.) twig sample from Norway *HS-SPME sampling*: fiber: 2 cm Stableflex 50/30 µm divinylbenzene (DVB)-Carboxen-PDMS (Supelco, Bellafonte, USA); sample amount: 20 mg, vial volume: 20 ml; sampling time: 10 min, temperature: 50 °C. *Peak identification* 1. α-pinene 2. α-thujene, 3. β-pinene 4. sabinene, 5. δ-3-carene, 6. α-phellandrene+myrcene, 7. α-phellandrene, 8. limonene, 9. β-phellandrene, 10. γ-terpinene, 11. β-ocimene, 12. p-cymene, 13. α-terpinolene, 14. terpinen-4-ol acetate, 15. γ-muurolene, 16. germacrene B, 17. myrtenyl acetate. (a) enantiomers (+), (b) enantiomers (−)

run [19.93–95]. Figure 19.5 reports the HS-SPME heart-cut GC-GC profile of the volatile fraction of a juniper (*Juniperus communis* L.) twig sample from Norway *HS-SPME* with an HP-5 (25 m × 0.25 mm d_c × 0.25 mm d_f) as first column and 30% 6^{I-VII}-*O*-TBDMS-2^{I-VII}-3^{I-VII}-*O*-ethyl-β-CD in PS086 (25 m × 0.25 mm d_c × 0.25 mm d_f mm) as second column; for analysis conditions and peak identification.

2. Two-dimensional comprehensive GC (GC × GC) is suitable for highly complex mixtures, in which several components are to be submitted to chiral recognition. In GC × GC, *each component* eluting from the first column is on-line and automatically trapped, refocused, and reinjected into the second column, in a fixed time (4–8 s) by a thermal or valve-based focusing device (modulator) [19.96]. Unlike heart-cut GC-GC, the GC × GC system for chiral recognition consists of a chiral column of conventional i.d. and length (^{1}D) connected to a very short narrow-bore (NB) column (^{2}D) coated with a conventional stationary phase, to enable fast analyses to be run during the time of one modulation. The chiral column must be in the first dimension because of the high efficiency required for effective enantiomer separation [19.97, 98]. Figure 19.6 reports the GC × GC contour plots of lavender essential oil.

As already mentioned, enantiomers have indistinguishable mass spectra, making chromatographic data indispensable for their reliable identification in chiral recognition. The most widely used and reliable methods to identify sample components through gas chromatographic data are based on retention indices (I_s^T) or, alternatively, on the retention time locking (RTL) approach. Either of these systems makes identification more reliable than the simpler retention time or relative retention time approaches, since they overcome most of their limits, in terms of precision and repeatability. However, correct identification of an analyte in a sample, through chromatographic data, always implies the availability of pure reference standards; this is particularly significant in the case of Es-GC in which, to establish the correct order of elution of each enantiomer within a pair, it is indispensable to have a single enantiomer standard, or one or more real-world samples in which one precisely identified member of the pair is present as such, or in a well-known enantiomeric excess.

Retention indices were first introduced by *Kováts* [19.88] for isothermal analysis (I_s); their use was later extended to temperature-programmed analysis (linear retention indices (I_s^T)) by *Van den Dool* et al. [19.89, 90]. I^T is a number that expresses the entity of the analyte/stationary phase interaction relative to a reference standard mixture (homologous series of hydrocarbons or fatty acid methyl or ethyl esters) and provides its unequivocal position in the chromatogram, provided that rigorously standardized analysis conditions are applied. In agreement with the authors' experience, I_s^T with the same stationary phases are indispensable in Es-GC to identify an enantiomer in a sample. This approach implies that the enantiomer is located in the chromatogram through its mass spectrum, and then identified through its I^T by comparison to that of the reference standard determined on the same column. I_s^T in essential oil analysis were reviewed by *d'Acampora Zellner* et al. [19.99] and their importance in plant volatile fraction discussed by *Rubiolo* et al. [19.100].

In general, the identification potential of GC is somewhat neglected because of the power of MS when used as detector for GC. Few GC-MS software packages, therefore, include I_s^T information to help component identification, while some of them only report I_s^T values in the library as *blind* or *inactive* data, making them only useful for additional confirmation [19.101–107]. On the contrary, the *interactive* use of I_s^T values (their use as an active identification parameter) can actually be highly effective, since it provides a second

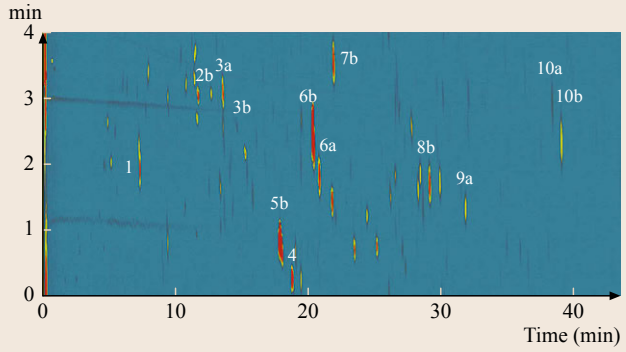


Fig. 19.6 GC × GC contour plot of lavender essential oil (*Lavandula angustifolia* P. Mill).
Analysis conditions: GC × GC-MS system: Agilent 6890 GC - Agilent 5975 MSD ionization mode: EI 70 eV (Agilent, Little Falls, DE, USA); transfer line temp.: 280 °C, scan range: m/z 35–250 in fast scanning mode (12 500 amu/s). GC × GC interface: KT 2004 loop modulator (Zoex Corporation, Houston, TX, USA), modulation time: 4 s. Column set: ^{1}D: 30% 6^{I-VII}-O-TBDMS-2^{I-VII}-3^{I-VII}-O-ethyl-β-CD in PS086 (25 m, d_c: 0.25 mm, d_f: 0.15 µm), ^{2}D: OV1701 column (1 m, d_c: 0.10 mm, d_f: 0.10 µm) (MEGA – Legnano (Milan)-Italy) Peak identification: 1: 1,8-cineol, 2: β-phellandrene, 3: limonene, 4: 1-octen-3-ol, 5: camphor, 6: linalool, 7: borneol, 8: linalyl acetate, 9: 4-terpineol, 10: lavandulyl acetate; a: (*S*) enantiomer, b: (*R*) enantiomer

orthogonal tool to identify a compound, operating in parallel to MS spectra.

An MS library specific for the identification of enantiomer components in the flavor and fragrance field was developed by the authors, using I_s^T values *interactively* in parallel to MS spectra [19.108]. The library was built at an interlaboratory level with the collaboration of two research groups, to increase result reliability. It is based on the interactive I^T/mass spectrum system [19.103] developed by *Costa* et al. [19.109] for the flavor and fragrance field, where I_s^T are automatically calculated and incorporated as an active part of the matching criteria together with mass spectra. The correct identification of an analyte is assured by the range within which its I^T must fall (retention index allowance (RIA) see below), which must be determined preliminarily.

The library consists of 134 racemates whose I^T values were determined on four CD coated with different CD chiral selectors. Table 19.2 reports the list of racemates included in the first version of the library.

Table 19.2 List of compounds included in the library (after [19.108])

Hydrocarbons
α-Phellandrene
α-Pinene
β-Citronellene
β-Citronellene
β-Phellandrene
β-Pinene
Camphene
Caryophyllene
Limonene
Sabinene
Heterocyles
Ambroxide
Menthofuran
Rose oxide
Esters
α-Terpinyl acetate
Bornyl acetate
Bornyl isovalerate
Butyl butyrolactate
cis-2-Methyl-3-hexenylbutyrate
cis-Carvyl acetate
Dihydrocarvyl acetate
Dimethyl methylsuccinate
Ethyl 2-methylbutyrate
Ethyl 2-phenylbutyrate
Ethyl 3-hydroxybutyrate
Ethyl 3-hydroxyhexanoate
Ethyl 3-methyl-3-phenylglicidate
Isobornyl acetate
Isobornyl isobutyrate

Table 19.2 (continued)

Esters (continued)
Lavandulyl acetate
Linalyl acetate
Linalyl cinnamate
Linalyl propionate
Menthyl acetate
Methyl 3-hydroxyhexanoate
Methyl dihydrofarnesoate
Neomenthyl acetate
Nopyl acetate
Propylene glycolbutyrate
Styrallyl acetate
Lactones
Aerangis lactone
3-Methyl-γ-decalactone
δ-Decalactone
δ-Dodecalactone
δ-Heptalactone
δ-Hexalactone
δ-Nonalactone
δ-Octalactone
δ-Undecalactone
ε-Decalactone
ε-Dodecalactone
γ-Decalactone
γ-Dodecalactone
γ-Heptalactone
γ-Hexalactone
γ-Nonalactone
γ-Octalactone
γ-Pentadecalactone
γ-Pentalactone
γ-Tetradecalactone
γ-Undecalactone
Massoia decalactone
Massoia dodecalactone
Whiskey lactone
Ketones
1,8-Epoxy *p*-menthan-3-one
3,6-Dimethylocta 2-en-6-one
3-Methylcyclohexanone
3-Oxocineole
α-Damascone
α-Ionone
β-Irone
Camphor
Camphorquinone
Carvone
Fenchone
Isomenthone
Menthone
Methylcyclopentenolone
Nootkatone
Piperitone

Table 19.2 (continued)

Ketones (continued)
Pulegone
Verbenone
Aldehydes
Citronellal
Cyclamen aldehyde
Hydroxycitronellal
Myrtenal
Perillyl aldehyde
Alcohol
α-Bisabolol
1-Octen-3-ol
1-Phenyl ethanol
1-Phenyl-1-propanol
1-Phenyl-2-pentanol
2-Butanol
2-Heptanol
2-Hexanol
2-Methylbutanol
2-Octanol
2-Pentanol
2-Phenyl-1-propanol
3-Hexanol
3-Octanol
4-Methyl-1-phenylpentanol
6-Methyl-5-hepten-2-ol
α-Terpineol
Borneol

Table 19.2 (continued)

Alcohol (continued)
cis- Myrtanol
Citronellol
Fenchyl alcohol
Geosmin
Isoborneol
Isomenthol
Isopinocampheol
Isopulegol
Lavandulol
Linalool
Linalool oxide
Menthol
Neoisomenthol
Neomenthol
Nerolidol
Octan-1,3-diol
Patchouli alcohol
Perillyl alcohol
Terpinen-4-ol
Tetrahydrolinalool
trans- Myrtanol
Viridiflorol
Acids
Citronellic acid
2-Methylbutyric acid
2-Phenylpropionic acid
Chrysanthemic acid

Four cyclodextrin derivatives diluted at 30% in PS-086 had to be used to obtain the separation of most of the racemates usually analyzed in the flavor and fragrance field, in particular:

- 6^{I-VII}-*O*-methyl-3^{I-VII}-*O*-pentyl-2^{I-VII}-*O*-methyl-β-cyclodextrin (2,6DM3PEN-β-CD) [19.110, 111]
- 6^{I-VII}-*O*-TBDMS-3^{I-VII}-*O*-methyl-2^{I-VII}-*O*-methyl-β-cyclodextrin (2,3DM6TBDMS-β-CD) [19.112]
- 6^{I-VII}-O-TBDMS-3^{I-VII}-O-ethyl-2^{I-VII}-O-ethyl-β-cyclodextrin (2,3DE6TBDMS-β-CD) [19.113]
- 6^{I-VII}-O-TBDMS-3^{I-VII}-O-acetyl-2^{I-VII}-O-acetyl-β-cyclodextrin (2,3DA6TBDMS-β-CD) [19.112]

The determination of the RIA window, the range within which the I^T of an analyte has to fall to be correctly identified, is a key point for univocal identification. An effectively operating library should be based on a single RIA window to be applied automatically to all enantiomers analyzed on all columns investigated. The ideal RIA should be *narrow* enough to include only one of the two enantiomers, but at the same time *wide* enough to avoid the I^T of a given analyte falling outside the range because of retention variation. A reliable RIA therefore requires baseline enantiomer separation and highly stable I^T values, the latter being obtainable, among others, by highly standardized analysis, constantly tested inert columns, and corrected injection volumes.

The single RIA for this library was obtained from the average RIA of each class of compounds, in its turn determined from the individual RIA of each of the enantiomers of the 134 racemates investigated, analyzed on the four enantioselective columns adopted. A single average RIA value of -1 and $+2$ for all analytes analyzed on all the columns investigated was adopted.

Automatic mass spectral deconvolution (AMDIS) [19.101] is another software package, developed by the National Institute of Standards and Technology (USA), that actively uses I_s^T, often in combination with NIST Mass Spectral Libraries.

Retention time locking (RTL) [19.91, 92] is a different approach for reliably identifying an analyte from its GC retention data in programmed temperature analysis. The principle underlying RTL involves determining

the adjustment of inlet pressure necessary to achieve the desired match in retention time(s) of an analyte(s) with similarly configured GC systems.

19.5.2 Mass Spectrometry and Reliability of Chiral Recognition

Clearly, the recent advances in MS can dramatically increase the diagnostic power of GC-MS, thanks to the routine use of tandem mass spectrometry or MS/MS (mass spectrometry/mass spectrometry or MS^n). These systems consist not only of the last generation of well-established triple quadrupole (QqQ – tandem-in-space) and ion trap (IT-MS – tandem-in-time) MS/MS analyzers, but also include the most recent hybrid mass spectrometers that are based on high-resolution time of flight (TOF)-MS analyzers, as such or as a second MS unit in combination with a quadrupole or an ion-trap analyzer (Q-TOF or IT-TOF, respectively). The resulting hybrid system, in combination with soft ionization ion sources, thus combines the advantages of tandem MS with those of high-resolution analyzers; these advantages include, for instance, the determination of the exact mass of molecular ions and/or fragments, at an accuracy below 5 ppm. However, Es-GC alone is sometimes not sufficient to detect the authenticity of a sample, in particular when racemates naturally occur, or when they derive from processing and/or storage or, especially, from adulteration with a synthetic enantiomer. A decisive step toward achieving a unequivocal definition of sample authenticity is combining Es-GC (or even better Es-GC-GC) with isotope ratio mass spectrometry (IRMS). The effectiveness of Es-GC-IRMS is based on the consideration that the ratio between stable isotopes of enantiomers from the same natural source may be expected to be the same (δ-C^{13}) even when a partial enantiomer racemization has occurred, since both enantiomers are generally formed through the same biosynthetic pathway in the same natural organism. In a GC-IRMS system, analytes eluting from the GC column are on-line combusted to CO_2 in a dedicated oven, from where the combusted product is directly introduced into an IRMS system. The abundances of 44 ($^{12}C^{16}O_2$), 45 ($^{13}C^{16}O_2$, $^{12}C^{16}O^{17}O_2$), and 46 ($^{12}C^{16}O^{18}O$) ions in the nmole range are then simultaneously measured with high precision (≤ 0.3 ‰), and the peak areas ratio of the two isotopic peaks compared to a standard reference value. A detailed discussion of this technique is outside the scope of this chapter [19.114].

19.6 Fast Enantioselective GC Analysis

A further important limit often conditioning the routine use of Es-GC is the long analysis time, due to the small difference in the energy of association between each selectand (enantiomer) and the CD chiral selectors; this means that very high chromatographic efficiency is required to obtain enantiomer separation. As shown in the above paragraphs, Es-GC separation of enantiomers with CDs as chiral selectors is based on fast kinetics, and is entirely governed by thermodynamics; as a consequence, it closely depends on temperature. Long analysis times are therefore to be expected, since long columns and low temperature rates must in general be applied.

Routine applications require the development of fast Es-GC methods, in order to satisfy the large number of control analyses required. Routine fast-GC can in general be obtained by acting on column length, inner diameter, and/or flow-rate, and has resulted in the adoption of NB columns [19.115]. In fast-Es-GC, NB columns not only increase analysis speed and analyte detectability, because of peak sharpening [19.116], but also reduce the enantiomer elution temperature; this results in a gain of enantioselectivity that compensates (in full or in part) for the loss of efficiency (N) due to column shortening. Enantiomer separations with CDs with short columns were already under study in the early 1990s; they afford separation even in a few seconds [19.117–119]. *Schurig* and *Czesla* [19.120] studied the basis for speeding up ES-GC in depth, and concluded that short, conventional 0.25 –mm d_c columns would have to be used for fast Es-GC, because of their good loadability, integration characteristics, use of conventional instrumentation, and lower consumption of carrier gas. However, conventional d_c short columns can only be used successfully for monodimensional fast Es-GC when chiral compounds have to be recognized in low-complexity samples and/or when a limited number of enantiomers are to be analyzed simultaneously. With medium-to-high complexity samples, as is often the case in the flavor and fragrance field, a highly efficient separation system combined with single- or multiple-ion monitoring-MS detection (SIM-MS or MIM-MS) is necessary to determine ee and/or er correctly (see Sect. 20.3). This paragraph critically discusses the two complementary methods developed and adopted in the authors' laboratory to speed up routine Es-GC analyses [19.121, 122]. The two methods will now be briefly described and illustrated through the analysis of a lavender essential oil. Lavender essential oil is used as an example for both

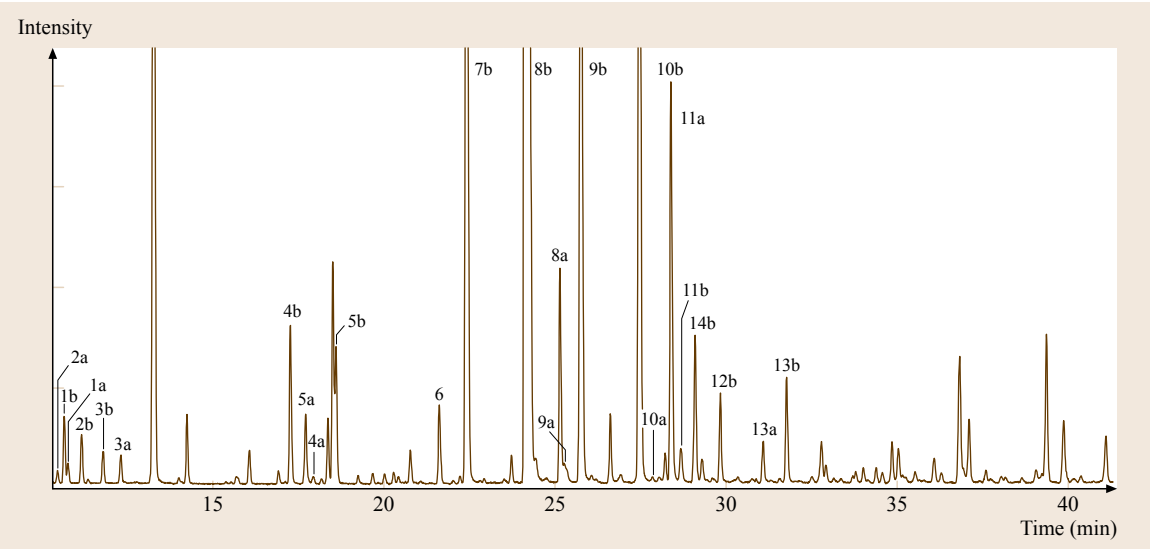


Fig. 19.7 The Es-GC-MS profiles of the lavender essential oil analyzed with the reference column, 30% $6^{I–VII}$-*O*-TBDMS-$2^{I–VII}$-$3^{I–VII}$-*O*-ethyl-β-CD in PS086 (25 m × 25*mm* d_c, 25*mm* d_f). For analysis conditions see Table 19.3. Peak identification: 1: α-pinene, 2: camphene, 3: β-pinene, 4: β-phellandrene, 5: limonene, 6: 1-octen-3-ol, 7: camphor, 8: linalool, 9: borneol, 10: linalyl acetate, 11: 4-terpineol, 12: lavandulol, 13: α-terpineol, 14: lavandulyl acetate; a: (*R*) enantiomer, b: (*S*) enantiomer, 6: not separated; 7a, 12a and 14a: not detected [19.122]

approaches, because it is characterized by several chiral markers whose enantiomeric composition is reliably described in the literature [19.123, 124]. Lavender essential oil contains several optically active components: α- and β-pinene, camphene, β-phellandrene, limonene, 1-octen-3-ol, camphor, linalool, borneol, linalyl acetate, terpinen-4-ol, lavandulol, α-terpineol, and lavandulyl acetate.

MS plays a crucial role in the first approach [19.122]. This consists of searching for the best trade-off between speed of analysis and loss of resolution of chiral compounds, even at the expenses of separating the other sample components. This is valid since the separated enantiomers can be highlighted by operating in extract-ion, SIM- or MIM-MS modes. Analysis time can therefore be reduced by exploiting the excess of resolution that columns coated with the last generation of CD derivatives can provide for several chiral compounds, by acting on column dimension, flow rate, and temperature rate. The essential prerequisite is to maintain baseline separation of the enantiomers of the chiral compound(s) investigated, so as to afford correct ee or er determination. With this approach, co-elution of the chiral components with other sample components, due to column shortening and increased heating rates, is to be expected but should not interfere with enantiomeric recognition of the chiral marker(s), because MS as a second dimension in detection, in extract-ion mode (or SIM- or MIM-MS) with diagnostic and specific ions, reliably discriminates them from other co-eluting peaks.

Figure 19.7 reports the Es-GC-MS profiles of the lavender essential oil analysed with the reference conventional column, together with the identification of peaks of chiral components.

Table 19.3 compares enantiomer resolutions of chiral components, analysis time, and % analysis time reduction, when lavender essential oil was analysed with the reference conventional column, and with the 10, 5 and 2 m narrow bore columns. Column length and analysis conditions were considered adoptable for routine analysis only when enantiomer resolutions of all chiral compounds were above 1.5.

The results (Fig. 19.8) show that:

1. The 10 m NB column can only be used at 2 °C/min, because at higher rates terpinen-4-ol, linalyl acetate and borneol are not baselxine separated.
2. The highest temperature rate for the 5 m NB column was 5 °C/min, because at 10 °C/min resolution of terpinen-4-ol was only 1.2, as is clear from its ion profiles at m/z 71, 111, 154.
3. Similar considerations can be made for 2 m NB columns.

Under these conditions, the analysis time was reduced from about 40 min with the reference column to about 14 min with the 5 m NB column. Figure 19.8

Table 19.3 Enantiomer resolutions of chiral components, analysis time and % analysis time reduction and separation measure *S* obtained for a lavender essential oil analysed with the 10, 5 and 2 m NB columns in comparison with those of the reference column (Reference)

Temperature rate (°C/min)		Reference	10 m NB				5 m NB				2 m NB			
Analysis time (min)		2	2	3.5	5	10	2	3.5	5	10	2	3.5	5	10
% Analysis time reduction		40.67	29.35	19.60	14.77	8.67	26.81	17.77	13.60	8.01	28.83	16.57	12.73	7.56
			27.8	51.8	63.7	78.7	34.1	56.3	66.6	80.3	29.1	59.2	68.7	81.4
	I^{T} [a]	Resolution												
α-Pinene (1)	(*R*)921/(*S*)923	1.2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Camphene (2)	(−)917/(+)923	6.8	2.2	1.9	1.8	1.7	6.7	5.6	5.6	4.9	3.9	3.5	3.4	3.5
β-Pinene (3)	(+)944/(−)955	5.0	1.8	1.5	1.4	1.2	5.3	4.8	4.4	3.5	3.3	3.3	2.9	2.6
β-Phellandrene (4)	(−)1049/(+)1060	6.1	2.1	1.8	1.7	1.5	6.2	5.3	5.1	4.5	3.1	2.8	2.6	2.4
Limonene (5)	(*S*)1056/(*R*)1072	9.1	2.8	2.5	1.9	1.9	9.0	8.2	8.0	5.0	5.4	4.2	3.8	2.9
1-Octen-3-ol (6)	1126	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Camphor (7)	(*S*)1133/(*R*)1141	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E
Linalool (8)	(*R*)1174/(*S*)1189	6.1	2.6	2.4	2.3	1.9	3.1	3.3	3.2	2.5	3.1	3.4	3.0	3.0
Borneol (9)	(*S*)1192/(*R*)1200	3.0	1.6	1.2	1.2	1.0	2.9	2.5	2.0	1.9	2.1	2.0	1.7	1.6
Linalyl acetate (10)	(*R*)1231/(*S*)1237	3.2	2.6	2.5	1.6	NS	3.8	3.0	2.8	2.4	4.1	3.8	3.1	1.9
Terpinen-4-ol (11)	(*S*)1248/(*R*)1253	2.2	1.6	1.0	NS	NS	2.4	2.1	2.0	1.2	2.6	2.3	2.0	1.3
Lavandulol (12)	(*S*)1250/(*R*)1273	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E
α-Terpineol (13)	(*S*)1296/(*R*)1309	6.0	3.2	2.8	2.5	1.9	6.9	5.6	5.5	4.2	6.5	5.6	4.7	4.0
Lavandulyl acetate (14)	(*R*)1259/(*S*)1263	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E

[a] Obtained on the reference column
NS: not baseline separated
1E: only (*R*) enantiomer found

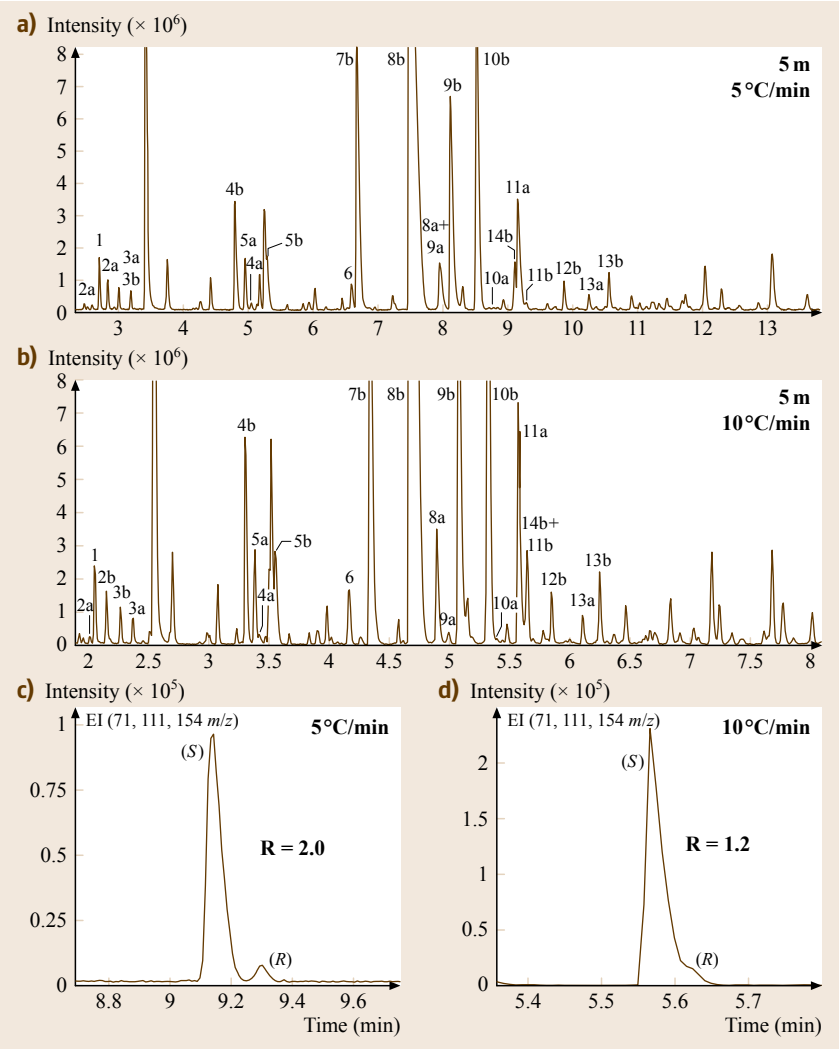


Fig. 19.8a–d The Es-GC-MS profiles of the lavender essential oil analyzed with the 5 m NB column at 5 **(a)** and 10 °C/min **(b)**. Extract ion profiles of terpinen-4-ol (71, 111, 154 m/z) at 5 **(c)** and 10 °C/min **(d)**. For analysis conditions see Table 19.3. For peak identification, see caption to Fig. 19.7 [19.122]

reports the Es-GC-MS profiles of the lavender essential oil analyzed with the 5 m NB column at 5 (A) and 10 °C/min (B) and the extract ion profiles of terpinen-4-ol (71, 111, 154 m/z) at 5 (C) and 10 °C/min (D).

The second approach [19.121] is based on the opposite strategy – shortening analysis time by seeking the maximum separation efficiency of the chromatographic system, by optimizing analysis conditions. The routine analyses of a large number of different samples in a single field (aromas from different matrices) are in general carried out under the same standardized GC conditions, partly because of the possibility of automatically identifying peaks from chromatographic data (relative retention times, linear retention indices, RTL, etc.). Usually, satisfactory separations are obtained under nonspecific routine conditions, thanks to much-higher-than-required efficiency of the chromatographic system, to the detriment of analysis times. Optimization of analysis conditions for a specific sample, that is, tuning a dedicated method for each matrix, can successfully and dramatically speed up routine GC analyses. *Blumberg* and coworkers [19.125–129] investigated in depth the most important theoretical concepts required to optimize capillary GC methods and to obtain the best speed–separation trade-off, while preserving separation and peak elution order.

These studies resulted in the well-known GC method translation [19.130], a short description of which will now be given. This approach implies optimizing the chromatographic conditions to give the best speed–separation trade-off with a conventional d_c column, and then transferring the optimized method to a shorter NB column. The parameters influencing a GC analysis are divided into two main groups: *translatable*

Table 19.4 Retention time(t_R) and resolution (R_s) of the enantiomers of the lavender essential oil chiral markers under several single-ramp heating rates. Condition: Conventional column, FID

Initial flow rate (ml/min)		1		2		2		2		2	
Heating rate (°C/min)		2.0		2.0		2.6		3.3		5.0	
Compound		t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s
1a	(*S*)-α-Pinene	13.14	1.0	9.84	1.0	8.70	1.0	7.85	0.9	6.45	0.6
1b	(*R*)-α-Pinene	13.02	1.0	9.74	1.0	8.62	1.0	7.78	0.9	6.41[a]	0.6
2a	(*S*)-Camphene	12.90	7.5	9.52	6.9	8.49	6.2	7.70	6.8	6.41[a]	5.0
2b	(*R*)-Camphene	13.65	7.5	10.21	6.9	9.04	8.49	8.15	7.70	6.71	5.0
3a	(*S*)-β-Pinene	14.93	4.5	11.33	4.9	9.96	4.4	8.92	4.0	7.27	3.3
3b	(*R*)-β-Pinene	14.40	4.5	10.82	4.5	9.56	4.4	8.60	4.0	7.06	3.3
4a	(*S*)-β-Phellandrene	20.85	5.1	16.88	5.9	14.38	5.5	12.49	4.9	9.64[b]	2.7
4b	(*R*)-β-Phellandrene	20.22	5.1	16.20	5.9	13.87	5.5	12.11	4.9	9.48	2.7
5a	(*S*)-Limonene	20.65	6.5	16.65	6.8	14.21	6.7	12.37	6.5	9.64[b]	6.0
5b	(*R*)-Limonene	21.55	6.5	17.48	6.8	14.91	6.7	12.92	6.5	9.98	6.0
6	1-Octen-3-ol	24.43	NR	20.67	NR	17.22	NR	14.67	NR	11.04	NR
7b	(*R*)-Camphor	25.86	1E	21.40	1E	18.02	1E	15.51	1E	11.88	1E
8a	(*S*)-Linalool	28.23	6.3	24.26[a]	7.0	20.05	6.1	16.96	5.5	12.63	4.1
8b	(*R*)-Linalool	27.28	6.3	23.27	7.0	19.28	6.1	16.37	5.5	12.23	4.1
9a	(*S*)-Borneol	28.86	2.9	24.26[a]	4.5	20.30	3.5	17.33	2.7	13.10	2.3
9b	(*R*)-Borneol	29.30	2.9	24.80	4.5	20.66	3.5	17.59	2.7	13.25	2.3
10a	(*S*)-Linalyl acetate	31.54	2.0	26.92	3.0	22.34	2.6	18.99	2.0	14.07[c]	NR
10b	(*R*)-Linalyl acetate	31.20	2.0	26.53	3.0	22.06	2.6	18.74	2.0	14.07[c]	NR
11a	(*S*)-Terpinen-4-ol	31.84	2.0	27.50	2.2	22.68	2.2	19.14	1.9	14.21	1.6
11b	(*R*)-Terpinen-4-ol	32.12	2.0	27.80	2.2	22.91	2.2	19.31	1.9	14.32	1.6
12b	(*R*)-Lavandulyl acetate	33.02[a]	1E	28.17	1E	23.37	1E	19.83	1E	14.84	1E
13b	(*R*)-Lavandulol	33.02[a]	1E	29.02	1E	23.74	1E	19.87	1E	14.59	1E
14a	(*S*)-α-Terpineol	34.53	5.0	30.24	6.1	24.78	5.5	20.77	4.9	15.27	3.9
14b	(*R*)-α-Terpineol	35.19	5.0	30.96	6.1	25.30	5.5	21.17	4.9	15.52	3.9

[a,b,c] Coeluting peaks, 1E – only one enantiomer detected, NR – not resolved

(column dimension (d_c and length), outlet pressure (1 atm for flame ionization detector (FID), vacuum for MS, etc.), carrier gas and flow rate) and *nontranslatable* (stationary phase type and phase ratio). The crucial operative parameter is hold-up time, which is taken as time unit to express all time-related parameters (duration of temperature plateau(s) and heating rate(s)) and to determine the normalized temperature program. Two methods are translatable only when nontranslatable parameters and normalized temperature programs are identical. The method-translation principles applied to a given temperature-programmed analysis enable either the flow rate to be optimized, producing the highest efficiency (i. e., the plate number) of a given column (efficiency-optimized flow, EOF), or a combination of flow rate, column dimensions, and carrier gas to be determined that corresponds to the shortest analysis time for a given required plate number (speed-optimized flow, SOF [19.127]). Method translation software can be downloaded for free from the Internet [19.130].

Optimization of Es-GC analysis conditions of lavender essential oil with a conventional 25 m × 0.25 mm column consisted of three main steps:

1. Choice of initial conditions to be optimized
2. Determination of optimal multirate temperature program for a predetermined fixed column pressure
3. Determination of optimal pressure (i. e., flow-rate) for the normalized optimal multirate temperature program.

The lavender essential oil was first analyzed with the conventional d_c column, under the temperature and flow conditions applied to routine analyses, that is, helium flow rate 1 ml/min and 2 °C/min heating rate. Under these conditions, the chiral markers were well separated in an analysis time of 35.2 min. Table 19.4 reports order of elution, retention times (t_R), and resolutions (R_S) of the enantiomers of the chiral markers investigated. Figure 19.9a reports the Es-GC pattern of the lavender essential oil investigated, analyzed un-

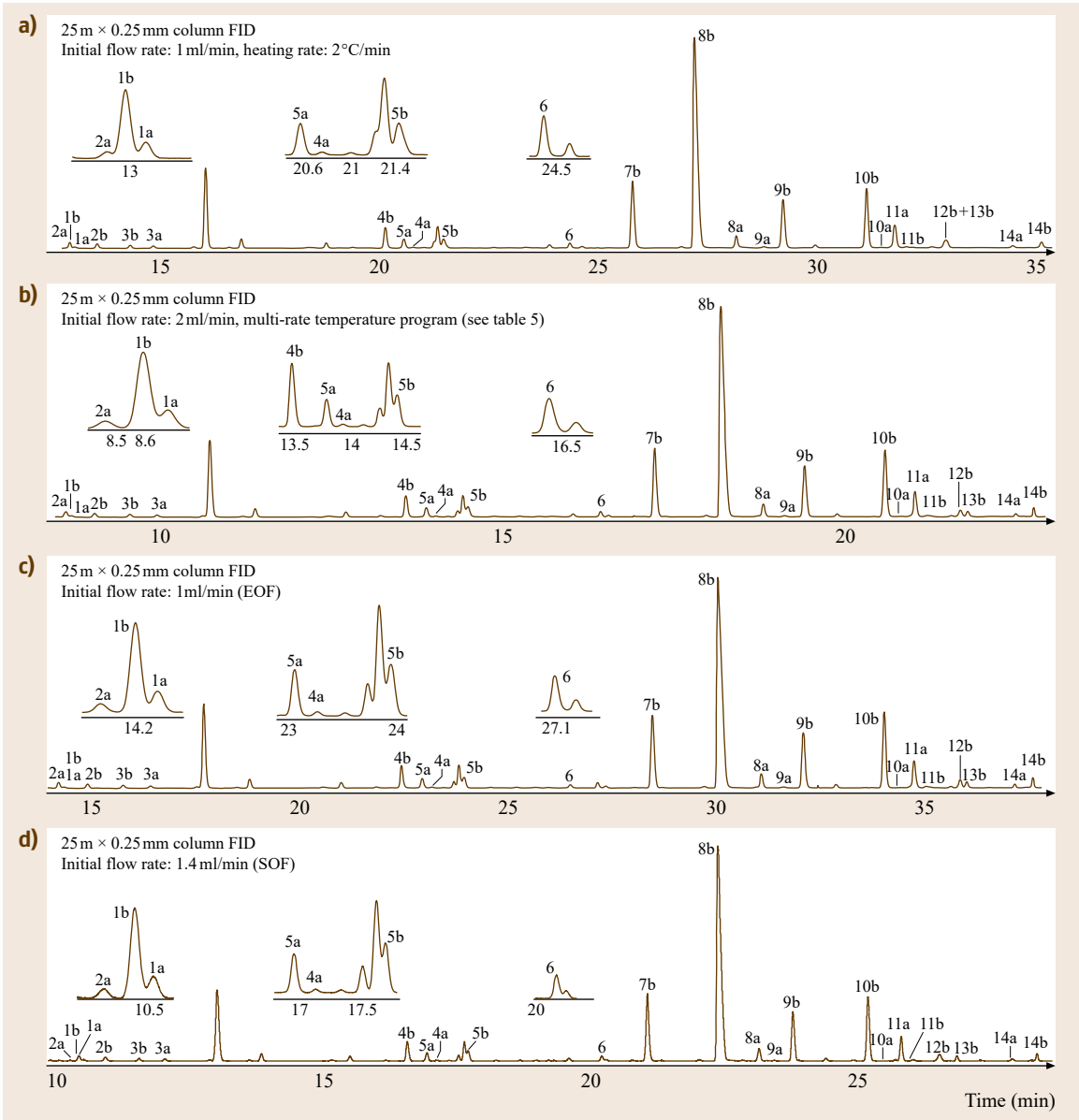


Fig. 19.9a–d Es-GC-MS profile of the lavender essential oil analyzed under different conditions with a conventional d_c column. For analysis conditions see text and Table 19.3. For peak identification, see caption to Fig. 19.7 [19.121]

der routine analysis conditions. This CD column and these conditions produced baseline separation of all chiral compounds, with the exception of α-pinene (1) enantiomers (R_S around 1) and of 1-octen-3-ol (6) enantiomers (not separated at all), while the (*S*)-enantiomers of camphor (7), lavandulol (13), and lavandulyl acetate (12) were not detectable, and (*R*)-lavandulol (13b) and (*R*)-lavandulyl acetate (12b) coeluted. As the starting point for method optimization, the initial flow rate was doubled to 2 ml/min (4) to reduce the time needed for method development, because this choice did not affect the final optimal conditions.

The optimal multirate temperature program at a fixed initial flow rate was obtained by applying a set of different single-ramp heating rates, namely 2.6, 3.3, and 5 °C/min (°C/t_m) (Table 19.4). The most satisfactory separation was obtained at 2.6 °C/min rate, which resulted in an analysis time of about 25.5 min. These experiments showed that, besides the separation of the enantiomers of chiral markers, lavender essential

Table 19.5 Method parameters (initial flow rates and translated hearing rates) and measured parameters (analysis times and resloutions of α-pinene enantiomers)

Column dimensions (detector)	25 m × 0.25 mm (FID)											10 m × 0.1 mm (FID)	10 m × 0.1 mm (MS)
Initial flow rate (ml/min)	2.0	0.3	0.5	0.7	1.0 (EOF)	1.4 (SOF)	1.7	2.3	2.5	2.8	4.0	0.56 (SOF)	0.56 (SOF)
Temperature program													
Initial temperature (°C)	50	50	50	50	50	50	50	50	50	50	50	50	50
Heating rate 1 (°C/min)	2.60	0.58	0.90	1.19	1.57	2.02	2.32	2.86	3.02	3.25	4.08	5.53	5.90
Intermediate T_1 (°C)	74	74	74	74	74	74	74	74	74	74	74	74	74
Heating rate 2 (°C/min)	3.30	0.74	1.14	1.51	2.00	2.57	2.95	3.63	3.83	4.13	5.17	7.04	7.50
Intermediate T_2 (°C)	115	115	115	115	115	115	115	115	115	115	115	115	115
Heating rate 3 (°C/min)	15.00	3.34	5.20	6.87	9.08	11.67	13.40	16.49	17.43	18.77	23.52	31.96	34.10
Final temperature (°C)	220	220	220	220	220	220	220	220	220	220	220	220	220
Final time (min)	2.00	8.98	5.77	4.37	3.30	2.57	2.24	1.82	1.72	1.60	1.27	0.94	0.90
Analysis time (min)	22.76	102.13	65.93	49.82	37.65	29.28	25.49	20.69	19.59	18.19	14.50	10.78	10.09
Resolution of α-pinene	0.96	1.01	1.04	1.06	1.07	1.04	1.00	0.93	0.90	0.88	0.72	1.09	1.10

Table 19.6 Retention time (t_R), reslution (R_s) and s values of the enantiomers of the lavender essential oil chiral markers analysed under different conditions. Legends: 1E = only one enantiomer detexted; NR = not resloved

Column, initial flow rate		25 m, 1 ml/min (EOF)			25 m, 1.4 ml/min (SOF)			10 m, 0.56 ml/min (SOF)					
								FID			MS		
	Compound	t_R	σ	R_s	t_R	σ	R_s	t_R	σ	R_R	t_S	σ	R_S
1a	(*S*)-α-Pinene	14.43	1.91	1.1	11.21	1.54	1.0	4.07	0.48	1.1	3.81	0.47	1.0
1b	(*R*)-α-Pinene	14.29	1.95		11.10	1.56		4.04	0.51		3.78	0.47	
2a	(*S*)-Camphene	14.07	1.96	6.8	10.93	1.64	6.5	3.97	0.53	7.5	3.71	0.55	6.9
2b	(*R*)-Camphene	14.98	2.04		11.64	1.63		4.23	0.53		3.97	0.55	
3a	(*S*)-β-Pinene	16.50	2.11	4.7	12.82	1.67	4.7	4.66	0.54	5.4	4.37	0.48	5.4
3b	(*R*)-β-Pinene	15.84	2.05		12.31	1.60		4.47	0.51		4.19	0.50	
4a	(*S*)-β-Phellandrene	23.24	2.00	5.5	18.06	1.55	5.5	6.61	0.53	6.0	6.19	0.41	6.2
4b	(*R*)-β-Phellandrene	22.51	2.00		17.49	1.55		6.40	0.55		6.00	0.52	
5a	(*S*)-Limonene	23.01	1.99	7.3	17.88	1.55	7.3	6.54	0.52	7.8	6.13	0.47	8.4
5b	(*R*)-Limonene	24.01	2.17		18.66	1.63		6.84	0.60		6.40	0.51	
6	1-Octen-3-ol	27.21	1.87	NR	21.16	1.47	NR	7.79	0.53	NR	7.29	0.46	NR
7b	(*R*)-Camphor	28.53	2.26	1E	22.18	1.71	1E	8.13	0.67	1E	7.62	0.59	1E
8a	(*S*)-Linalool	31.14	1.90	6.0	24.21	1.49	6.0	8.93	0.51	5.6	8.36	0.47	5.8
8b	(*R*)-Linalool	30.09	3.32		23.41	2.45		8.61	1.17		8.07	1.00	
9a	(*S*)-Borneol	31.67	2.47	3.1	24.62	1.82	3.1	9.05	0.73	3.1	8.48	0.82	2.6
9b	(*R*)-Borneol	32.14	2.26		24.99	1.75		9.19	0.64		8.60	0.60	
10a	(*S*)-Linalyl acetate	34.46	1.64	3.0	26.79	1.30	3.0	9.84	0.48	3.1	9.20	0.51	2.7
10b	(*R*)-Linalyl acetate	34.09	2.17		26.50	1.65		9.73	0.61		9.11	0.55	
11a	(*S*)-Terpinen-4-ol	34.80	2.12	2.1	27.06	1.65	2.0	9.98	0.60	2.0	9.34	0.49	2.2
11b	(*R*)-Terpinen-4-ol	35.09	2.03		27.28	1.72		10.06	0.56		9.41	0.52	
12b	(*R*)-Lavandulyl acetate	35.90	2.06	1E	27.91	1.65	1E	10.25	0.53	1E	9.59	0.56	1E
13b	(*R*)-Lavandulol	36.06	1.82	1E	28.04	1.47	1E	10.33	0.44	1E	9.67	0.43	1E
14a	(*S*)-α-Terpineol	37.22	1.45	4.6	28.94	1.15	4.5	10.66	0.36	5.6	9.97	0.35	5.3
14b	(*R*)-α-Terpineol	37.65	1.36		29.28	1.08		10.78	0.32		10.09	0.31	

Note: All t_R values are in minutes, all σ values are in seconds

oil presented three critical pairs of components: α-pinene (1)/camphene (2), 1-octen-3ol (6)/γ-terpinene, and (*R*)-lavandulol (13b)/(*R*)-lavandulyl acetate (12b), that are separated at different heating rates (2.6, 3.3, and 2.6 °C/min, respectively). A multiramp temperature program was therefore applied to obtain the best resolution of critical pairs in the shortest time; in particular, from 50 to 74 °C (elution temperature of (*R*)-α-pinene (1b), retention time 8.62 min) at 2.6 °C/min, then to 115 °C (elution temperature of (*R*)-lavandulol

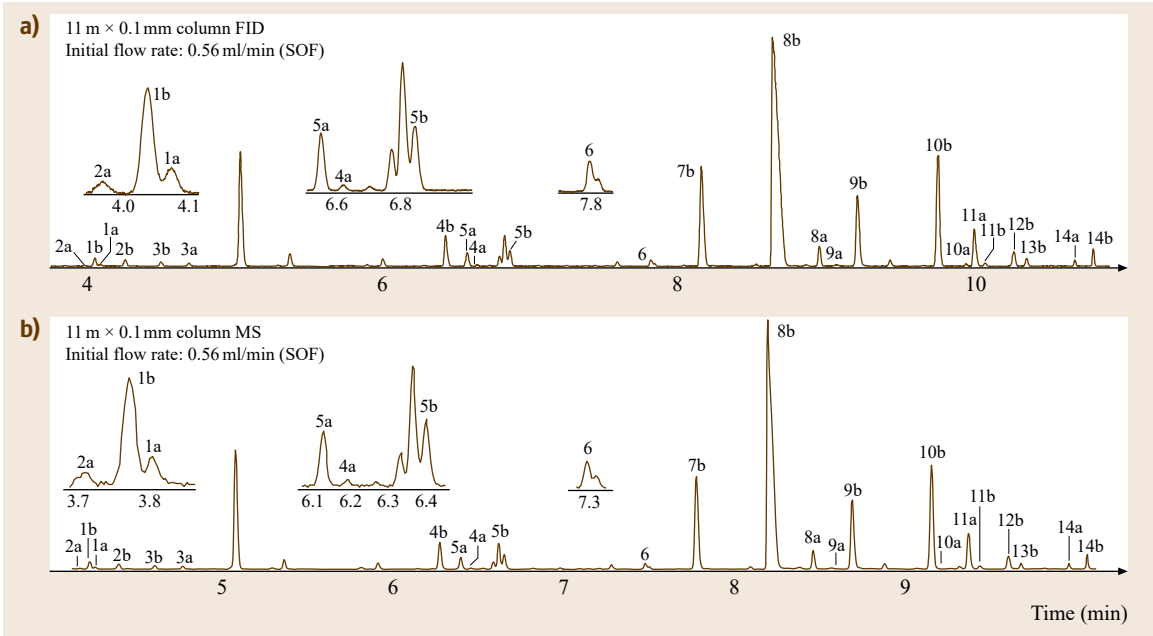


Fig. 19.10a,b Es-GC-MS profiles of the lavender essential oil analyzed under SOF conditions with the NB column. For analysis conditions, see text and Table 19.5. For peak identification, see caption to Fig. 19.7 [19.121]

(13b), retention time 21.79) at 3.3 °C/min, then to 220 °C at 15 °C/min to clear the column. Figure 19.9b reports the Es-GC pattern of the lavender essential oil analyzed under the optimized multirate temperature program.

The next step was to optimize the flow rate by determining the initial EOF (initial flow that maximizes column efficiency and peak resolution) and calculating the initial SOF (initial flow that minimizes analysis time at fixed efficiency) [19.125]. A reasonable compromise to optimize the flow rate is to apply the initial value that is optimal for the most critical pair (or the most important pair, in this case α-pinene). This can be EOF, which causes the highest column efficiency for a given solute pair and, as a result, its highest resolution (see below), but it can also be the initial SOF, which causes the shortest analysis time for a given resolution of the critical pair. Ten different pressures were applied to the column, resulting in different initial flow rates. The GC method translator was used to translate the temperature program for each pressure, in order to maintain the same normalized temperature program in all cases. Table 19.5 reports the initial flow rates, the corresponding translated temperature programs, and the resulting analysis times. SOF can be calculated from EOF as SOF $= \sqrt{2}$ EOF [19.127], that is, in this study, the initial EOF was 1 ml/min so that the initial SOF was 1.4 ml/min, and the corresponding first two heating rates were 2.02 and 2.57 °C/min (Table 19.5). Under these conditions, the analysis time was 29.3 min.

Table 19.6 reports retention times, enantiomer resolution, and σ values of chiral markers of the lavender essential oil analyzed, under the optimal conditions determined. The lavender essential oil profiles at EOF and SOF are shown in Fig. 19.9c,d respectively.

The optimized SOF method with a conventional (25 m × 0.25 mm × 0.25 µm) column was then translated to the corresponding NB column (11.13 m × 0.1 mm × 0.1 µm). Parameters of the translated method are shown in Table 19.5, and the essential oil profile in Fig. 19.10a. The flow rate was reduced in proportion with the column d_c, from 1.4 to 0.56 ml/min, thus assuring SOF operation of the NB column. Under these conditions, and because both columns had very similar length/d_c ratio, translation did not affect resolution of any peak pair (Table. 19.6), but analysis time was reduced to about a third (29.50 versus 10.78 min), without loss of peak resolution.

The SOF analysis conditions with ES-GC-FID were then translated to the Es-GC-MS for the same lavender essential oil analysis. Translation from conventional column at SOF to the MS method with NB column at SOF reduced analysis time overall to about a third (retention time of the last peak was reduced from 29.28 to 10.09 min).

19.7 Total Analysis Systems and Real-World Sample Analysis

The above paragraphs show that Es-GC is very successful as a technique for chiral odor recognition in the flavor and fragrance field, in particular when considered within the modern strategies of analysis based on fully automatic systems, better known as *Total Analysis Systems* (TASs); these are systems in which the three main steps of the analytical process (sample preparation, analysis, and data processing) are on-line integrated into a single step [19.131, 132]. The success of TAS systems is one of the factors that has greatly contributed to the radical change of strategies that began in the early 1990s in analysing volatiles in the flavor and fragrance fields [19.133].

The adoption of these systems in general, and in particular in chiral recognition, has been made possible by the parallel improvements achieved not only in Es-GC but also in sample preparation and mass spectrometry as detector (Sect. 19.5.2). In spite of the number of effective distillation and extraction techniques introduced into sample preparation over this period, the volatile nature of most odorants in the flavor and fragrance field makes headspace sampling, when applicable, the technique of choice for these analyses [19.100, 134, 135]. HS techniques have the advantages of being solvent-free, fast, simple, reliable, and, above all, easy to automate; they can also be combined on-line to GC-MS systems. These considerations include both conventional (static (S-HS) or dynamic (D-HS)) modes, and high concentration capacity headspace techniques (HCC-HS) [19.134–136]. HCC-HS are recently developed techniques that act as a *bridge* between S-HS and D-HS techniques: volatiles are statically or dynamically accumulated on polymers, operating in sorption and/or adsorption modes or, less frequently, on solvents (headspace solid-phase microextraction (HS-SPME), headspace sorptive extraction (HSSE), headspace sorptive tape extraction (HS-STE), headspace liquid-phase microextraction (HS-LPME), etc.). The development of these techniques was favoured by the success of polydimethylsiloxane (PDMS) as an accumulation material for volatiles [19.137].

Figure 19.11 reports an example of a TAS where the genuineness of a peach juice is determined by a one-step analysis by HS-SPME-Fast-GC-MS and (SIM-MS) [19.81].

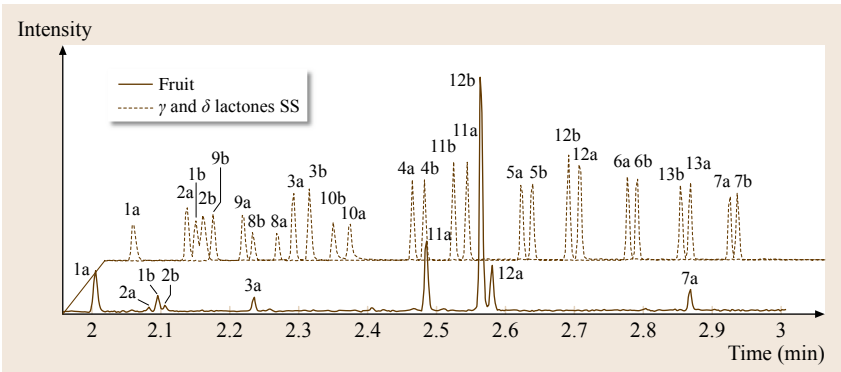


Fig. 19.11 HS-SPME-Fast-Es-GC-SIM-MS profiles of peach juice HS and γ-C6-C12 and δ-C6-C12 (—) standard solutions on a 30% 6^{I-VII}-*O*-TBDMS-$2^{I-VII-}3^{I-VII}$-*O*-acetyl-β-CD in PS086 (5 m, d_c: 0.10 mm, d_f: 0.10 mm) Sampling conditions: fiber: 2 cm Stableflex 50/30 µm DVB-Carboxen-PDMS (Supelco, Bellefonte, PA, USA); sample amount: 20 mg; vial volume: 20 ml; sampling time: 20 min, temperature: 60 °C. Analysis conditions: injection mode: split; split ratio: 1 : 50. Inj. temperature: 230 °C, transfer line: 250 °C; ion source: 200 °C; ionization mode: EI at 70 eV. Scan range: 35–350 m/z. Temperature program, 90 °C/24.0/140 °C/60.0/220 °C. Peak identification: 1: γ-hexalactone, 2: γ-heptalactone, 3: γ-octalactone, 4: γ-nonalactone, 5: γ-decalactone, 6: γ-undecalactone, 7:γ-dodecalactone, 8: δ-hexalactone; 9: δ-octalactone, 10: δ-nonalactone, 11: δ-decalactone, 12: δ-undecalactone, 13: δ-dodecalactone; (a) *(R)*-enantiomer, (b) *(S)*-enantiomer

19.8 Conclusions

Cyclodextrin derivatives are nowadays the most effective chiral stationary phases available for Es-GC in the flavor and fragrance field. This consideration is not only justified by the number of CD derivatives with high enantioselectivity and good chromatographic properties offering the possibility to separate most enantiomer pairs without derivatization to the corresponding diastereoisomers, but also to the performance of the resulting GC columns, also thanks to the dilution in moderately polar polysiloxanes, in particular:

- High stability and separation repeatability maintained for hundredth of injections.
- Efficiency and analysis times comparable to those of conventional columns.
- High inertness with classes of compounds, within a relatively wide range of polarity.
- Extended operative temperature range (20–250°). As a result, very few enantiomer pairs in this field cannot be separated with these chiral selectors provided that a suitable derivative is adopted.

However, in view of an ever increasing demand of chiral recognition, a lot of work has still to be done, mainly on two complementary directions: a) the first one concerning an ever better understanding of the mechanisms that are the basis of enantiomer recognition with CD derivatives in vapour phase thus allowing the operator to design a separation and avoiding to proceed by trial and error, as it is often the case with new enantiomer pairs, and b) the latter implying introduction of a new generation of CD derivatives with a more universal enantioselectivity enabling to extend their use to the separation of highly polar chiral compounds.

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20. Stable Isotope Ratio Analysis for Authenticity Control

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This chapter summarizes terms, definitions, and reference materials used for stable isotope ratio analysis (SIRA) of the bioelements hydrogen, carbon, and oxygen. The principles of biotic and abiotic fractionation in biomolecules like flavor compounds are explained. A short review of the common methods for the determination of isotope ratios $^{2}H/^{1}H$, $^{13}C/^{12}C$, and $^{18}O/^{16}O$, using isotope ratio mass spectrometry (IRMS) and nuclear magnetic resonance spectrometry (NMR) of hydrogen and carbon (^{2}H- and ^{13}C-NMR) are introduced. Further the focus is set on selected applications of authentication control of flavor compound and flavorings using isotope ratio analysis. Examples of benzaldehyde, vanillin, vanilla flavorings and vanilla extracts, butanoic acid, isoprenoids, and essential oils as well as fruity flavor compounds like γ- and δ-lactones are presented. Potentials and limitations of SIRA are discussed taking the analytical requirements into consideration, as well as representative databases and suitable guidelines for authenticity assessment.

Authenticity of a single flavoring substance, a flavoring, or an essential oil can be defined as the genuineness of the described purity and source of origin. The regulation of *the European Parliament and Council* on flavorings lays down the criteria for the use and labeling of flavorings and certain food ingredients with flavoring properties for intended food use. Flavorings should, in particular, not be used in a way as to mislead the consumer about issues related to the nature, quality of ingredients used, genuineness, or the production processes. The *International Organization of the Flavor Industry (IOFI)* has established the *IOFI Code of Practice* corresponding to official regulations, which describes best practices and finds full acknowledgment by all member associations and member companies. Thus the proof of authenticity of flavorings represents an important task regarding consumer protection against fraud and also regarding the legitimate interest of quality assurance departments in the flavor and fragrance as well as the food industry. In combination

with the correct labeling of products, the confirmation of a justifiable cost-to-benefit ratio reflects the commercial basis of fair trade in many industries besides the food industry like the pharmaceutical industry or animal nutrition industry.

Many commercially important flavoring substances can be produced via different routes like chemical synthesis, traditional food preparation methods, and enzymatic or microbiological processes from various sources. Due to the high demand for natural flavorings analytical methods have to be constantly optimized in order to detect sophisticated adulterations. The detection of adulterations or the proof of authenticity of flavorings involves the following three main analytical focus points according to EU legislation [20.1]:

- Is the flavoring substance or flavoring exclusively originating from the natural source or material referred to?
- Is the indication of the geographic origin and botanical variety of the source of the flavoring appropriate?
- Are flavoring substances derived from microbiological, enzymatic, or physical processes from natural and approved raw materials or are they synthesized by chemical processing?

Gas chromatography combined with mass spectrometry (GC-MS) is a standard analytical method not only for the determination of the qualitative and quantitative composition of a complex flavoring, often containing more than 100 flavor substances, but also for the proof of the purity of a single flavoring substance. The application of GC-MS methods usually provides a first qualitative and quantitative overview as well as a first insight regarding the potential source of a flavoring mixture including suspicious findings [20.2–4]. Enantioselective gas chromatography on chiral phases to determine the enantiomeric distribution may only help to prove the natural character of a few chiral flavor substances [20.5]. In consequence, a significant proof of authenticity, which also provides sufficient evidence in court, is not always possible with these methods only.

Since more than 30 years, the stable isotope ratio analysis (SIRA) of the bioelements hydrogen, carbon, and oxygen is used in laboratories of the flavor industry, but also in laboratories responsible for official consumer protection, for a more significant authenticity control of flavor compounds and flavorings [20.6–10]. Currently, the various possibilities of SIRA using IRMS, also coupled on-line with multidimensional gas chromatography [20.11] and enantioselective analysis [20.5], as well as nuclear magnetic resonance spectroscopy (NMR) are mainly focused on a significant assessment of authenticity of a flavor compound [20.12, 13].

20.1 Fundamentals

Each element is defined by the composition of its atomic nucleus, which contains a specific number of protons and neutrons. The number of protons is identical with the number in the periodic system of an element and defines the name of the element (1 for hydrogen, 6 for carbon, or 8 for oxygen). The sum of the protons and neutrons is responsible for the relative isotopic mass or the atomic mass.

Isotopes are variants of a particular chemical element which only differ in their number of neutrons. An additional neutron of an element defines an additional isotope of this element. For example, the predominant percentage of carbon ($\approx 99\%$) has 6 protons and 6 neutrons and is reported as ^{12}C to define its atomic mass. However, about 1.1% of the carbon in the Earth's biosphere has 6 protons and 7 neutrons and is the heavier stable isotope reported as ^{13}C. *Stable isotopes* do not decay into other elements. In contrast, *radioactive isotopes* (e.g., ^{14}C) are unstable and will decay into other elements [20.14, 15].

The stable isotope ratios of the bioelements carbon ($^{13}C/^{12}C$), hydrogen ($^{2}H/^{1}H$), and oxygen ($^{18}O/^{16}O$) are usually expressed in the so-called *delta notation* ($\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$). For the hydrogen isotope ^{2}H also the term *deuterium* with its abbreviation *D* is used. In case of isotope ratio of *isotopomers* the term (D/H) is used.

Isotopomers or *isotopic isomers* have the same number of each isotopic atom but have different positions of the heavier isotope in the molecule. For example, ethanol is composed of three isotopomers with deuterium occurring in the three positions (CH_2DCH_2-OH, CH_3CHDOH, and CH_3CH_2OD) [20.16]. *Isotopologues* are molecules that differ only in their isotopic composition. Water, the best known example, can be differentiated between *light water* (HOH), *semi-heavy water* with the deuterium isotope in equal proportion to the proton (HDO), and *heavy water* with two deuterium isotopes of hydrogen per molecule (D_2O or $^{2}H_2O$). Oxygen-related isotopologs of water include the heavy-oxygen

water ($H_2^{18}O$) and the more difficult to separate version with the ^{17}O-isotope.

20.1.1 Terms and Definitions

In order to establish an isotopic profile or signature for a material, the ratios of the stable isotopes of elements, such as $^2H/^1H$, $^{18}O/^{16}O$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{34}S/^{32}S$ can be measured. Variations in the natural abundance of stable isotopes are expressed using the delta (δ) notation ($\delta^{13}C$) as shown in Eq. (20.1). δ-values are commonly expressed in parts per thousand (per mil or ‰). Isotope ratios are measured relative to international standards that define the measurement scale for particular isotopes (Table 20.1). The *International Atomic Energy Agency* (IAEA) has defined these scales by reference to natural or virtual materials identified by the V prefix:

$$\delta^{13}C[‰]_{V\text{-}PDB} = \left(\frac{R_{Sample}}{R_{Standard}} - 1\right) \cdot 1000\,, \qquad (20.1)$$

where R_{Sample} is a ratio ($[^{13}C]/[^{12}C]$) of the sample, $R_{Standard}$ is a ratio ($[^{13}C]/[^{12}C]$) of the international standard.

20.1.2 International Standards

The calibration and intercomparison materials typically consist of natural minerals and compounds commonly studied in isotope geochemistry, having the desired characteristics of isotopic composition, homogeneity, chemical purity, and stability. The IAEA distributes calibration and intercomparison materials for the interlaboratory calibration of measurements of stable isotope ratio variations in natural compounds. A comprehensive review of stable isotope scales and reference materials is the *IAEA-TECDOC-825*, available from the IAEA in Vienna [20.18]. Standards, calibration, and intercomparison materials can be grouped in primary reference standards, calibration materials, and intercomparison materials.

The primary reference standards used to express natural variations of isotopic composition of hydrogen, oxygen, carbon, nitrogen, and sulfur are SMOW, PDB, Air, and CDT. SMOW (*standard mean ocean water*) was used since it was defined by Craig in 1961 as reference to express the relative variations of $^2H/^1H$- and $^{18}O/^{16}O$-ratios in natural waters [20.19]. The definition of SMOW was based on a water standard of the *National Bureau of Standards* (NBS-1). PDB (*Peedee Belemnite*) consisted of calcium carbonate from the rostrum of a Cretaceous belemnite, *Belemnitella americana*, from the Peedee formation of South Carolina. The CO_2 evolved from PDB, by treatment with phosphoric acid, was adopted as the zero point for oxygen and carbon isotopic measurements. AIR (atmospheric nitrogen), with a very homogeneous isotopic composition all around the world [20.20], is the primary reference standard for nitrogen stable isotope variations. Atmosphere is the largest terrestrial reservoir of nitrogen, and it is also the main pool of this element taking part in natural and industrial processes (production of fertilizers). CDT (*Canyon Diablo Troilite*) consisted of FeS (Troilite) present in the iron meteorite of Canyon Diablo, Arizona. Meteoritic sulfur was taken as reference standard because its $^{34}S/^{32}S$ isotopic ratio exhibits only small variations and corresponds quite well to the average isotopic ratio of terrestrial sulfur [20.21]. CDT has a $^{32}S/^{34}S$-ratio of 22.22 $‰_{V\text{-}CDT}$ [20.22], and is considerably depleted in ^{34}S (about +20 $‰_{V\text{-}CDT}$) with respect to marine sulfate, which is isotopically the most homogeneous sulfur reservoir on the earth crust. From these primary reference standards SMOW, was selected as a theoretical calibration standard while PDB and CDT were used as such for long time.

The IAEA distributes these materials to laboratories around the world. All reference materials are carefully calibrated versus the primary reference standards based on internationally agreed and adopted calibration values. Both water reference materials V-SMOW (*Vienna Standard Mean Ocean Water*) and SLAP (*Standard Light Antarctic Precipitation*) are distributed for the calibration of the $^2H/^1H$, $^{18}O/^{16}O$, and $^{17}O/^{16}O$ variation measurements. V-SMOW was prepared by blending distilled ocean water with small amounts of other waters to produce an isotopic composition close to the

Table 20.1 International isotope ratio scales (after [20.17])

Element	International scale	Isotope ratio	Accepted ratio[a]
Hydrogen	Vienna Standard Mean Ocean Water (V-SMOW)	$^2H/^1H$	0.00015575
Oxygen	Vienna Standard Mean Ocean Water (V-SMOW)	$^{18}O/^{16}O$	0.0020052
Carbon	Vienna PeeDee Belemnite (V-PDB)	$^{13}C/^{12}C$	0.0111802
Nitrogen	AIR	$^{15}N/^{14}N$	0.0036782
Sulfur	Vienna Canyon Diablo Troilite (V-CDT)	$^{34}S/^{32}S$	0.0441509

[a] [Heavy isotope]/[light isotope]

Table 20.2 Reference materials

Name	Nature	Isotope	δ ‰ (±SD)[a]
GISP	Water	^{2}H	-189.5 ± 1.2
		^{18}O	-24.76 ± 0.09
IAEA-CH-7	Polyethylene	^{2}H	-100.3 ± 2.0
		^{13}C	-32.151 ± 0.050
IAEA-N-1	Ammonium sulfate	^{15}N	$+0.4 \pm 0.2$
NBS-18	Calcite	^{18}O	-23.2 ± 0.1
		^{13}C	-5.014 ± 0.035
IAEA-CH-6	Sucrose	^{13}C	-10.449 ± 0.033
NBS-127	Barium sulfate	^{34}S	$+20.3 \pm 0.4$
		^{18}O	$+9.3 \pm 0.4$

[a] The isotopic ratio data are reported in per mil (‰) deviation from the international isotope standards V-SMOW for oxygen and hydrogen, V-PDB for carbon, Air for nitrogen, and V-CDT for sulfur. The δ-values and SD are those reported by IAEA (compiled 2013) under: http://nucleus.iaea.org/rpst/ReferenceProducts/ReferenceMaterials/Stable_Isotopes/index.htm

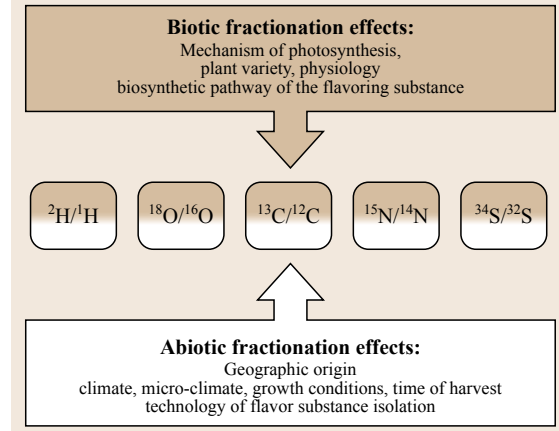


Fig. 20.1 Parameters, influencing biotic and abiotic stable isotope fractionation of bioelements in flavorings from plant sources

definition of SMOW. V-SMOW has an isotopic composition practically identical to the SMOW defined by Craig, while SLAP, which was prepared from South Pole firn, is considerably depleted in heavy isotopes with respect to V-SMOW. Now V-SMOW has been superseded by V-SMOW2 which was prepared at the IAEA Isotope Hydrology Laboratory in 2006 to replace the V-SMOW material [20.23]. PDB has isotopic ratios close to those of limestone of marine origin, and is considerably enriched in ^{13}C with respect to organic carbon compounds. Therefore, NBS-19 (or TS-Limestone) was distributed for the calibration of the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ variation determinations. NBS-19 has been indirectly calibrated versus PDB. By international agreement, the isotopic composition of NBS-19 versus a hypothetical V-PDB (Vienna-PDB), supposed identical to PDB, has been fixed to $\delta^{13}C = +1.95\,‰_{\text{V-PDB}}$ and $\delta^{18}O = -2.20\,‰_{\text{V-SMOW}}$ [20.24]. The absolute isotopic ratios of NBS-19 have not been determined. Improved measurement precision has revealed that CDT was not isotopically homogeneous to be continued as the primary reference material and [20.25], as a consequence, the CDT scale has been replaced with V-CDT. V-CDT is defined by assigning a $\delta^{34}S$-value of $-0.3\,‰$ to IAEA-S-1 (silver sulfide) [20.26].

Intercomparison materials are natural and synthetic compounds, which provide the means for laboratories to periodically check the overall quality of the measurements performed, including the long-term reproducibility of sample preparation from a variety of materials, in comparison with those obtained by other laboratories. The δ-values of these materials (distributed by IAEA) are agreed and adopted internationally but, in contrast to the primary reference standards, have uncertainty associated with the δ-values (Table 20.2).

20.1.3 Principles of Stable Isotope Discrimination and Fractionation of Bioelements

Many studies on the inter- and intramolecular nonstatistical distribution of heavy stable isotopes of the bioelements ^{13}C, ^{2}H, ^{18}O, ^{15}N, and ^{34}S in plant material ingredients have proved that their distribution is controlled by physical and chemical principles, which result in a characteristic pattern and intrinsic correlation of stable isotope ratios. In plant materials, this pattern is mainly assigned by different biochemical mechanisms in different plant varieties and different physicochemical effects induced by the geographic locations and conditions of growth, harvest, or processing [20.14, 15, 27].

The so-called *stable isotope discrimination or fractionation* can be divided into *biotic* and *abiotic* processes, which both have main influences on the stable isotope distribution of hydrogen, carbon, oxygen, and nitrogen in an organic molecule like a flavoring substance. In addition, isotope fractionation effects, which may occur due to environmental influences (*anthropogenic fractionation*) as well as *geological fractionation* are affecting the composition of the soil where, for example, a plant was grown [20.14, 15]. This influence is prominent for sulfur and nitrogen but less relevant for the main bioelements hydrogen, carbon, and oxygen of flavor substances. The final isotopic pattern of components from a plant material integrates both biotic and abiotic factors.

Thus, the knowledge of the characteristic patterns and the mechanisms responsible is a powerful tool for authentication of individual flavoring substances.

Figure 20.1 shows the main influences on biotic and abiotic stable isotope fractionation.

20.1.4 Biotic Fractionation (Kinetic Fractionation Effects)

The best known and important biotic fractionation effect is the carbon-isotope discrimination, which is a result of the different primary *CO_2-fixation processes* by photosynthesis in terrestrial plants and which shows different ^{13}C-isotope fractionation, in the so-called *C_3-*, *C_4-plants*, and *CAM-plants* [20.28, 29]. The C_3-*plants* – the name is derived from the number of carbon atoms of phosphoglyceric acid, the first photosynthetic product – have a larger isotope effect on the ribulose-bisphosphate-carboxylase reaction, with the result of a depletion of the ^{13}C-isotopes and δ^{13}C-values between -37 and $-24\,‰_{\text{V-PDB}}$. C_3-plants represent the most abundant group of plants and comprise vegetable flavoring sources like pomaceous fruits, stone fruits, berry fruits, grapes, wheat, grain, or sugar beet [20.30].

C_4-plants – the name is derived from the four C-atoms of the oxalacetate, the photosynthetic product of the phosphoenolpyruvate-carboxylase reaction. Due to a smaller isotope effect, more ^{13}C is accumulated resulting in δ^{13}C-values between -16 and $-10\,‰_{\text{V-PDB}}$. The best known C_4-plants used in agricultural industry are sugar cane, corn, or millet.

Products from *CAM-plants* (crassulacean acid metabolism), which are characteristic for some succulents, orchids, and tropical grasses, have both CO_2-fixing reactions. Therefore the δ^{13}C-values range in between these areas of the C_3- and C_4-plants. Products of CAM-plants also show a higher enrichment of deuterium in carbohydrates and in some substances of their secondary metabolism, like aromatic compounds. CAM-plants with important flavorings are pineapple, vanilla, or agave [20.12, 14].

Fractionation of hydrogen and deuterium is also influenced by photosynthetic processes. Thus also deuterium is depleted in C_3-plants and accumulated in C_4-plants. Deuterium is also fractionated during alcoholic fermentation of sugar by yeasts. The corresponding biotic intramolecular fractionation effects induced by different kinetic and enzymatic mechanisms during biosynthesis and metabolism can also be used for authenticity assessment of a flavor substance. Due to the complex biosynthetic correlations in the formation and the extremely complicated analytical demands of the analysis of ^{13}C-patterns, only ^{2}H- and ^{18}O-patterns are of practical importance with regard to the following general rules [20.12]:

^{2}H-patterns:

- Natural aromatic compounds descending from the shikimic acid pathway show relative positional ^{2}H-abundances in the sequence $p > o \geq m$; when the *p*-position is hydroxylated, the sequence is inverted into $m > o$.
- Isoprenoids from the mevalonate pathway show in general a ^{2}H-depletion of the methylene groups relative to the methyl and vinyl positions, while those from the desoxyxylulose-phosphate pathway are characterised by a distinct relative ^{2}H-depletion of the vinyl groups.
- Chains of fatty acids and descendants or acetogenins show an alternating ^{2}H-abundance with relative ^{2}H-depletions in the odd (C = O-deriving) positions.

^{18}O-patterns:

- In natural compounds, the δ^{18}O-values of hydroxyl groups originating from monooxygenase reactions (most phenolic OH-groups, some OH-groups in isoprenoids) range between $+3$ and $+7\,‰_{\text{V-SMOW}}$.
- Oxygen functions introduced from water by a lyase reaction must be ^{18}O-depleted relative to this water, with water isotopically equilibrated carbonyl or carboxyl groups are relatively ^{18}O-enriched by about $+28$ and $\approx +19\,‰_{\text{V-SMOW}}$, respectively.
- Natural but also certain nature identical esters are characterized by an extreme ^{18}O-enrichment (up to $+40$ to $+60\,‰_{\text{V-SMOW}}$) of the carbonyl-O and a modest ^{18}O-content of the ether-O ($< +10\,‰_{\text{V-SMOW}}$).

20.1.5 Abiotic Fractionation (Thermodynamic Fractionation)

Abiotic fractionation processes are mainly induced by thermodynamic isotope effects during water evaporation and condensation, and the isotope equilibration between water and CO_2. There is a predictable linear relationship between ^{2}H and ^{18}O in water, which is referred to as *meteoric water line* and allows an approximation of ^{2}H based on measurements of ^{18}O and vice versa [20.19, 31, 32]. The following general rules have to be taken into consideration with regard to enrichment or depletion of heavier stable isotopes ^{2}H and ^{18}O in precipitation water:

- *Altitude effect*: the δ^{18}O- and δ^{2}H-values of precipitation decrease with increasing altitude. Typical

gradients are $-0.5\,‰_{\text{V-SMOW}}$ to $-0.5\,‰_{\text{V-SMOW}}$ per 100 m for ^{18}O, and $-1.5\,‰_{\text{V-SMOW}}$ to $-4\,‰_{\text{V-SMOW}}$ per 100 m for ^{2}H.

- *Latitude effect*: the $\delta^{18}O$- and $\delta^{2}H$-values decrease with increasing latitude because of the increasing degree of *rain-out* [20.33].
- *Continental effect*: Isotopic ratios decrease inland from the coast.
- *Amount effect*: The greater the amount of rainfall, the lower the $\delta^{18}O$- and $\delta^{2}H$-values of the rainfall.

Subsequently physical fractionation of water in a plant or in a fruit is the fractionation of plant or leaf water. Evaporation through the stomata or through the skin of a fruit or a vegetable enriches both ^{18}O and ^{2}H in fruit or leaf water. The extent of the enrichment depends on factors like microclimatic influences, location, water sustenance, but also physiological parameters like type of plant and finally agricultural conditions like period maturation of a fruit involving its different annual meteorological conditions and the date of harvest.

20.2 Methods for the Determination of Isotope Ratios

The ratio of stable isotopes of the bioelements H, C, O, S, and N in biomolecules like flavoring substances can be determined with IRMS. In analysis of volatile compounds like flavor substances or complex mixture of flavorings IRMS combined with gas chromatography is the method of choice.

Hydrogen or carbon isotope ratios of isotopomers of different positions or a functional group of a molecule can be determined either with ^{2}H-NMR or ^{13}C-NMR or using IRMS after specific degradation of a molecule.

20.2.1 Isotope Ratio Mass Spectrometry

Isotope ratio mass spectrometers are specifically designed to measure precisely small differences in the abundances of isotopes, such as $^{2}H/^{1}H$, $^{18}O/^{16}O$, and $^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{34}S/^{32}S$.

There are two common instrument configurations used – continuous flow IRMS (CF-IRMS) and dual-inlet IRMS (DI-IRMS). In dual inlet systems, the gases are generated off-line, and admitted into the sample bellows of the system. A second bellow contains the reference gas, and sample and reference are alternatively admitted into the ion source. In continuous-flow instruments, the dual inlet system is replaced by a carrier gas flow, commonly helium. This type of design is particularly suitable for the use of on-line preparation systems, such as elemental analyzers, gas chromatographs, or equilibrium devices. For isotope ratio measurement, the analyte must be converted into a simple gas (H_2, CO, CO_2, N_2, and SO_2) before entering the ion source of an isotope ratio mass spectrometer. The isotope ratio mass spectrometer measures the ratio of ions that correspond to these gases. For example, in the analysis of carbon isotope ratios, the mass spectrometer monitors ions with mass to charge ratios (m/z) of 44, 45, and 46, which correspond to the ions produced from CO_2 molecules containing ^{12}C, ^{13}C, ^{16}O, ^{17}O, and ^{18}O in various combinations. Ionization of the analyte gases is achieved using electron ionization (EI). The ionized gases are separated in a single magnetic sector analyzer by virtue of their momentum and are detected by an array of Faraday cups the output from which is used to calculate the final stable isotope ratio. This is calculated relative to a standard of known isotopic composition and expressed using the δ-notation.

The sample gas is produced in a range of automated preparation systems that can lead to *bulk* (BSIA) or *compound-specific* (CSIA) isotopic analysis.

An elemental analyzer coupled to an isotope ratio mass spectrometer (EA-IRMS) gives the bulk isotopic composition of the sample. Depending on the specific set up, it is possible to measure isotopic ratios of H, C, N, O and S in a range of solid and liquid matrices. At first the continuous flow elemental analyzer technique was suitable for the measurement of ^{13}C- and ^{15}N-isotopes in organic samples [20.34], and some years later for ^{34}S-isotopes in organic samples [20.35]. In combustion mode, C-, N-, and S-containing materials are converted into the measuring gases carbon dioxide, nitrogen, and sulfur dioxide for on-line isotopic analysis. The high-temperature conversion (*pyrolysis*) mode is employed for the analysis of isotope ratio of hydrogen and oxygen. Samples are loaded into silver capsules and dropped into a ceramic reactor lined by a glassy carbon tube (filled with glassy carbon) in the inside, where the sample is pyrolyzed [20.36–39]

On-line, high-precision CSIA was first demonstrated in 1978 by *Matthews* and *Hayes* [20.40–42], using a single-collector high-precision isotope ratio instrument. The compounds eluted from the chromatography column are carried by the helium flow; combusted in the interface, and carried into the source of the mass spectrometer. Initially, only ^{13}C could be determined [20.43]. Later modifications were made that allow measurement of N, O, and H isotope ratios [20.44, 45]. Carbon and nitrogen compounds eluting from the

chromatographic column then pass through a combustion reactor (an alumina tube containing Cu, Ni and, Pt wires maintained at 940 °C) where they are oxidatively combusted. This is followed by a reduction reactor (an alumina tube containing three Cu wires maintained at 600 °C) to reduce any nitrogen oxides to nitrogen [20.46]. For hydrogen and oxygen, a high-temperature thermal conversion reactor is required [20.47]. Following GC separation, the hydrogen bound in organic compounds must be quantitatively converted into H_2-gas prior to analysis in the IRMS. Quantitative conversion is achieved by high-temperature conversion (TC) at temperatures > 1400 °C [20.45].

The premise for gas chromatography isotope ratio mass spectrometry (GC-IRMS) is that the compounds constituting the sample mixture are amenable to GC, that is, they are suitably volatile and thermally stable. Polar compounds may require further chemical modification (derivatization) and in such cases the relative stable isotope ratio of the derivatization agent must also be determined. Coupling a liquid chromatograph to the IRMS (LC-IRMS) may overcome some of these problems [20.48]. However, LC-IRMS requires the elimination of the mobile phase either before or after oxidation of the sample of interest. The technique is useful to analyze high molecular weight compounds not well suited for GC-IRMS, such as sugars, amino acids, or active components of pharmaceuticals and drugs.

20.2.2 Nuclear Magnetic Resonance

Nuclear magnetic resonance spectroscopy (NMR) is a powerful analytical technique for determining the structure or purity of organic compounds and it is more and more frequently used in food analysis [20.32, 49, 50]. NMR is based on the fact that many nuclei, such as for example ^{1}H, ^{2}H and ^{13}C, possess a nuclear spin and thus have a nuclear magnetic moment. When placed in a magnetic field, the sample's nuclear magnetic moments align along the magnetic field's axis, resulting in a macroscopic magnetization, whose magnitude is proportional to the number of spins. By applying a pulsed magnetic field, oscillating at the observed isotope's specific resonance frequency, the magnetization can be rotated into the plane perpendicular to the magnetic field. There, it will precess around the field's axis, inducing an electrical current in the receiver coil, where it is picked up as the NMR-signal, the so-called *free induction decay* (FID). The distribution of frequencies contributing to the FID can be analyzed by Fourier-transformation. The normalized frequency (*chemical shift*) is characteristic of the chemical environment of the nucleus, as for example the protons of a methyl group possess a chemical shift of about 1 ppm.

The main disadvantages of NMR analysis for flavorings are on one hand a significantly lower sensitivity compared to mass spectrometry and on the other hand the lack of on-line separation techniques for volatile organic compounds. Whereas ^{1}H-spectra with signal-to-noise ratios of > 150 can be achieved within a few minutes, i. e. less than 50 scans for pure substances, more than 2000 scans are necessary to provide corresponding ^{2}H-NMR spectra, since the relative low natural abundance of ^{2}H with only 0.0145% of the hydrogen atoms has to be taken into account. In consequence, analytical applications with NMR stable isotope analysis are restricted to ^{2}H- and ^{13}C-NMR of single flavoring substances or pre-separated, concentrated and purified extracts of flavorings or flavored products only. The main advantage of NMR is that this technique is non-destructive [20.49].

20.2.3 ^{2}H–Nuclear Magnetic Resonance

^{2}H-Nuclear magnetic resonance was initially applied to the detection and official control of grape must and wine chaptalization with beet sugar via the nonstatistical distribution of deuterium in different sites of ethanol. The *site specific isotope fractionation NMR* (SNIF-NMR) is an official analytical method of wine control since 1990 [20.16, 50, 51].

Activities to use ^{2}H-NMR for characterizing the origin and authenticity of other biomolecules were soon focussed on single flavor substances [20.10, 51]. However, quantitative ^{2}H-NMR is limited to the application of pure or purified flavor substances. As for instance in case of vanilla flavoring or vanilla flavored products, ^{2}H-NMR analysis is only possible after extraction, purification and recrystallization of vanillin and a rather long measurement period of several hours with more than 3000 scans [20.52].

Besides vanillin, specific ^{2}H-NMR analytical protocols and spectral data are actually available for flavor substances like anethol, benzaldehyde, estragol, thymol, different lactones, monoterpenes, and terpenoid substances as well as essential oils esters [20.12, 53].

20.2.4 ^{13}C–Nuclear Magnetic Resonance

Due to the fact that the relative sensitivity of NMR for ^{13}C is theoretically higher by two orders of magnitude as compared to deuterium, a quantitative evaluation for this nucleus actually has only been performed in scientific studies [20.54–56]. The results have shown that carbon isotope effects are much smaller than hydrogen isotope effects, and in consequence that the relative differences between given positions are expected to be lower. Furthermore, in contrast to ^{2}H-NMR, ^{13}C-NMR

requires longer relaxation delays and measuring time for quantitative analysis. Nevertheless, the main feasibility of site-specific quantitative ^{13}C-NMR has been shown and characteristic ^{13}C-distributions in natural and synthetic vanillin originating from different sources have been obtained [20.54, 55].

20.3 Selected Applications of Authentication of Odorants

Isotope ratio analysis is the key method in the authentication of genuine flavorings as well as individual flavoring substances in quality control and assurance in food industries as well as for consumer protection purposes. In this chapter, a selected number of important applications in food industry is presented.

20.3.1 Benzaldehyde

The essential oils of bitter almond are important flavors used in the food and beverage industry. Bitter almond oil, which contains high amounts of benzaldehyde, is most relevant in numerous fruit and beverage applications [20.57]. The essential oil of bitter almond is obtained by water steam distillation of bitter almonds, apricots or other prunus seeds and contains up to 90% benzaldehyde. In order to distinguish between the different sources of benzaldehyd, for example, obtained by the oxidation of toluene, the alkaline hydrolysis of benzal chloride or from cinnamomum cassia different isotope ratio analyses are conducted.

For example, the δ^{13}C-values for benzaldehyde of botanical extracts (-27.9 ± 0.4 ‰$_{\text{V-PDB}}$) and natural oils (-28.6 ± 0.7 ‰$_{\text{V-PDB}}$) fall within a narrower range, this suggests a similar process or the generation from a botanical precursor. The δ^{13}C-values for benzaldehyde obtained from chemical synthesis (via benzalchloride) are depleted in ^{13}C relative to the natural samples (-29.2 ± 0.8 ‰$_{\text{V-PDB}}$), whereas synthesized material (via toluene oxidation) is enriched in ^{13}C by nearly 2‰ with respect to the natural extracts (-26.1 ± 0.6 ‰$_{\text{V-PDB}}$) [20.58]. The carbon isotope abundances are obviously not indicative for the origin of benzaldehyde, but the hydrogen isotope abundances seem to be more typical. Benzaldehyde from natural sources shows a mean δ^2H-value of -125 ± 14 ‰$_{\text{V-SMOW}}$. δ^2H-values of synthetic benzaldehyde depend on the manufacturing process. Products synthesized from benzal chloride have δ^2H-mean values of -40 ± 21 ‰$_{\text{V-SMOW}}$ and those derived from catalytic oxidation of toluene $+777 \pm 20$ ‰$_{\text{V-SMOW}}$ [20.59].

An important natural source of benzaldehyde is cassia oil. At the moment, it is impossible to differentiate the product derived from cassia oil from natural benzaldehyde by means of δ^2H-values [20.12, 62].

These examples show that in order to differentiate benzaldehyde from different origin via GC-IRMS multielement analysis is required. Table 20.3 indicates the ranges of δ^2H-, δ^{18}O-, and δ^{13}C-values of benzaldehyde and Fig. 20.2a,b illustrates examples of a multiele-

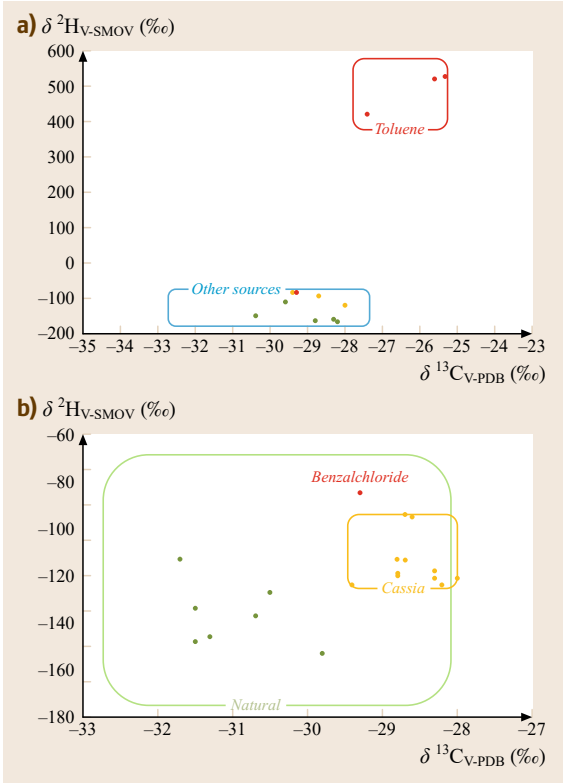


Fig. 20.2a,b Multielement IRMS analysis of different sources of benzaldehyde. **(a)** Gives an overview of benzaldehyde sources ex toluene (*red*) and other sources (*blue*), while **(b)** outlines the cluster for other sources as various natural sources (*green*), benzalchloride (*red*), and ex cassia (*yellow*) (unpublished data, Symrise AG)

Table 20.3 Ranges of δ^2H-, δ^{18}O- and δ^{13}C-values of benzaldehyde (after [20.12, 60])

Benzaldehyde	Origin	δ^2H ‰$_{V\text{-}SMOW}$	δ^{18}O ‰$_{V\text{-}SMOW}$	δ^{13}C ‰$_{V\text{-}PDB}$
	Bitter almond	−152 to −82	+6.3 to +19.3	−31.7 to −27.1
	Apricot kernel	−84 to −86	+8.7	−27.5 to −28.0
	Cinnamon cassia	−150 to −68	+2.2 to +18.1	−29.8 to −26.0
	Toluene	+380 to +802	+14.1 to +19.3	−28.6 to −24.6
	Benzalchloride	−78 to −11	+5.0 to +9.4	−30.4 to −26.4

Table 20.4 Relative ^{2}H-abundances in benzaldehyde (after [20.61])

Benzaldehyde	Origin	Relative ^{2}H-abundances at position			
		-CHO	*ortho-*	*meta-*	*para-*
	Bitter almond	0.82	0.76	0.77	1.00
	Apricot kernel	0.86	0.83	0.83	1.00
	Cinnamon cassia	0.91	0.95[a]	0.85	1.00
	Toluene	5.62	1.06	1.13	1.00
	Benzalchloride	1.12	0.92	0.96	1.00

[a] Unpublished data of Symrise AG number of samples $n = 7$

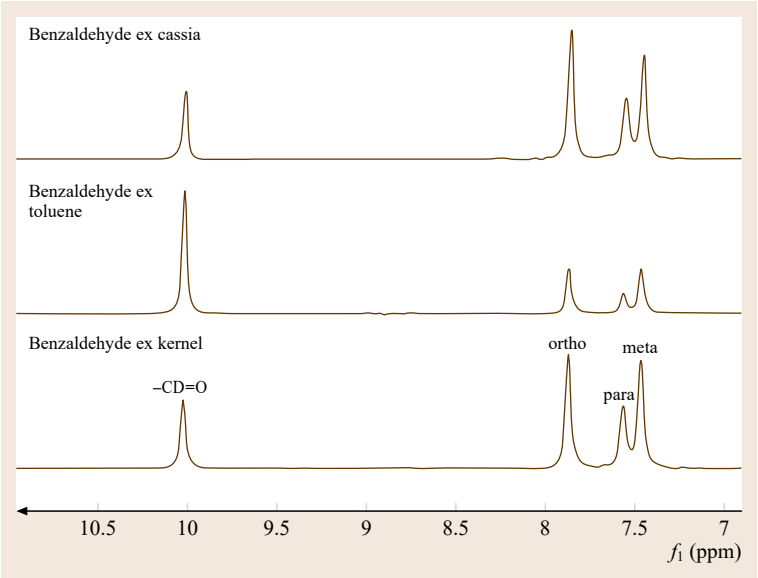


Fig. 20.3 Quantitative ^{2}H-NMR spectra of different benzaldehyde sources as cassia, toluene, and bitter almond kernel (unpublished data, Symrise AG)

ment IRMS analysis of benzaldehyde, exhibiting that ex toluene can be distinguished from the remaining sources. The combination of δ^2H- and δ^{18}O-values presented in Table 20.3 provides further indication of the origin [20.12, 60].

For the unambiguous assignment of the origin of benzaldehyde the complementary use of quantitative ^{2}H-NMR data is essential, which is also known as SNIF-NMR in specific applications. Benzaldehyde is synthesized via the biosynthetic shikimic acid pathway, which leads to a different relative abundance of ^{2}H at the *ortho–meta–para* position at the aromatic ring [20.30]. The ^{2}H-NMR provides a practical means of determining the site-specific distribution of deuterium in benzaldehyde as well as differentiating petrochemical, cassia, and bitter almond oil benzaldehydes [20.63]. Figure 20.3 presents quantitative ^{2}H-NMR spectra of different benzaldehyde sources and Table 20.4 gives the relative abundances of ^{2}H at different positions of benzaldehyde depending on the origin in natural and nature identical benzaldehyde [20.30]. The repeatability of the ^{2}H-NMR measurements of benzaldehyde and the capability for both providing and quantifying adulterations have been estimated [20.64]. An unambiguous assignment of the benzaldehyde source by quantitative deuterium NMR data seem to be feasible as the examples in the 3-D plot in Fig. 20.4 show.

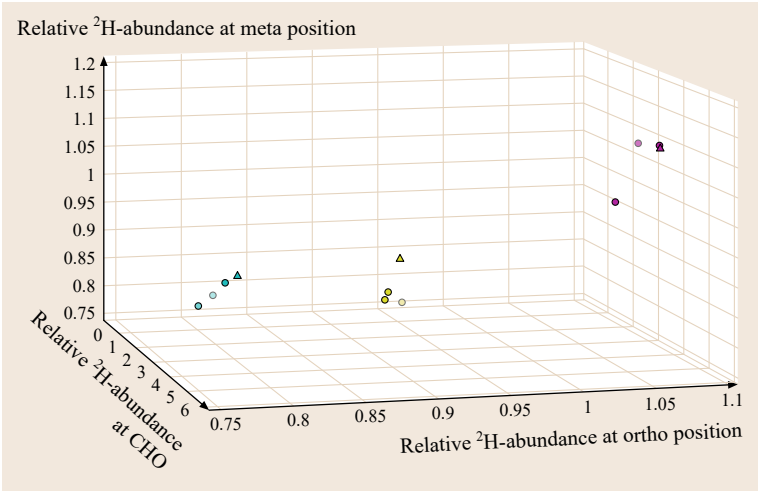
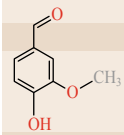


Fig. 20.4 Relative ^{2}H-abundances at different positions in benzaldehyde from different origins (*blue* ex bitter almond; *green* ex cassia; *purple* ex toluene; *triangle* symbol: literature values, *circles* unpublished data Symrise AG)

Table 20.5 Overview over the range of δ^2H-, δ^{18}O-, and δ^{13}C-values of vanillin (after [20.65])

Vanillin	Origin	δ^2H ‰$_{V\text{-}SMOW}$	δ^{18}O ‰$_{V\text{-}SMOW}$	δ^{13}C ‰$_{V\text{-}PDB}$
	Ex beans *Vanilla planifolia*		+6.7 to +12.4	−21.5 to −19.2
	Ex beans *Vanilla tahitiensis*			−19.7 to −15.9
	Ex beans	−115 to −52	+12.2 to +14.0 +8.1 to +10.7	−20.4 to −20.2 > −21.5 −21.5 to −16.8
	Ex guaiacol	−23 to −17	−3.1 to −2.5	−26.1 to −24.9
	Ex eugenol	−87	+11.8 to +13.3	−31.7 to −29.9
	Ex lignin	−204 to −170 −195 to −178	+6.1 to +6.8 +6.0 to +9.8	−28.7 to −26.5
	Ex ferulic acid ex rice bran	−168 to −165	+12.4 to +13.2 +10.7 to +11.2	−37.0 to −36.0 −36.4 to −33.5

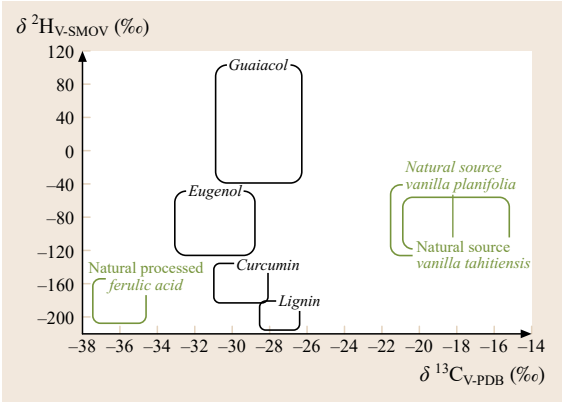


Fig. 20.5 Multielement IRMS range of value depending on the source of vanillin (unpublished data Symrise AG)

20.3.2 Vanillin, Vanilla Flavorings, Vanilla Extracts

Vanilla is an important natural flavor ingredient with major business relevance in the food and beverage industry [20.66, 67]. The worldwide production of natural vanilla extracts, commonly from vanilla beans of the species *Vanilla planifolia* and *Vanilla tahitensis* or *Vanilla pompona*, ranges at around 2000 metric tons per year while the consumption of vanillin (mainly in its synthetic form), the main flavoring ingredient in vanilla extracts, amounts to more than 12 000 metric tons per year. Due to the high demand and the relatively scarce availability, consumers' preferences for natural products and the dependence on the harvest of the natural crop, vanilla extracts command a high price that can vary considerably from season to season. Hence the differentiation of authentic vanillin from vanilla beans versus the inexpensive synthetic vanillin or other alternative natural sources converted into vanillin by biotransformation of lignin or ferulic acid is of high importance [20.65, 68]. For this reason, considerable effort has been directed toward the development of reliable methods for the detection of such adulteration. Among these efforts, the analysis of marker compounds and analysis of stable ratios of vanillin using isotopic ratio mass spectrometry or site specific natural isotopic

Table 20.6 Overview over the published δ^{13}C-values of vanillin and corresponding references

Source	δ^{13}C ‰$_{V\text{-}PDB}$ Mean value ± SD	Reference
Ex vanilla		
Vanilla planifolia		
Madagascar	−20.4 ± 0.2	[20.69]
Madagascar	−21.1	[20.66]
Java	−18.7 ± 0.4	[20.69]
Java	−19.8	[20.66]
Mexico	−20.3 ± 0.1	[20.69]
Different origins: Ex beans	−21.5 to −19.2	[20.70]
Vanilla tahitensis	−16.8 ± 0.2	[20.69]
	−19.7 to −15.9	[20.70]
	−18.5	[20.66]
Other sources		
Ex eugenol (clove oil)	−30.8	[20.69]
Ex guaiacol	−32.7	[20.69]
Ex lignin	−27.0 ± 0.2	[20.69]
Ex lignin	−27.3	[20.66]

fractionation by nuclear magnetic resonance or techniques play an important role [20.65].

Table 20.5 gives an overview over IRMS δ^{2}H-, δ^{18}O-, and δ^{13}C-values of vanillin from different origins. Hoffman and Salb have published δ^{13}C-values of vanillin from most of the major growing areas and of vanillin from all three other sources (Table 20.6) [20.69]. Most vanillin qualities from other sources yield δ^{13}C-values more negative than −27.0 ‰$_{V\text{-}PDB}$. As a result of their experience, Hoffman and Salb set a limit of −21.0 ‰$_{V\text{-}PDB}$ as a standard of identity for natural vanillin. Any vanillin that gives a δ^{13}C-value more negative than −21.0 ‰$_{V\text{-}PDB}$ must be considered to contain vanillin from a source other than vanilla beans. Figure 20.5 shows the multielement analysis of numerous samples of vanillin from different sources and gives a good impression of how potent the IRMS analysis for the authentication of vanillin is representing different range of values depending on the origin of vanillin.

Another powerful tool for the authentication of vanillin is the quantitative ^{2}H-NMR spectroscopy. A results of a collaborative study performed with pure vanillin showed that the method allows a satisfactory discrimination between different vanillin

Table 20.7 Relative ^{2}H-abundances in vanillin (after [20.61])

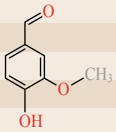
Vanillin	Origin	Relative ^{2}H-abundances at position			
		−CHO	*ortho-*	*meta-*	-OCH$_3$
	Ex beans	0.67	0.80	1.00	0.65
	Ex lignin	0.63	0.77	1.00	0.65
	Ex eugenol	0.73	0.84	1.00	0.71
	Ex ferulic acid	0.84	0.88	1.00	0.85
	Ex guaiacol	2.30	0.95	1.00	0.99

Fig. 20.6 Quantitative ^{2}H-NMR spectra of vanillin from different sources as ferulic acid, guaiacol, lignin, and from vanilla beans (unpublished data, Symrise AG)

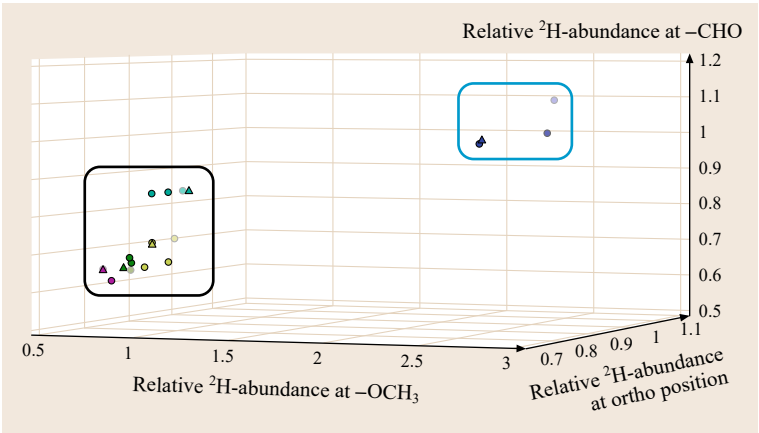


Fig. 20.7 Relative ^{2}H-abundance at different positions in vanillin originating from different sources. An overview including ex guaiacol in *dark blue* and other sources in the *black circle* (*purple* ex lignin; *green* ex bean, *light green* ex eugenol; *light blue* ex ferulic acid; *blue triangle* symbol: literature values, *circles* unpublished data Symrise AG)

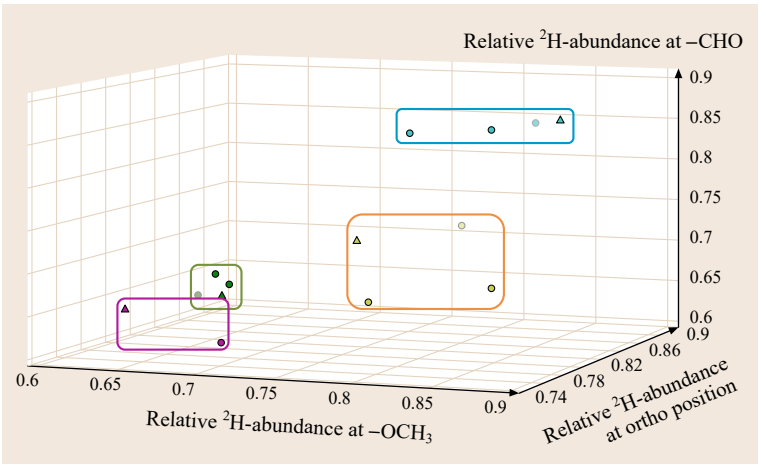
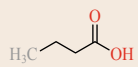


Fig. 20.8 Relative ^{2}H-abundance at the different positions in vanillin originating from different sources (*purple* ex lignin; *green* ex bean, *orange* ex eugenol; *light blue* ex ferulic acid; *triangle* literature value, *circles* unpublished data Symrise AG)

sources [20.71]. The relative ^{2}H-abundances in vanillin at different positions are presented in Table 20.7, while Fig. 20.6 shows quantitative deuterium NMR spectra from different vanillin sources. Vanillin from ex guaiacol sources are easy to distinguish from natural and other source vanillin due to the high relative ^{2}H-abundance of the aldehyde as the overview of several quantitative deuterium NMR experiments in Fig. 20.7 illustrate. But also the clustering of different vanillin sources in the 3-D plot in Fig. 20.8 indicates a potential differentiation of the remaining origins.

Table 20.8 Range of δ^2H- and δ^{13}C-IRMS values of butanoic acid (after [20.72])

Butanoic acid	Origin	δ^2H ‰$_{V\text{-}SMOW}$	δ^{13}C ‰$_{V\text{-}PDB}$
	Other source butanoic acid	+63	−22.9
	Natural butanoic acid	−232	−14.5

20.3.3 Butanoic Acid

Butanoic acid is found in numerous milk products and especially the triglyceride of butanoic acid is used in many food and feed applications. The hydrolysis of the triglyceride and the release of butanoic acid itself are responsible for the unpleasant odor of rancid butter for example. Butanoic acid as educt for the production of various butyrate esters is manufactured for industries by fermentation of sugar or starch as natural sources or by synthesis.

Table 20.8 shows the δ^2H- and δ^{13}C-values of butanoic acid as it was found in the research of the Isotopic Studies Committee [20.72]. Emad Ehtesham just recently showed that the δ^2H- and δ^{13}C-values of four fatty acids (C4:0, C14:0, C16:0, C18:1) and bulk milk powder were found to be correlated with the regional production area of milk powder in New Zealand. In

Table 20.9 Range of δ^2H-, δ^{18}O- and δ^{13}C-values of carvacrol in origanum oils (after [20.73])

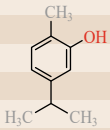
Carvacrol	Origin	δ^2H ‰$_{V\text{-}SMOW}$	δ^{18}O ‰$_{V\text{-}SMOW}$	δ^{13}C ‰$_{V\text{-}PDB}$
	Authentic origanum oils	−262 to −306	+15.3 to +20.1	−25.2 to −28.5
	Commercial origanum oils	−162 to −294	+12.1 to +18.5	−24.7 to −30.2
	Other source standards	−106 to −262	−13.0 to +19.5	−26.1 to −28.3
	Authentic savory oils	−274 to −295	+13.0 to +16.5	−25.1 to −28.9
	Commercial savory oils	−266 to −280	+14.6 to +15.4	−24.8 to −26.8
	Other source savory standards	−10 to −262	−13.0 to +19.5	−26.1 to −28.3

Table 20.10 Range of δ^2H- and δ^{13}C-values of γ- and δ-Lactones from different origins (after [20.74])

γ-Decalactone	Origin	δ^2H ‰$_{V\text{-}SMOW}$	δ^{13}C ‰$_{V\text{-}PDB}$
	Other source	−184	−28.3
	Other source	−151	−27.4
	Natural	−214	−28.1
	Natural	−230	−29.2
	Natural	−247	−29.2
	Natural	−192	−28.3
	Biosynthetic	−286	−29.0
δ-Decalactone		**δ^2H ‰$_{V\text{-}SMOW}$**	**δ^{13}C ‰$_{V\text{-}PDB}$**
	Other source	−171	−28.2
	Natural	−203	−30.1
	Natural	−230	−27.7
	Biosynthetic	−185	−29.3

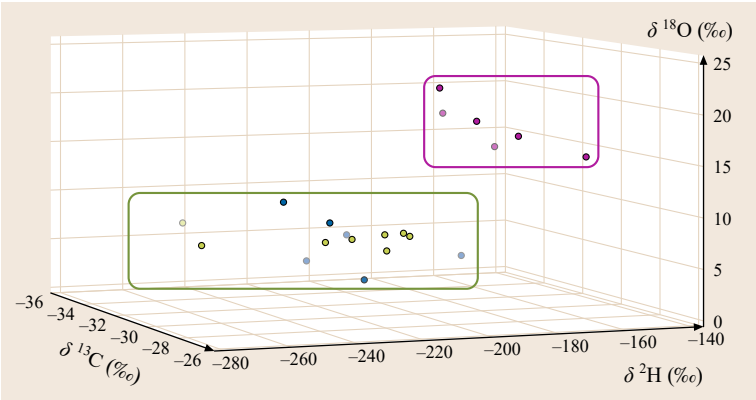


Fig. 20.9 Multi-element IRMS analysis of different sources of γ-decalactone (*purple*: other source, *blue* and *green*: natural source; unpublished data Symrise AG)

this study, the δ^{13}C-value of butanoic acid was found to cover a wide range of −31.0 to −42.5 ‰$_{V\text{-}PDB}$, while the δ^2H-values showed a range between −130 and −170 ‰$_{V\text{-}SMOW}$ [20.75].

20.3.4 Isoprenoids and Essential Oils

Isoprenoids are widely spread in nature and comprise commercially important compound classes like mono, sesqui-, di- and triterpenes, iridoides, carotinoides, and steroides. There are numerous reviews citing important criteria for the quality control of isoprenoid compounds in essential oils [20.12, 76, 77].

Respectively, isoprenoids are produced in different compartments of cells and bacteria via the precursors mevalonic acid and deoxyxylulosephosphate [20.27]. For these reasons, the expected global δ^{13}C- and δ^2H-values show significant differences depending on the corresponding biosynthetic pathway [20.30, 78, 79]. In this chapter, the analysis of origanum oil and the main ingredient carvacrol is discussed based on Multicomponent-/Multielement-IRMS data. The enantiomeric distribution of the chiral minor compounds of the essential oil like pinene, limonene, and terpinen-4-ol can be achieved via chiral gas chromatography [20.73, 80]. The enantiomeric distribution of the minor compounds provides a first insight. A potential adulteration of the essential oil, based on the addition of the main value adding ingredient carvacrol is highly relevant.

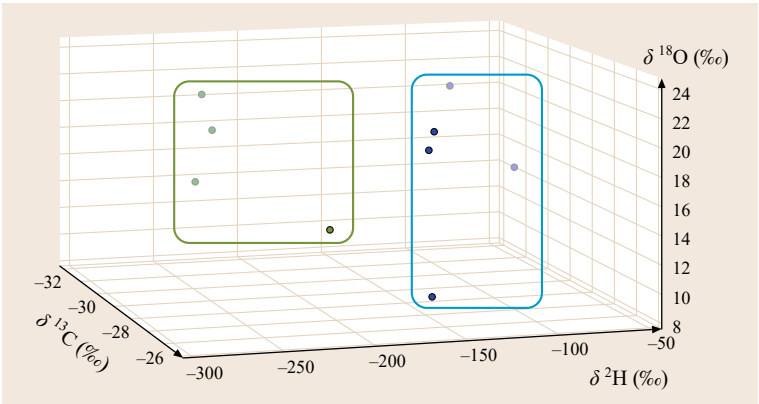


Fig. 20.10 Multi-element IRMS analysis of different sources of δ-decalactone (*green*: natural source, *blue*: other source; unpublished data Symrise AG)

Table 20.9 presents the ranges of δ^2H-, δ^{18}O-, and δ^{13}C-values of the aromatic monoterpene alcohol carvacrol in origanum oils [20.73]. This example illustrates that 3-D multielement IRMS analysis does not provide a complete resolution of all commercially relevant sources in this specific case.

20.3.5 Fruit Flavors (γ- and δ-Decalactones)

A prominent example for the authenticity control of natural flavorings in food products is the application of IRMS analysis of γ- and δ-decalactones. Chiral γ- and δ-decalactones are flavoring substances with high sensory importance for various fruit flavors with strawberry, raspberry, peach, apricot, mango, passion fruit, plum, and coconut profile [20.81]. The authenticity of the natural sources of γ- and δ-decalactones can be accessed by their enantiomeric composition [20.82]. Several synthesis and biosynthesis routes have been established to create these flavoring substances [20.83–87].

In the study of *Tamura* et al., γ- and δ-decalactones were analyzed in fruits of *Prunus* species, for example, peach, apricot, and nectarine from different geographical origin [20.74]. The results of the study and the data shown in Fig. 20.9 illustrate that a differentiation between the different species cannot be achieved using δ^2H and δ^{13}C IRMS data. On the other side, samples from natural and other sources show significant differences (Table 20.10). *Tamura* et al. also reported IRMS data for γ- and δ-decalactones from biosynthetic origin [20.74]. The presented data for γ-decalactones (Fig. 20.9) show within the given scope of natural variation a differentiation of other source material (purple box) from the samples produced via biosynthetic route. The distinction between natural and biosynthetic origin (green box), however, seems not to be possible in this case. δ-Decalactones from other source origin (blue box) and natural sources (green box) can be distinguished as shown in Fig. 20.10.

20.4 Requirements and Guidelines for Authentication

Stable isotope ratio analysis is the method with high relevance for the quality control in flavor industry. In official food control laboratories, SIRA is mostly used in specific cases, if suspicious facts are evident or suspicious findings have to be confirmed. The application of SIRA for authentication of flavorings in official food control finds its limitations in the fact that primarily flavored products have to be analyzed in food, which always requires rather elaborate sample clean-up techniques for the final characterization of flavor substances via SIRA. In both responsibilities, the check for purity of a single flavor substance or the qualitative and quantitative composition of a complex flavoring is determined using GC, GC-MS, and high pressure liquid chromatography (HPLC) at the beginning of a flavor analysis. For the investigation of the natural status of a flavoring substance, additional methods like enantioselective analysis of chiral flavor compounds are used. However, in order to get a definite proof of authenticity, SIRA is the only method of choice.

The use of stable isotope data for assessment of authenticity of flavor substances and flavorings has to meet the same requirements as for the conventional

analytical data of ingredients of natural materials. Validated analytical techniques, reliable and representative reference samples and validated data are the basis for the assessment as well as mathematical, statistical, and multivariate methodologies for calculation and evaluation of the relevant analytical parameters with regard to a final significant result and assessment of authenticity. Examples for corresponding statistical and multivariate procedures are discussed by example of authentication of wines using stable isotope data or elements [20.88–90].

20.4.1 Analytical Requirements

The sophisticated methods of SIRA require high efforts in isolation and analytical instrumentation thus the following essentials are important for acquisition of reliable and comparable stable isotope data:

- Stable isotope fractionation and discrimination has to be avoided during isolation, concentration, and measurement of a flavor substance.
- Validated procedures of SIRA for the target flavor compound with defined analytical data of uncertainty and reproducibility must be available.
- Participation in ring tests or proficiency tests including the isolation from the representative matrix is indispensable.

20.4.2 Requirements of Representative Reference Data

Isotope patterns of a natural flavoring source and its flavor substances are characterized by the natural variations due to the biotic and abiotic fractionation, and biosynthetic effects. Further effects are different processing and isolation techniques of flavorings or single flavorings from the source materials, especially if physical, enzymatic, or fermentative processes are involved. Since the evaluation of analytical data from natural sources is almost based on reference data, the following aspects have to be taken into consideration:

- Stable isotope data of the flavor compound originating from authentic reference samples must be available.
- The meta data of the sample to be analyzed and reference sample(s) like source, botanical cultivar or variety, geographic origin, year, or even perhaps the time of harvest need to be available as accurate as possible.
- The significance of the assessment depends on the question whether sufficient representative and validated data are available, preferably in a validated data bank.

20.4.3 Guidelines for Interpretation and Evaluation of Isotope Data

The authentication of a flavor substance or a flavoring by SIRA has to provide convincing evidence in a court case and therefore should be based on the following guidelines for interpretation and evaluation:

In case of a single stable isotope ratio of a single flavoring substance, it has to be evaluated for its genuineness and first of all be checked, whether the isotope value or ratio is within the known and expected range of reference samples or data published in literature. If the value outranges the expected natural range, the value can be assessed as a so-called *cut-off value* for which no extensive reference material is necessary. As an example, a δ^{13}C-value of $-35\,‰_{\text{V-PDB}}$ for vanillin can be assessed as a cut-off value for genuine vanillin from vanilla bean, since the minimum δ^{13}C-value is $-23\,‰_{\text{V-PDB}}$ (taking into account uncertainty of measurement and preparation). If the value is near the authentic range the variation of the natural distribution should be investigated. The confidence interval of the reference value depends on the number and standard deviation of the data available. In addition, uncertainty of the measurement has to be taken into consideration as well.

Using the multielement approach, typical correlations can be used for authentication of a single flavor substance. The combination of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) and gas chromatography-pyrolysis-isotope ratio mass spectrometry (GC-P-IRMS) is applied to the authenticity assessment of flavor compounds from various sources. The combination of on-line capillary GC-C-IRMS and GC-P-IRMS to determine δ^{13}C- and δ^{18}O-values of estragole and methyl eugenol showed that the δ^{18}O-values enabled differentiation even if δ^{13}C-values of products from natural and synthetic origin are rather similar [20.91]. By correlation of the δ^{2}H- and δ^{13}C-values of cinnamaldehyde, characteristic authenticity ranges were defined for Ceylon, cassia, and wood cinnamon [20.92]. An important substance in raspberry flavor is α-ionone which is available as natural, natural-identical, and biotechnologically produced compound. The δ^{13}C-value of natural α-ionone is -29.0 to $-35.1\,‰_{\text{V-PDB}}$, of the natural-identical -24.3 to $-27.1\,‰_{\text{V-PDB}}$ and of the biotechnologically produced -10.2 to $-31.7\,‰_{\text{V-PDB}}$. The δ^{2}H-values of natural (-190 to $-214\,‰_{\text{V-SMOW}}$) and biotechnological (-205 to $-296\,‰_{\text{V-SMOW}}$) α-ionone are more negative than the

δ^2H-values of the natural-identical α-ionone [20.93]. A combination of δ^2H- and δ^{13}C-values allows a secure proof of the origin of α-ionone.

Using δ^{13}C-, δ^{18}O-, and δ^2H-data of the multielement analysis as well as enantioselective multidimensional GC-MS of linalool and linalyl acetate, a differentiation of the synthetic and natural compound is possible as well as an authenticity assessment of lavender oil [20.94].

The isotope ratio analysis provides multielement as well as multicomponent data, which provides information about the authenticity of a flavoring with much more higher significance, even with a limited number of reference samples available.

20.5 Conclusion

The authenticity control of natural flavoring substances, natural flavoring preparations and in particular essential oils must be focused on two main areas. The authentication of the source material and the verification of the application of legally permitted production methods.

In both areas, the pattern of stable isotopes like ^{13}C-, ^{2}H-, and ^{18}O-values of the source materials allow in many cases the assignment to source specific multielement data clusters, which provide a more detailed differentiation than the assignment to the pool of C_3-, C_4-, and CAM-plants only. In this context, the availability of authentic reference compounds is of utmost importance and will require additional studies in the next years in order to capture the full spectrum of potential source materials.

The second focus area of process-specific indicators requires also high efforts, since natural flavor compounds may be produced either by extraction and isolation of natural sources, by microbiological and enzymatic (biosynthetic) procedures, or by appropriate physical and traditional food preparation processes. Thus it is necessary to fully understand all process-derived isotope shifts and subsequent modifications of the stable isotopes patterns. In order to achieve these aims, a close scientific cooperation between academia, industry, and government authorities will be required.

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21. Machine Olfaction

Brian Guthrie

It has been a longstanding goal of many research groups to replicate human olfactory sense with instruments. Sensor technology aims not only to replace the traditional analytic methods that are mostly focused on individual chemical identification and quantitation, but also to predict the human perceptions of smell, odor recognition and odor hedonics, thus replacing human sensory evaluation. Sensors have progressed from early gas sensors, to e-noses and e-tongues to biosensors and bio-e-noses that utilize elements from natural signal transduction to gain better sensitivity and selectivity. There has recently been a rapid increase in research and development of advanced sensor technologies and enabling technologies such as nanotechnology, cellular biology, wireless communication, and neural computing methods that have helped overcome the sensitivity, selectivity, portability and recognition problems of early sensor systems. Much of this development comes in response to global bioterrorism and other security threats. The activities in the various areas enabled by machine olfaction are poised to impact many industries not only as potential enablers of competitive advantage, but also through international standards development and enforcement. However, while machine olfaction instruments and sensors systems have been under development for more than 30 years, they still cannot completely replace the human senses for sensitivity, selectivity, and speed. While complete replacement of human sensory perception is not yet possible, certain sensor arrays provide fast, cheap, portable, networkable, low-expertise alternatives in some applications where simple detection is required. Nevertheless, current machine olfaction devices can provide a low-sample preparation approach that significantly reduces the amount of human sensory and advanced chemical testing needed.

Machine olfaction is the instrumental replication of the human olfactory sense. Many research groups have worked in this area since the applications for machine olfaction are extremely broad. The instruments, sensors and approaches for machine olfaction, or artificial olfaction, have been reviewed by *Sankaran* et al. [21.1] and *Bartlett* et al. [21.2]. Many of these will be discussed below with a particular focus on agriculture and the food industry.

There are many types of sensors, each with individual strengths and weaknesses. In general, compared to the traditional analytical methods or human sensory

testing, they can be faster, lower cost (instrument, sample preparation), require lower expertise, and can be portable and networkable. The emergence of biosensor technology has significantly increased the selectivity and sensitivity of electronic sensors. The technologies that enable these devices are still improving.

There is evidence that the leaders in the food industry and other industries have begun to incorporate instruments for machine olfaction into normal operations and research and development efforts. For example, industrial researchers reported e-nose applications in spices and flavorings [21.3], clinical diagnosis [21.4], food quality control (QC) [21.5], body odor sensors for personal care applications [21.6], bad breath sensors [21.7], as well as gas sensors and ambient mass spectrometer-based sensor systems, across several applications, such as milk analysis, vitamin analysis, process monitoring, rapid analytics, packaging analysis, and sensory testing replacement [21.8]. They report the use of proton transfer reaction and laser mass spectrometer (MS) e-nose systems for the detection of oxidation in infant formula [21.9], for process monitoring in coffee roasting [21.10, 11], milk quality assessment [21.12], detection of contaminants [21.13, 14], volatile release [21.15], off flavor and packaging taint analysis and quality assessment of cocoa liquor to name a few [21.16–19]. The needs in many broad application areas will surely continue to drive the development of sensor technology and data analytics that enable machine olfaction.

21.1 Chemical Senses

The chemical sense of taste (gustation) and smell (olfaction) and their interaction (flavor) are highly evolved and extremely sensitive. Humans use these senses to find acceptable food, to recognize mating partnerships, to detect disease and many other things that are vital to survival. These senses are especially important in industries related to food and food quality. *Sankan* et al. [21.1] and *Dymerski* et al. [21.20] reviewed the cell biology of olfaction and gustation.

21.1.1 Olfaction

Several reviews of biological olfactory processes can be found in the literature [21.21–24].

If one were to break the cell biology of olfaction down and express the various subprocesses in terms of instrumental counterparts, the following elements would be represented:

- Two sampling points (*ortho*- and *retro*-nasal olfaction, with *retro*-nasal olfaction providing dynamic, repeated sampling during eating)
- A preconcentration/absorbent system (olfactory binding proteins)
- Rapid clearing, broadly tuned sensor array (mucus, olfactory receptor proteins, olfactory neurons)
- A self-calibrating signal processor and pattern recognition system [21.25].

These elements are often targets for instrumental reproduction as elements in the electronic sensor systems.

21.1.2 Gustation

Recently, large advances have been made in the understanding of gustatory processes from the identification of receptors at the periphery to the integration of gustatory signals with other signals in the cortex and other regions of the brain. These have been extensively reviewed [21.26–30].

The processes of gustation are similar in the most part to olfaction. Gustatory information is however more closely associated with information from oral processing and trigeminal information. Also, gustatory sensation is compiled with much more information, such as nutritional status, than olfactory information before it reaches the cognitive processing centers of the brain. These elements, that are unique to gustation, are difficult to imitate with artificial taste sensors.

21.1.3 Sensory Perceptual and Cognitive Processing

After many years of research, the mechanisms involved in olfactory perception and cognition are still not well known. The rules that govern the perception of chemical mixtures are even less understood. Brain activation studies are only recently starting to reveal some of the information processing and connections that occur in the brain [21.31, 32]. While rapid advances in the understanding of these processes are expected, it is currently not completely possible to build biomimetic data processing models directly for artificial olfaction and gustation.

21.1.4 Instrumental Surrogates for Human Chemical Senses

Information Goals

Analytics and sensor systems strive to reproduce portions of, or complete, human olfactory systems. It is

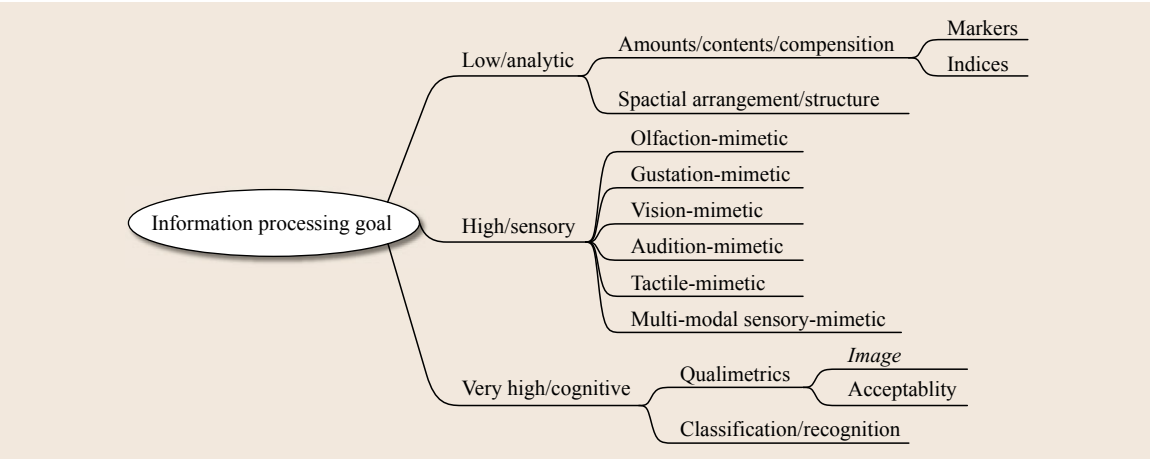

Fig. 21.1 Proposed information processing scheme of analytical and sensor systems

helpful to understand the information generated by each. The information processing goals of these methods are outlined below in Fig. 21.1.

Many analytical methods that are used for quality control and trade specifications are focused on the determination of the amounts of certain chemicals, marker chemicals, compositions, or some indices of quality that are well known. These assays can also be focused on the determination of the chemical structure of target compounds or other spatial information. These methods are considered to have somewhat low information goals relative to human perception of quality and class. They have been validated where single compounds or sets of compounds have shown a good correlation with human sensory perception. For example, hexanal is often used as an analytical marker for the human perception of rancidity, or low quality (nontypical sensory impressions). However, satisfactory simple analytical level predictors of human perception are rare.

A generation of sensor systems have attempted to serve as instrumental replacements for individual senses, called here *sense-mimetic*. These were the early e-nose and e-tongue instruments focused at reproducing the selectivity and sensitivity of human olfaction and gustation respectively. However, this high sensory information goal was rarely met. This is not surprising given that the transduction of information in the human systems is only poorly understood today and human senses do not act in isolation.

Researchers next attempted to develop systems with the very high information goals of replacing human perception and cognition, representing an even more challenging task. Pattern recognition procedures were first attached to the early within-mode sensors and sensor arrays. The next step to achieve a high level of information processing followed discoveries in human psychophysics with a greater array of sensors being mixed with even more types of signal processing, pattern recognition and information theoretics being applied. These systems often included e-nose sensor arrays plus e-tongue sensor arrays and other sensor types to mirror the data and thought processes used by humans to make determinations such as *quality*, *class*, and *acceptability*.

One could argue whether it is rational to attempt such a high information goal with instruments since the principles of human sensory perception and recognition of mixed chemical stimuli are not well understood [21.33–36] thus tuning of these systems cannot proceed from first principles but must be generated from data-driven empirical observations with the usual data analytic problems of overfitting, low generalization of results to other systems or species, etc. Only recently have *odor spaces*, or a comprehensive understanding of the multidimensional *tuning* of odor quality, intensity and duration, been explored for lower organisms such as flies [21.37]. In this work, researchers used Drosophila antenna responses to 100 odorants to establish an *odor space* for this organism. Such an understanding of the human odor space, along with the added understanding of human cognitive processing of chemical stimulants, would be required to develop machine olfaction systems with high-level information processing capabilities such as the prediction of food quality.

The ideal system for the replacement of the human chemical senses would include the following items listed below. Some artificial systems are more successful than others. The ideal sensor system would:

- Handle sampling and sample preparation and/or concentration

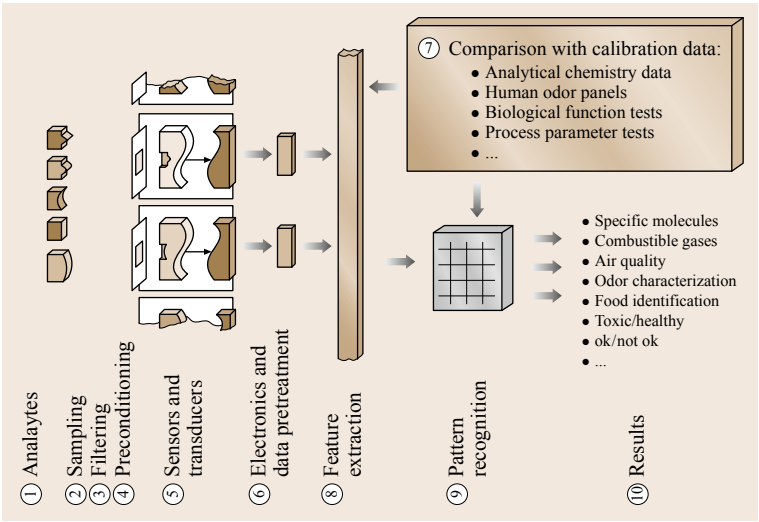

Fig. 21.2 Typical sensor system setup (after [21.38])

- Have fast responses or data acquisition and data processing response times
- Have rapid pattern recognition and classification capabilities
- Have sensors that are rapidly reset with little drift and not be troubled by high levels of interfering compounds
- Would be portable with low energy consumption and networkable
- Would be of low cost, but achieve long life.

Systems with characteristics relating to each of these features are discussed further below.

21.1.5 Machine Olfaction

Many research groups have worked to replicate human olfaction. The sensors and approaches for machine olfaction, or artificial olfaction, have been reviewed by *Sankaran* et al. [21.1] and *Bartlett* et al. [21.2]. Typical sensor arrangements, whether for e-nose or e-tongue, are shown below in Fig. 21.2 [21.38].

Most systems begin with elements for sample introduction, often with preconcentration or filtering. Analytes then interact with sensors or arrays of sensors. There are a large number of possible sensors and combinations. These will be discussed further below. The sensors transduce the interactions of the chemicals into electric signals that are usually pretreated before further signal processing. Data systems, after extensive calibration, will extract the important features of the signals and then, with the use of pattern recognition algorithms, attempt to interpret the sensor response. The interpretation that is desired is captured by training a pattern recognition system using either supervised classification (*good* versus *bad*, location 1 versus location 2, age 1 versus age 2, etc.), unsupervised classification (sample groupings) or detection (below a certain level) approaches.

It should be noted that the calibrations require large numbers of samples and must include all known interferences or contaminants. Initial calibrations can take a lot of effort to establish, and they take a long period of maintenance and adjustment until all noise can be tuned out of the calibrations.

However, researchers have found that machine olfaction can only work, considering the current sensor technology, for a limited set of conditions. *Rock* et al. [21.38] developed a scheme shown below to capture this (Fig. 21.3).

According to this scheme, the ideal case for reliable measurement is when the volatiles responsible for the odor of interest are in high concentration while the odorless or interfering volatiles are not (case 1). If the odorless or interfering volatiles are correlated with the odorous volatiles, then a limited odor measurement is possible (case 2). Otherwise, odorless volatiles can mask the target volatiles (case 3) and no odor measurement is possible. If odorless volatiles can be masked (case 4), then odor measurements are possible. For trace levels of odor volatiles, a limited odor measurement capability is possible if they are correlated with odorless volatiles (case 5), if not (case 6), odor measurement is not possible. Much of the work, when establishing a sensor-based method, is to determine the situation for the case under development. However, it is well known that key food volatiles are usually present in only trace amounts. Therefore, the research is usually focused on the extent of correlation to odorless volatiles (e.g., case 6 or case 5) and the extent of the odor

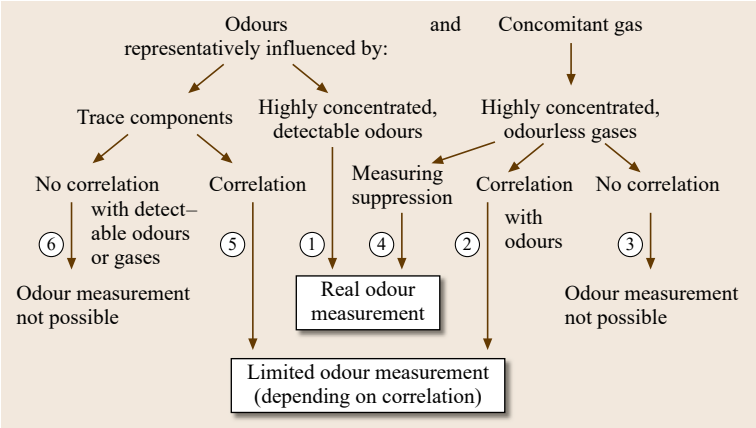

Fig. 21.3 Scheme showing possible conditions for reliable odor measurements (after [21.38])

measurement possible given the trace levels of target analytes.

Quantitative odor–structure relationships have been found [21.39]. Human odor spaces and primary odor dimensions have been hypothesized. However, these have not led to the development of better machine olfaction systems. Odor perception has been found to be more of a function of perceptual processing rather than receptor events at the periphery of the nervous system.

21.1.6 Chemical Senses Perception–Cognition Coding

The neurobiology involved with the transduction and coding of the chemical senses is critical in the development of instrumental sensor systems that reproduce the highest information capabilities of the human chemical senses. *Hoare* et al. [21.40] hypothesized that flies use fuzzy coding in olfaction. Fuzzy methods are being used today as a part of the data analytics used with several sensor systems discussed below. *Haddad* [21.41] reviewed the information processing involved in the neurobiology of human olfaction where the physical space of odorant molecules is transformed through a neural space of information processing, and then into a perceptual space of smell. They reported that odor measurement metrics significantly hinder an expanded understanding of human olfactory coding.

While the exact information processes are not known, many data analytic methods attempt to imitate the neural processing found in the human chemical senses to replicate perceptual and cognitive processes in humans.

21.2 Sensor Types

21.2.1 Overview

The different sensors used in machine olfaction and gustation and rapid food analysis are described below. The different types can include simple gas sensors that have either single or multiple reactivity (*cross-reactivity*) to detect single or multiple gases and vapors. These consist of high temperature sensors with reactive surfaces and low temperature polymeric sensors that can have adsorptive coatings for increased selectivity. Optical gas and vapor sensors consist of probes coated with arrays of different dyes that produce colored response patterns that are linked with wave guides or optical fibers to detectors where patterns can be recorded and converted to a response. Gas and vapor sensors can also utilize ionization with mass separation and detection as a type of sensor array.

Liquid sensors exist that include various types of electrodes and spectrophotometric devices. More recently, a large number of biosensors have been developed for liquid samples that consist of combining binding elements found in nature (DNA, RNA, enzyme, immunoglobulins, antibodies, proteins, etc.), giving high selectivity, with a micro- or nanoscale optical or electronic transduction system that provides high sensitivity.

It is important to note that, like the chemical senses, the goal of sensor systems is to generate a pattern or fingerprint that can be recognized, rather than traditional analytic chemistry that is to quantitate individual chemical species. To that end, sensor systems have evolved using arrays of single or multiple types of sensors as a way to capture the most diverse pattern possible to increase the chances of successful recognition in data

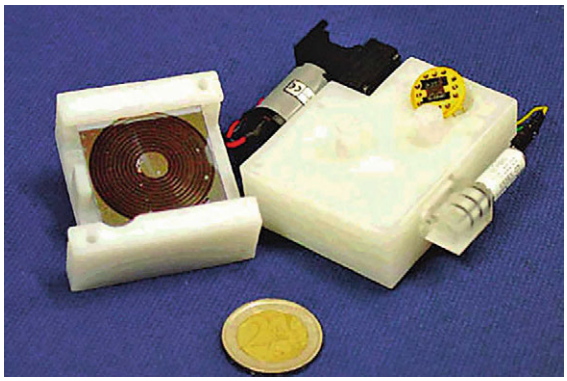

Fig. 21.4 Example of a portable hybrid microsystem GC sensor system: zero grade air unit, GC column, MOX sensor (after [21.42], Copyright 2005 Elsevier)

analysis. Thus, many sensor systems today contain mixtures of different types of sensors arranged as arrays.

Early and significant problem areas for sensor systems involved sample concentration and miniaturization and portability. In response to these problems, more recent sensor system designs commonly combine sensors and arrays with sample introduction systems for collection and concentration, such as microfluidic devices, with sensors and sensor arrays in a microsystem format. One manufacturer has designed a complete gas chromatography system with a metal oxide (MOX) detection system that fits in one hand and can run on batteries [21.42] (Fig. 21.4).

Each of these sensor types will be discussed in further detail below.

In addition to sensor-based systems, methods for nondestructive food testing have been developed and are frequently reported in the literature. These tend to utilize spectral detection in combination with pattern recognition. High selectivity was reported using methods such as nuclear magnetic resonance (NMR) imaging, near-infrared spectroscopy (NIR), mid-infrared spectroscopy (Mid-IR) and fluorescence. One example of such an approach was reported by *Lin* et al. [21.43] who used NIR and support vector machines (SVM) to determine the freshness of eggs. These methods are outside the scope of this review and will only briefly be discussed further below.

21.2.2 Chemical Sensors

The early chemical sensors were designed for detection of single gas vapors or for single components of liquid systems and not initially for pattern recognition. Many of these are found in the literature. *Nanto* [21.44] reviewed the various types of chemical sensors. These were the original types of sensors used in first generation e-nose instruments [21.45]. A summary of the types and chemical sensors is shown below in Table 21.1.

Cross-reactive gas sensors were developed to generate complex patterns for better selectivity of materials with complex odors from mixtures of volatile chemicals. *Albert* et al. [21.46] reviewed cross-reactive gas sensors. These sensors are designed to react differently when exposed to different odorants. A common design for sensing volatiles involves the use of arrays of thin-film cross-reactive chemoresistive vapor sensors. One type is called a carbon-black polymer composite (CBPC) sensor. The group of Nathan Lewis from Caltech has been very active in the development of these types of sensors. These commonly utilize different types of polymers in the sensor array, such as poly-(ethylene-vinyl acetate) (PEVA), each with carbon black additives for conductivity. The conductivity of each polymer changes differently as it absorbs vapors and swells. Arrays with different polymer components allow the generation of a spatiotemporal conductivity pattern [21.47]. These have been designed for specific sensing applications such as fatty acid sensing [21.48] and many other applications. Arrays have been compared to human olfaction [21.49]. The CyranNose e-nose system was developed using arrays based on these sensors with chemometric pattern recognition analysis [21.50].

21.2.3 E-Nose

Electronic nose (e-nose) technology has been under development for close to 30 years. Several families of sensors have been developed and then arrays of different sensors have been assembled to better approximate the sensitivity and selectivity of human olfaction. Recently, e-nose technology has been reviewed for safety and quality determinations in food, and pharmaceuticals [21.20, 51–58], applications in agriculture and forestry [21.59, 60], developments in sensor and instrument technology [21.61–68], and advances in data processing and analysis [21.69].

Many different e-nose platform technologies have evolved over the last 30 years of development. Most evolutions and platforms were attempts to make improvements from the previous technology. Inexpensive electrical sensors, such as gas metal oxide semiconductor (MOS) and metal oxide semiconductor field effect transistors (MOSFET), and gravimetrical sensors, such as surface acoustic wave, bulk acoustic wave and microcantilever systems, were used in the first e-nose instruments. The electrical sensors required high temperatures and had significant drift. Their performance was also significantly impacted by vapor interferences

Table 21.1 Overview of Chemosensors (after [21.44]). Classification of chemosensors that have been exploited so far. Metal oxide semiconductor (MOS), MOS field effect transistor (MOSFET); quartz crystal microbalance (QCM), surface acoustic wave (SAW), surface plasmon resonance (SPR)

Principle	Measurand	Sensor type		Fabrication methods	Availability/ sensitivity	Advantages	Disadvantages
Conducto-metric	Conductance	Chemo-resistor	MOS	Microfabricated, Sputtering	Commercial, many types, 5−500 ppm	Inexpensive, microfabricated	Operates at high temperature
			Conducting polymer	Microfabricated, electroplating, plasma CVD, screen printing, spin coating	Commercial, many types, 0.1−100 ppm	Operates at room temperature, microfabricated	Very sensitive to humidity
Capacitive	Capacitance	Chemo-capacitor	Polymer	Microfabricated, Spin coating	Research	Applicable to CMOS-based chemosensor	Very sensitive to humidity
Potentio-metric	Volt-age/e.m.f.	Chemdiode	Schottky Diode	Microfabricated	Research	Integrated, Applicable to CMOS-based chemosensor	Needs Pd, Pt, Au, Ir (expensive)
	I-V/C-V	Chemotran-sistor	MOSFET	Microfabricated	Commercial, special order only, ppm	Integrated, Applicable to CMOS-based chemosensor	Odorant reac-tionproduct must penetrate gate
Calorimetric	Temperature	Thermal chemosensor	Thertmister (pyroolec-tric)	Microfabricated, Ceramic fab.	Research	Low cost	Slow response
			Pellistor	Microfabricated	Research	Low cost	Slow response
			Thermocou-ple	Microfabricated	Research	Low cost	Slow response
Gravimetric	Piezoelec-tricity	Mass-sensitive chemosensor	QCM	Microfabricated, Screen printing, Dip coating, Spin coating	Commercial, several types, 1.0 ng mass change	Well understood technology	MEMs fabrication interface electron-ics?
			SAW	Microfabricated, Screen printing, Dip coating, Spin coating	Commercial, several types, 1.0 ng mass change	Differential devices can be quite sensitive	Interface electron-ics?
Optical	Refractive index	Resonant-type chemosensor	SPR	Microfabricated, Screen printing, Dip coating, Spin coating	Research	High electrical noise immunity	Expensive
	Interisity/ spectrum	Fiber-optic chemosensor	Fluores-cence, chemolu-minescence	Dip coating	Research	High electrical noise immunity	Restricted avail-ability of light sources
Amperometry	current	Toxic Gas Sensor	Electrocata-lyst	Composite Electrode	Commercial ppb-ppm	Low cost noRh interference	Size

such a water vapor, ethanol, and others. Lower temperature polymer sensors were developed to improve the drift and interference stability of e-nose instruments. Both these and the gas sensors lacked adequate selectivity for high information goals. Many different modifications such as coatings, molecular imprinting of polymers, and advanced pattern recognition methods were employed to overcome these problems. These resulted in improvements; however, they were still not able to provide acceptable results for many high information applications. As a result of this and due to the need for better sensitivity and selectivity and reproducibility, new technology platforms were developed.

As the next step in their evolution, e-nose systems based on mass spectrometry were developed to gain more selectivity. The mass-spectrometer-based e-noses

utilize various types of soft chemical ionization with mass separation. This creates virtual sensors at each measured mass-to-charge ratio. Optical e-nose systems based on dyes and color or fluorescence have also been developed in an effort to generate more diverse response patterns thus enabling more sensitivity and selectivity.

E-nose developers then looked to human physiology and incorporated biomimetic elements into their sensor design. For example, several researchers have attempted to increase the performance of e-nose systems by imitating the dynamic spatiotemporal separation of high vapor pressure and low vapor pressure volatiles that occurs in the turbinate regions of human sinuses during normal human *ortho*-nasal olfaction. *Woodka* et al. [21.70] reported such a biomimetic approach using a spatiotemporal sensor system containing a carbon black conducting polymer (CBCP) sensor array arranged spatially with the sample flow. *Gardner* et al. [21.71] also attempted to imitate the dynamics of odorant reception on the human nasal mucosa during olfaction, which they called *nasal chromatography*, where the olfactory mucosa and its absorptive properties in time and space are imitated using a planner chromatography system and sensors to collect responses from layers of volatiles as they pass broadly tuned sensors. Others have attempted to use a nasal flow pattern to increase the sensitivity of the sensor arrays [21.72].

In the latest biomimetic evolution of the e-nose, called bio e-noses, actual elements from nature, such as enzymes, proteins, antibodies and other elements are being developed to further enhance the capabilities of e-nose sensor systems. These will be discussed further below. The following sections review the different components used in various e-nose sensor systems.

E-nose sensor performance and applications are extremely broad. Many different types have been developed for different types of volatiles and different applications. The sensor types are discussed below. Many applications are also reviewed. A vast number of volatile chemicals have been targeted by these devices. This review focuses on the applications of the various systems and sensors rather than the volatile classes and detailed detection mechanisms that are described in the references.

Electrical Sensors

Electrical sensors can be classified into the following categories according to the circuitry used and measurement principle: conductance, potentiometry, capacitance, and amperometry.

Conductivity-Based Sensors. Metal oxide semiconductor (MOS) sensors are one of the common types of conductivity-based electric sensors. The food and e-nose applications of these have been reviewed by *Berna* [21.53]. These sensors have the advantage of being easy and inexpensive to make with many different types of metal oxides available. The film thickness can be varied to have a slow response time (thick film) or fast response time (thin film) depending on the application requirements. These sensors, however, lack sensitivity, require high operational temperatures and are extremely sensitive to inferences from water vapor and other volatiles.

There are two types of sensors based on the chemical reactions that occur at the surface. N-Type MOS sensors are sensitive to reducing gases such as H_2, CH_4, CO, H_2S, and C_2H_5. SnO_2 sensors were amongst the earliest and most common N-type MOS sensors used. Many modifications have been tried to increase the sensitivity of these sensors including surface modification [21.73], doping (Taguchi sensors, [21.74]) and the use of carbon nanotubes and nanowires [21.75–77]. ZnO sensors are often used to monitor ethanol and acetic acid in spoilage, industrial, and occupational monitoring. Improvements have also been developed for these such as the modification with porphyrins [21.78]. Other types of N-type MOS include those of the following oxides: TiO, TgO, gallium Ox, Fe(III)Ox, and WO_3. These sensors are in use and are effective in applications to monitor the gases mentioned. They have some use in modern e-nose systems, but they are usually combined with other types of sensors.

P-Type MOS sensors are sensitive to oxidizing gases such as O_2, NO_2, and Cl_2. Several common materials of construction are NiO, CoO, and CuO. P-Type MOS sensors have found use in the detection of specific volatiles of importance in food such as for monitoring trimethylamine to determine fish freshness [21.79].

These types of sensors are often combined with other types of sensors in arrays to overcome their inherent weaknesses such as drift, limited specificity, and interference from common volatiles. For example ZnO MOS sensors were combined with surface acoustic wave detector and polymer coatings for increased selectivity to classify wine [21.80, 81] and many other applications discussed further below.

Further development of these types of sensors continues today. Complementary MOS (CMOS) has become a common platform for chemical microsensor systems [21.82]. Advances in sensor substrate materials and methods, such as hydrogel technology and substrate patterning, look promising for improved performance [21.83].

Conducting polymer sensors are the next major type of conductivity-based electric sensors. Their use as sensors has been the subject of several reviews [21.84–87]. They operate at much lower temperatures than MOS

sensors. The conductivity of these sensors changes as they adsorb vapors. The two types are intrinsically conductive polymers (ICP) and composite conducting polymer sensors.

The ICPs are still very sensitive to water vapor and drift. They also have been known to oxidize with time and change their responses. The most common base polymer used for ICP sensors are lanthanide bis-phthalocyanines [21.88], which commonly contain modifiers to improve performance. The following materials have also been used in ICP sensors: metallophthalocyanines, phthalocyanines [21.89], and polymers of pyrrole, aniline, thiophene, acetylene, phenylvinylene, 3,4-ethylenedioxythiophene, N-vinylcarbazone, and ethinylenevinylene. Polymer materials are easily structured. Nanostructuring of thin film ICPs has been reported to improve their performance as sensors [21.90]. Sensors with large numbers of different polymers can be further generated using new approaches such as inkjet printing [21.91].

Composite conductive polymers (CP) are another class of material used in the construction of conductivity-based sensor design. The group of *Lewis* [21.49] has reported extensively on the development and application of carbon black polymer composite (CBCP) sensors and arrays. The base polymers can be varied to create arrays with different swelling properties. Polystyrene, acrylate, vinyl alcohol and others have been used in these sensors [21.92]. Composites with dopants such as zinc phthalocyanine have shown improved performance [21.93]. The polymeric nature of these sensors allows the fabrication of different physical forms. Conductive foams were also studied in an attempt to obtain a better conductive polymer sensor and to improve performance [21.94].

More recently, single walled carbon nanotubes (SWNT) have been used in the design of conductive polymer sensors. *Kong* et al. [21.95] report their use in the detection of NH_3 and NO_2.

Potentiometric Sensors. Potentiometric sensors measure the change in voltage. They were an attempt to achieve a lower operating temperature than MOS-based sensors. While they have lower operational temperatures than MOS sensors, they are still troubled by significant drift.

The most common type of potentiometric sensor is the metal oxide field effect transistor (MOSFET). These sensors use the same material as found in MOS sensors, but they are arranged on a chip in a manner to enable the measurement of voltage changes. New types of field effect transistors are beginning to be developed. Tunnel-field effect transistors [21.96] are reported to have greatly improved sensitivity.

Capacitance Sensors. Single walled carbon nanotubes (SWNT) have been used in capacitance circuits as chemical sensors that work well in the detection of polar analytes [21.97]. *Philip* et al. [21.98] developed composite thin films of polymethylmethacrylate (PMMA) with multiwalled carbon nanotubes (CNTs) and surface-modified multiwalled carbon nanotubes (f-CNTs) for solvent vapor-sensing applications. *Star* et al. [21.99] used chemical vapor deposition to apply metal nanoparticles to SWNT for toxic gas sensor applications.

CBCP have also been used in capacitance circuits as gas sensors. Amino-terminated dendrimer-carbon black composite sensors showed excellent sensitivity in the detection of butyl amine [21.48]. Organic acid vapor sensors have also been designed using poly-(ethylenimine)-carbon black composites [21.100, 101].

Amperometric Sensors. Amperometric gas sensors have been used for the detection of harmful gases such as CO, NOS, H_2S, and SO_2. Catalytic bead sensors, containing a catalytic pellistor, are another type of sensor arrangement for the detection of harmful and flammable gas vapors. RAE Sensor Company offers these as personal and lightweight flammable gas monitors for industrial applications.

Electrochemical/Voltammetry Sensors. Electrochemical sensors contain various types of electrodes, such as Ag-carbon electrodes, where a voltage can be applied and a response measured as the result of an oxidation or reduction reaction. The common electrodes are often coated to increase performance. Langmuir–Blodgett (LB) or the Langmuir–Schaefer (LS) films of conductive phthalocyanine have been used as voltammetric sensors for the determination of antioxidants [21.102], but have limited application as volatile sensors in machine olfaction.

Gravimetric Sensors

Gravimetric sensors monitor the absorption of chemical vapors by measuring the frequency changes of reference waves. These are also called piezoelectric or acoustic sensors. These types of sensors have been developed as gas sensors and are found in e-nose arrays. The technology used in acoustic wave-type sensors has been reviewed [21.103, 104]. Acoustic wave sensors operate to measure either waves in the bulk of the sensor material or surface oriented waves. These are described further below. These types of sensors rely on the absorption of target volatiles by single polymers or polymer arrays. As with other sensor types, this limits their selectivity since many volatiles can have similar absorption and solubility properties in polymers. The

adsorption and solubility is mainly based on polarity with volatiles of similar polarity adsorbing with similar patterns. These sensors are also limited in that volatiles can often take significant time to desorb from the polymers, making the response of the system slow and the duty cycle less than desirable.

Bulk Acoustic Wave (BAW) Sensors. Quartz crystal microbalances (QCM) are often used as BAW sensors. The QCM surfaces are often modified with an additional adsorbent such as chromatography packing material [21.105] or an adsorbent polymer coating [21.106] to enhance the sensitivity. Molecular imprinted polymers (MIP) have shown improved results when used with these sensors [21.107].

Surface Acoustic Wave (SAW) Sensors. SAW sensors have a resonating substrate, such as ZnO, $LiNbO_3$, $LiTaO_3$ quartz, or SiO_2, as their core. Various reference waves such as Rayleigh waves, surface transverse waves, Bleustein–Gulyaev waves, or Lamb and Love waves are established across these substrates. The changes in frequencies can be related to the amount of analyte that is absorbed. Several different coating strategies can be used to treat the surfaces of SAW sensors to make them more selective. Polymer coatings [21.108, 109], Langmuir–Blodgett films (LB) [21.110] and self-assembled monolayers (SAM) have been reported as methods to improve the performance of SAW sensors.

Microcantilever Devices. Microcantilever devices were evaluated by *Wachter* et al. [21.111] to determine the possibility of using them as sensors. The microcantilevers are first coated with an absorbent polymer film and the changes in resonance frequency are measured with exposure and absorption of analyte vapors [21.112, 113]. Different polymer films and coatings can be used to change the sensitivity and selectivity of these types of sensors [21.114].

Optical Sensors

Optical sensors have been developed in an effort to increase the complexity of the response pattern to increase the selectivity of the sensors. These systems include a radiation source. Many of these use addressable optical fiber bundles or wave guides. Typical optical detectors are used with these systems to record the response. These include photodiodes, charge coupled devices (CCD), or CMOS detectors. The detection modes include absorbance, fluorescence, polarization, optical layer thickness and color. Static or dynamics responses are recorded.

Colorimetric Optical Sensor. Colored patterns can be generated using a variety of dyes, such as pH sensitive dyes and solvatochromic pigments. Chemical vapor sensors have been developed that utilized these for vapor detection. For example, metalloporphyrin dyes with LED or photodetectors have been used to monitor VOCs, such as ethanol and acetic acid for meat spoilage detection [21.115, 116]. Photonic crystals arranged as Bragg stacks with Bragg diffraction peaks within visible wavelengths have also been used as colorimetric optical sensors to monitor amines in the headspace of bacterial cultures [21.117].

Sensor arrays have been fabricated using a large number of different dyes for VOC and odor detection [21.118–121]. Colorimetric dye arrays are also combined with other types of sensors to have cross reactivity [21.122, 123]. Nanoparticles have also been used to increase the sensitivity of these types of sensors [21.124].

Fluorescence-Based Optical Sensors. Fluorescence sensors are based on the polymer absorption of organic vapor and the fluorescence enhancement of immobilized fluorophores. Many use fiber optics, microbeads [21.125, 126], or other wave guides to form sensor arrays. The fluorophores are usually solvatochromic dyes. Exposure to organic vapors causes responses such as spectral shifts, intensity changes, spectral shape variations and temporal responses that are measured and recorded [21.122]. The ability to obtain different types of responses and patterns has led several to use such devices as a model for human olfaction [21.127, 128]. Arrays of these types of device can be portable and sensitive [21.129].

Mass Spectrometer-Based E-Nose-Type Systems

Mass spectrometers have found renewed interest as rapid, highly selective and sensitive sensors systems for e-nose types of applications. Their use and advantages, as compared to other gas or vapor sensors, have been reviewed [21.20, 130].

Virtually any existing mass spectrometer could be used like an e-nose with the proper pattern recognition analysis and sample introduction system. Headspace (HS) extraction is often coupled with MS without chromatographic separation to perform a rapid *mass chromatography* or *fingerprint MS*. For example, *Vera* et al. [21.131] and *Kojima* et al. [21.132] used HS-MS to classify beer samples. This has become a fairly widespread alternative to integrated e-nose instruments. However, these systems still require a high level of expertise to operate and they require the develop-

ment of pattern recognition models and calibration models.

Makers of e-nose equipment added mass detectors to their existing arrays of MOS sensors in order to achieve more selectivity. The early e-nose systems using mass spectrometers used the typical analytical instruments with vacuum and the typical types of ionization commonly available such as chemical and electron impact ionization with the usual types of mass separation including quadruple, time of flight, ion trap and others.

The ability to perform ionization at atmospheric pressures (ambient MS) has led to the development of different systems that have greatly reduced sample introduction times while maintaining the selectivity and sensitivity of standard mass-spectrometry instruments [21.133, 134]. There are many different types of systems that are now capable of atmospheric ionization. They use a variety of ionization methods including chemical, laser, and photoionization. These systems are capable of sampling solids with the use of surface desorption where even large biomolecule measurement and surface mapping is possible [21.135, 136]. Several methods include: desorption atmospheric pressure photoionization (DAPPI), resonance-enhanced multiphoton ionization (REMPI), vacuum ultraviolet single-photon ionization (VUV-SPI), extractive electrospray ionization (EESI), electrospray-assisted laser desorption (ELDI), matrix-assisted laser desorption electrospray ionization (MALDI-ESI), laser-assisted electrospray ionization (LAESI), and atmospheric pressure laser ionization (APLI). Not all of these are available commercially as turnkey e-nose systems.

Atmospheric pressure chemical ionization (APCI) was one of the first ambient MS methods to be developed and then used for ambient mass-spectrometry e-nose types of applications. Selected ion flow tube (SIFT)-MS has been developed for e-nose applications [21.137]. This system allows different reagent gases to be used. Desorption electrospray ionization (DESI) can be used for rapid spot sampling of samples at atmospheric pressures. Proton-transfer reaction (PTR) ionization creates charged volatile water clusters as the ionization reagent. This ionizes analytes via proton transfer at ambient pressures. Direct analysis in real time (DART) [21.138] uses charged helium ions to ionize sample gases and vapors.

Ion Mobility spectrometry mass spectrometry (IMS-MS) has been found to be an excellent instrumental platform for rapid ambient mass-spectrometry-based detection such as e-nose types of applications. It has found broad use in security, military, antiterrorism applications, and food applications in quality and safety management tasks such as: the detection of metabolites from bacteria and mold for the identification and control of their growth; process control in food production and fermentation; quality control of raw materials or for the control of storage conditions; and the quality control of packaging materials [21.139]. High field asymmetric waveform IMS-MS (FAIMS) is one format that is especially useful and is a common platform for e-nose types of instruments [21.140–142]. The Owl-Stone Company [21.143] markets an ultraFAIMS for rapid sensing and e-nose applications.

Marsili [21.144] improved the performance of a mass-spectrometry e-nose system by using a solid phase microextraction (SPME) to concentrate aroma volatiles before introduction to the mass-spectrometry instrument.

Gas Chromatography–Based Systems

Many sensors and sensor arrays generate patterns using different polymers with dopants, coatings or absorbents; or, by separation of chemical fragments based on mass-to-charge ratios. The selectivity of these systems, while improved, was still a liability. Researchers then focused on using traditional analytical approaches, such as fast gas chromatography (GC), as a way to increase the dimensionality and diversity of patterns. Standard GC instruments have detectors that require gases, vacuum systems, and energy supplies. To overcome these problems, developers built integrated systems using GC technology to separate analytes and to create diverse patterns, but with nontraditional detectors that had fewer operational requirements [21.145]. A common combination was the integration of a fast-GC separator, often miniaturized, with a SAW detector [21.146–148]. The Znose [21.149] is a commercial version of this type of e-nose system.

Vibrational Spectroscopy–Based Systems

While not usually classified as e-nose technology, many researchers have evaluated different types of vibrational spectroscopy as rapid nondestructive ways to perform the same types of testing attempted by e-nose and e-tongue instruments, specifically replacing traditional analytical methods and human sensory and acceptability testing. Vibrational spectroscopy can provide results in less time with less expertise and sample preparation. Infrared spectroscopy (IR) methods, such as Fourier transform (FT)-IR, FT-mid range IR and vis-NIR, were the most commonly evaluated methods. These find much more utilization in biosensor systems that are discussed further below and will not be reviewed extensively here. However, a few examples of their use include monitoring nuisance odors from animal production using (FT)-IR [21.150], seafood freshness monitoring using (FT)-IR [21.151], FT-mid

range IR for beverages QC, and vis-NIR to discriminate wines [21.152]. Thermo Scientific offers a portable IR system, called MIRAN SapphIRe, using photoacoustic IR that is based on this type of approach.

Combined Technologies

Sensor researchers have started combining technologies in an effort to further improve the ability of these systems to respond like the human olfactory system, which is able to generate a huge number of patterns with extremely cross-reactive receptors. The developments in cross-reactive chemical sensors has been reviewed by *Albert* et al. [21.46]. Some e-nose developers looked at nature and attempted to develop bioinspired chemical sensors and e-noses. For example, *Al Yamani* et al. [21.153], used a bioinspired recognition approach with their design for the determination of VOCs.

Enhancing Technologies

Different technologies have been applied in an effort to improve the sensitivity and selectivity of sensors. The reduction of size and power requirements has also motivated the exploration of technologies to enhance the performance of sensors. Many of these are not simply focused on one type of sensor, but rather across sensors types. Several of these enhancing technologies are discussed below.

Coatings. Coating technology has the potential to make an impact across several different types of sensors. As an example, *Lima* et al. [21.154] reported a plasma treatment for thin polymer films that was able to preconcentrate VOCs for sensor applications.

Molecular Imprinted Polymers. Molecular imprinted polymer (MIP) technology has been used to build polymers that can be very selective in certain applications such as separation and purification. The use of MIP in sensor applications has been reviewed by *Shimizu* and *Stephenson* [21.155]. They report that this is a rapid and cheap way to increase the selectivity of polymer-based sensors and to decrease cross reactivity for improved pattern generation. MIPs have been especially helpful with gravimetric-based sensors. MIP and QCM technology has been used for high sensitivity detection of explosives [21.156], and VOCs [21.157]. *Dickert* et al. [21.158] reports that MIP-based *synthetic receptors* can provide very sensitive surfaces for the detection of small molecules and larger target analytes up to the length-scale of whole cells.

Nano-Enhancements. One of the central focuses of the nanotechnology revolution has been the development of advanced sensor technology. The nanotechnology-enabled toolkit allows the facile generation of sensor patterning, advanced wave-guide fabrication, high surface area nanoparticles, conducting carbon nanotubes, and other devices that are on track to deliver breakthroughs such as single molecule detection, extreme miniaturization and portability. The recent trend is to couple a nanotechnology-derived device with a biosensor system.

Sampling. Regardless of the sensor or sensor array used, one of the major weaknesses of e-nose systems is the sampling. Human olfaction has many orders of magnitude of differences in its sensitivities to certain key volatiles. For example, certain volatiles associated with tainted products can be detected in as low as parts per billion. Typical chemical analytical methods often require extensive isolation and concentration of volatiles before instrumental analysis. While sensor technology has made advances, the introduction of samples to detect trace amounts of volatiles, whether actively or passively, has been a problem, especially if there is an interfering vapor present. Sample concentration is laborious and manual. Traditional systems needed for sampling and sample concentration do not lend themselves to rapid sensitive analysis and eliminate many of the advantages of most e-nose systems. *Nakamoto* [21.159] reviewed the sampling methods used with e-nose systems.

21.2.4 Biosensors

E-noses and e-tongues have achieved some limited success in reproducing human sensory perception and replacing traditional analytical chemistry methods. Part of the problem was due to a trade-off between speed, low sample preparation and other advantageous features with the level of selectivity and sensitivity that was possible. Biosensors are the next evolutionary step towards the goal of replacing human sensory or traditional analytical methods. Biosensors combine a selective biological recognition element, such as enzymes, DNA, antibodies, receptors/proteins, membranes and even whole cells [21.160] and tissues, with a sensitive transducer. These biological recognition elements come directly from the cellular components involved with signal transduction, or they provide high detection selectivity for these components. They are versatile analytical tools applied more and more in different fields, such as medicine, food quality and safety control, environment pollution monitoring and many others [21.161]. They can provide very high levels of selectivity and sensitivity.

Their use in the replacement of traditional microbiological methods for the detection of pathogens have

been reviewed [21.162–168]. Several other areas of application, such as biomedical research [21.4], pollution monitoring [21.169] and food quality measurement and process control [21.170] have also been reviewed. *Velasco-Garcia* and *Mottram* [21.171] reviewed the use of biosensors in agriculture including detection of pollutants in crops and soils, detection and identification of infectious diseases in crops and livestock, online measurements of important food processing parameters, monitoring animal fertility and screening therapeutic drugs in veterinary testing. The biosensors most related to human olfaction are reviewed further below.

Modes of Action

As mentioned previously, biosensors are built around two main operational elements. They are constructed to contain an analyte recognition element, usually derived from a biological source. The recognition element is coupled to a transducer that is designed for extremely high sensitivity. These are discussed further below.

Recognition. The two general types of recognition elements are molecular-based and cell-based.

Antibodies are one of the most common molecular-based biosensor recognition elements used for immunoassays. *Skottrup* et al. [21.172] reviewed the use of surface immobilized antibodies for the detection of pathogens. Antibodies have been immobilized on a variety of other materials, such as magnetic beads in a magnetoresistive immunosensor for *E. coli* [21.173], quartz crystal microbalance for human immunoglobulin quantitation [21.174], and hydrogel particles in multiplex immunoassays [21.83]. These are usually employed as strategies to improve the transduction of the biosensors. These detect epitopes on target cells and thus are not usually included in e-nose systems focused on detection of olfactory-active volatiles.

Enzymes are another commonly used recognition element in biosensors. They are used in simple ethanol biosensors where immobilized alcohol oxidase generates hydrogen peroxide from ethanol vapors that can be detected with amperometric electrodes [21.175]. *Moyo* et al. [21.176] reviewed the different methods involving the polymer immobilization of enzymes for use in *enzyme electrodes* in biosensor applications.

Many other molecular-based recognition elements have been used in the design of biosensors. Biosensor recognition elements have been constructed using ion channels, nucleic acid/peptide nucleic acid (hybridization), genes, modified surfaces-SPR (biotin-silicon nanowires, streptavidin), immobilized olfactory receptors [21.177], ribozyme elements (Halfzyme, RiboReporters) and other biological themes of molecular interaction. For example, *Vidic* et al. [21.177] coexpressed a human olfactory receptor (OR) and G-proteins in Saccharomyces cerevisiae cells. The cells where used to prepare nanosomes and immobilized on a sensor chip. Using surface plasmon resonance they were able to quantitatively evaluate OR stimulation by an odorant, and G protein activation with discrimination between odorant ligands and unrelated odorants. There have been many recent developments using these types of approaches.

Biosensors have been designed with cell-based (cytosensor) recognition elements. Artificial cell membranes and tissues have been used. *Liu* et al. [21.178] used the olfactory tissue taken from a rat to make a biosensor for odors. They constructed this *neuron chip* by fixing the biological tissues onto the surface of a light-addressable potentiometric sensor (LAPS) [21.179]. They reported that the olfactory mucosa tissue of the rat was in a natural state with neuronal populations and functional receptor units of the cilia well preserved. The electrical properties of the tissue-semiconductor interface were analyzed by the volume conductor theory and the sheet conductor model. Then, the local field extracellular potentials of the receptor cells in the olfactory mucosa tissue were simulated and monitored. The results suggested that this tissue-semiconductor hybrid system was sensitive to odorants. They believed that the receptor cell-based biosensor technology is a valuable tool to record data with high information content with respect to odor stimulation in the intact cellular environment of the olfactory epithelium. While this approach had the advantages of intact epithelium, the preparation was complex and the durability of such a device is unknown. However, this could be a useful research tool when used with in vivo experiments.

Immobilized whole cells have also been studied as recognition elements in selective volatile sensors. Yeast clones (2,4-dinitrotoluene) [21.180], modified bacterial cells (napthlaene, salicylate, chlorophenols, cyanide) [21.181, 182], and GPCR-Hela/olfactory cells ((-)citronellal) [21.183, 184] have been reported to have benefits as recognition elements in biosensors. *Dickert* et al. [21.158] used biomolecular imprinting to make *synthetic* receptors that could be used with whole cell detection systems that where sensitive to coffee volatiles and others.

Bio-E-Nose

A class of biosensor attempts to closely mimic the human olfactory system. The researchers involved with this approach borrow different elements of the human olfactory system, such as receptors, tissue, or data processing as design elements. The area of the bio-e-nose

and the biomimetic approach to volatile sensing has been reviewed extensively [21.54, 185–190].

The construction of bio-e-nose devices has required the development of new materials. Sensitive coatings are formed using dip or drop coating, self-assembled monolayers (SAM) [21.191], Langmuir–Blodgett films [21.192], and others. Single walled carbon nanotube or PET polymers [21.193] and neural cell coated polymers [21.194] are some other examples. QCM and SPR are commonly used as detectors.

Odorants enter the human nasal cavity and are absorbed in the olfactory mucosa. These volatiles then are thought to interact with odor-binding proteins (OBP), however the exact details are somewhat controversial. Several bio-e-nose developers have used this initial binding event as the focus of their bio-e-nose designs. The efficacy of synthetic OBPs as sensor elements was explored using computer simulations of docking and scoring to calculate binding efficiencies [21.195, 196]. *Sankaran* et al. [21.197, 198] used synthetic odor-binding peptides in a bio-e-nose device to detect alcohols from Salmonella contamination. *Kruse* et al. [21.199] also used this technique to build an alcohol sensor. *Jaworski* et al. [21.200] used this approach to design a selective coating for the detection of volatiles associated with explosives. *McAlpine* et al. [21.201] attached odor-binding peptides to nanowires to increase their sensitivity.

After volatiles are bound to OBPs in the olfactory mucosa, they are thought to be transported to olfactory receptors where they start a cascade of enzymatic events that eventually lead to the generation of a series of nerve impulses. Many researchers have used cell-based approaches where olfactory receptor proteins (ORP) are either cloned or extracted and then expressed or implanted into heterologous cells that facilitate screening, or used directly. This has been reviewed by *Du* et al. [21.202]. The receptors can be extracted from animals such as rats, frogs, mice and humans. *Wu* [21.203] extracted bullfrog ORPs and used them directly in a piezoelectric biosensor. Several human cell lines are commonly used for cell-based screening with receptor clones. Human embryonic kidney 293 (HEK293) cells have been used with the expression of human ORPs [21.204], mouse olfactory receptor protein libraries [21.205], and with rat ORPs [21.206]. The human Xenopus laevis melanophore cell line has also been used to express an ORP sensitive to amines [21.207]. ORPs from *C. elegans* were expressed in *E. coli* [21.208]. *Minic* et al. [21.209] were able to express mammalian ORPs in yeast (*Saccharomyces cerevisiae*) for odor screening. *Vidic* et al. [21.177] expressed mammalian ORP in yeast (*Saccharomyces cerevisiae*) and then extracted them as membrane nanosomes to be attached to a sensor chip using SPR detection. These types of systems are still evolving. While potentially capable of very high specificity, the exact details about aspects such as detection limits, specificity, durability of these systems, especially for use as industrial sensors, are not reported.

Table 21.2 Examples of combined sensor platforms and applications

Sensor Combination	Application	References
E-nose/e-tongue	Tea	[21.210]
E-nose/e-tongue	Black tea	[21.211, 212]
E-nose/e-tongue	Beverages: water, orange juice and milks	[21.213]
E-nose/e-tongue	Port wine	[21.214]
E-nose/e-tongue	Wine	[21.215]
E-nose/e-tongue	Italian wine	[21.216]
E-nose/e-tongue	Honey	[21.217]
E-nose/vision	Beverages	[21.218]
E-nose/e-tongue/ e-eye	Wine aging	[21.219, 220]
E-nose/e-tongue/ e-eye	Olive oil	[21.221]
E-nose/e-tongue/ NIR/UV-vis	Italian red wine	[21.222]
E-nose/e-tongue/ UV-visible	beer	[21.131, 223]
E-tongue/ electromechanical microsensors	Wine	[21.224]
E-nose/e-tongue/ spectroscopy	Italian red wine	[21.225]
E-nose/e-tongue/ FT-NIR, FT-MIR	Wine aging	[21.226]
E-nose/GC (offline)	Fermented must	[21.227]
E-nose/GC	Grapes	[21.228]
E-nose/GC	VOC	[21.42]
E-nose-MS/ vis-NIR	Riesling wine	[21.229]
E-nose/GC/UV-vis	Wine aging	[21.230]
E-nose/MS	Wine origin	[21.231]

Moving further from the periphery of the olfactory system, some researchers are using the neural apparatus, such as elements that contain olfactory receptor neurons (ORN) from the olfactory system. The rationale is that the native reception and transduction is in place and that the nervous impulse would be easier to understand from a pattern recognition point of view. Endogenous tissues or whole cells were often used as elements in these types of bio-e-nose sensors and arrays. *Liu* et al. [21.232] used intact olfactory tissue from a rat to make a microelectrode array to detect odor patterns. *Liu* et al. [21.184] also used cultured human olfactory neurons to create a light-addressable potentiometric sensor (*neuron chip*) that was sensitive to acetic

acid. These devices are just beginning to be developed. They are expected to ultimately be capable of sensing many volatiles.

Along these lines, several research groups evaluated the construction of bio-e-noses from complete organs that also contained the ORN systems. Insect antennae were popular in these as well as parts from other animals. The rationale is that the olfaction of certain insects is tuned to certain volatiles such as amines for decaying animals, plants with infections and others. One group used antennae and ORN from blowflies (*Calliphora vicina*), to make sensitive detectors for biogenic amines [21.233, 234]. *Schutz* et al. [21.235] used the antennae from the Colorado potato beetle (*Leptinotarsa decemlineata*) to make a biofield effect transistor bio-e-nose to detect damaged plant volatiles. *Mead* [21.236] used similar logic and looked at antennae from lobsters to detect toxic chemicals.

21.2.5 Combination Sensor Systems

The human sense of flavor emerges from a cognitive fusion of gustatory and *retro*-olfactory signals. With e-nose, e-tongue and biosensors all evolving, much research has been focused on combining the different sensor platforms to gain the advantage of generating even more complex patterns and to attempt to reproduce human flavor sensations. This idea was often extended to include an instrumental surrogate for human vision using either instrumental colorimetry or spectral methods, and even electromechanical sensors to model mouthfeel, to increase the data available for fusion and analysis. *Ruiz–Altisent* et al. [21.60] and *Winquist* et al. [21.237] discussed the use of combined sensor platforms and their applications. Several examples of multisensor systems are listed in the Table 21.2 below. Hybrid sensor systems continue to be developed.

21.3 Biomimetic Data Analytic Approaches

Human sensory evaluation (cognition) of olfactory signals involves considerable information processing. An approach to data analytics applied to electronic sensor signals utilizes a biomimetic framework that imitates elements from biological olfaction and gustation in an attempt to achieve the higher information goals discussed earlier [21.238, 239]. *Martinelli* et al. [21.240] discussed the weakness of artificial olfactory sensors in light of the natural processing of olfactory information in biological systems. They used optical sensors to study biological olfaction. *Perera* et al. [21.241] proposed a new method reportedly inspired by the early olfactory receptor processes. Using data from two metal oxide sensors driven by a sinusoidal temperature profile, they performed data feature extraction and selection based on the projection of sensor features in *class space* using an algorithm suitable for high-dimensional feature vectors with robustness where only a small number of samples are available as a training dataset. They reported improved results since the approach accounted for sensor variance.

Raman et al. [21.242] focused on the first two stages in the olfactory transduction pathway: distributed coding with olfactory receptor neurons and chemotopic convergence onto glomerular units. They called the coding of olfactory receptor inputs the *chemotropic code* or a monotonic concentration-response model that maps sensor array inputs into a distributed activation pattern, similar to patterns found in the activation of a large population of neuroreceptors. Projection onto glomerular units in the olfactory bulb was simulated with a self-organizing model of chemotopic convergence. The pattern recognition performance of the model was characterized using a database of odor patterns from an array of temperature modulated chemical sensors. The chemotopic code achieved by the proposed model was shown to improve the signal-to-noise ratio available at the sensor inputs while being consistent with results from neurobiology. The authors [21.243] extended their model to capture on-off surround lateral interactions associated with convergence of the signals and validated their model using chemical sensors thereby enhancing signal contrast and separating identity information from intensity information.

Pioggia et al. [21.244] built an artificial data analytic model inspired by the mammalian cortex for the fusion of e-nose and e-tongue data using *neural* computing methods that aspire to capture the same neural network processing that likely happens in nature. These data analytic methods use layers of artificial *perceptrons*, or cross-linked transfer functions, that allow the tuning of complex and nonlinear responses from complex inputs such as those found in biology, after sufficient training or learning has occurred. The authors called this particular rendition a cortical-based artificial neural network (CANN). They reported better performance using CANN than other artificial neural networks methods, such as a multilayer perceptron (MLP), Kohonen self-organizing map (KSOM) and fuzzy Kohonen self-organizing map (FKSOM). *Robertsson* et al. [21.245] suggested the use of a similar approach that mimicked a human perception model

for sensory information processing applied to e-tongue sensor data.

Human olfaction produces information that is temporally and spatially resolved. Nasal flow rates tend to spread out the volatile impact zone across the olfactory tissues. *Gopel* [21.246] reviewed these ideas plus those from hyperspectral analysis from chemical imaging and discussed their application to e-nose and bio-e-nose systems. Further development of spatially-resolved sensor arrays is expected.

21.4 Machine Olfaction Applications

There are an incredible number of e-nose applications in the literature. This review will focus mainly on those of potential importance to the food industry and associated areas. These include food, environmental monitoring, clinical diagnostics, agriculture and supply chain, and industrial applications. These will be discussed in further detail below.

21.4.1 Food and Beverage Applications

For food and beverages, e-noses have been used to determine quality and safety. Moreover, they have been used to monitor food processing and shelf life. Also, they have been used to replace the usual human sensory testing tasks used in product development, and tasks such as measuring maturing and determining authenticity and origin. E-nose sensor applications in these types of food areas have been reviewed in several publications [21.56, 130, 247].

Determination of Quality, Identity or Origin

The determination of quality is very important in food and ingredients. It is directly related to value but extremely hard to measure instrumentally. Human panels are usually needed, specifically if characterizing the sensory quality of foods. This is extremely difficult in most production settings. The costs in time and people can be very high. Also, there is often a need to determine product origin and adulteration. In fact, researchers from the IFSH (Institute for Food Safety and Health, a lab associated with the Food and Drug Agency, USA) recently gave a presentation on olive oil authentication favorably comparing an e-nose's ability to detect adulteration versus LCMS. As a result, there have been a large number of reports focused on the evaluation of e-nose systems in this area. A list of these reports is shown below in Table 21.3.

E-nose sensors have also been used to test ingredients. These tests focus on the determination of quality, authenticity, adulteration and other applications much like those e-nose applications found for finished foods. Several examples of e-nose testing of food ingredients are listed below in Table 21.4.

Food Safety Methods

Food safety is perhaps the major application of electronic sensor technology. There is a constant need to be able to determine toxins and pathogens with speed, accuracy and sensitivity from the farm to the fork. Several reviews of work in this area have been reported. *Casalinuovo* et al. [21.381] reviewed the use of e-noses for the detection of the volatiles associated with food spoilage organisms and pathogens. These systems provide extremely rapid responses compared to the standard methods that require culturing and identifying microbial contaminants or traditional analytical methods for toxins. Steady improvements have been made using the volatile metabolic markers from organisms to confirm microbial identities and quantities. Their ultimate utility will be directly linked to the sensitivity that can be achieved. Work on single-molecule detection systems should greatly enable these. It is likely that these will serve as rapid surveillance methods to trigger subsequent standard testing.

Rapid detection of grain spoilage is another major area of potential for the application of e-nose technology. Mold growth on wet grain and subsequent mycotoxin production is a perennial problem. *Magan* and *Evans* [21.382] reviewed the use of e-noses for early detection of volatiles associated with fungal growth in grain. *Sahgal* et al. [21.383] discussed the possibility of using e-noses to discriminate between mycotoxigenic and nontoxigenic molds. Rapid methods are needed to support efficient supply chain management. This requires rapid, field-ready systems. While current systems are limited in sensitivity, there is ongoing research to develop new sensors to detect these contaminants. Nanotechnology, such as nanoscale construction of waveguides and other devices, should greatly enable these improvements.

Reports on the use of e-nose sensors for a variety of food safety applications are listed below in Table 21.5. The major areas include natural and manmade toxin detection, and pathogen detection.

Human Sensory Replacement

Human sensory testing is considered by some to be laborious and therefore expensive, subjective and slow.

Table 21.3 Selected applications of e-nose technology in finished foods

Food category	Product or application	References
Quality, grading, classification or origin applications		
Confection	Chocolates	[21.248]
Meat	Handling	[21.249]
Meat	Raw and cooked	[21.250]
Meat	Modified atmosphere packaging (MAP) poultry	[21.251]
Meat	Pizza meat	[21.252]
Fish	Freshness	[21.151, 253–256]
Fish	Salmon	[21.257]
Fish	Salmon, smoked	[21.258]
Fish	Sea bream	[21.259]
Fish	Cod, chilled	[21.257]
Fish	Cod, aged	[21.260]
Fish	Cod, roe ripeness	[21.261]
Fish	Puffer fish	[21.262]
Beer	Aging	[21.263]
Spirits	Quality control	[21.264]
Spirits	Taint	[21.265]
Beer	Nonalcoholic	[21.266]
Beer	Bitterness	[21.267]
Beer	Brewery applications	[21.268]
Beer	Quality	[21.269]
Beer	Portable sampling	[21.270]
Beer	Flavor	[21.271]
Wine	Classification	[21.80, 81]
Wine	Description	[21.272, 273]
Wine	Classify, portable	[21.274]
Wine	Spoilage	[21.275]
Wine	Origin	[21.276]
Wine	Red type, discrimination	[21.277]
Wine	Origin, vineyard	[21.278]
Wine	Calibration transfer	[21.279]
Wine	Description, thresholds	[21.280]
Wine	Classification	[21.281]
Wine	Origin	[21.231, 282]
Wine	Vintage year	[21.283]
Wine	Italian, type, origin	[21.284]
Vodka	Quality control	[21.264]
Vodka	Differentiation	[21.285]
Spirits	Aromas, ETOH	[21.286]
Beverages	Mineral water	[21.287]
Beverages	Soft drinks	[21.288]
Beverages	Juice, citrus	[21.289]
Beverages	Juice, orange	[21.290]
Beverages	Juice, fruit	[21.291]
Beverages	Coffee	[21.56, 292–296]
Beverages	Tea	[21.297, 298]
Beverages	Tea, green	[21.299, 300]
Beverages	Tea, black	[21.301, 302]
Fruit/vegetable	Apples	[21.303]
Fruit/vegetable	Apples, shelf life	[21.304]
Fruit/vegetable	Apples, defects	[21.305]
Fruit/vegetable	Peaches	[21.306, 307]
Fruit/vegetable	Peaches, ripeness	[21.308]
Fruit/vegetable	Pears	[21.309, 310]
Fruit/vegetable	Melons	[21.311]
Fruit/vegetable	Oranges	[21.312]
Fruit/vegetable	Bananas	[21.313]
Fruit/vegetable	Tomatoes	[21.314]
Fruit/vegetable	Tomatoes, maturity	[21.315]
Fruit/vegetable	Tomatoes, shelf life	[21.316]
Fruit/vegetable	Cucumber	[21.317]
Fruit/vegetable	Blueberries	[21.318, 319]
Fruit/vegetable	Blueberries, sorting	[21.320]
Fruit/vegetable	Mangoes	[21.321]
Fruit/vegetable	Pineapples, firmness	[21.322]
Fruit/vegetable	Mandarin orange	[21.323]
Fruit/vegetable	Olives	[21.324]
Fruit/vegetable	Apricots, varieties	[21.325]
Fruit/vegetable	Apricots	[21.326]
Fruit/vegetable	Fruity aroma	[21.327, 328]
Dairy	Review	[21.329]
Dairy	Milk, cultured products	[21.330]
Dairy	Milk, UHT	[21.331]
Dairy	Milk	[21.332–334]
Dairy	Off-notes	[21.335]
Dairy	Milk, chocolate	[21.336]
Dairy	Milk, spoilage	[21.337]
Dairy	Milk, UHT	[21.338]
Dairy	Milk, breast	[21.339]
Dairy	Milk, flavorings	[21.340]
Dairy	Milk, whole powder	[21.341]
Dairy	Milk, casein	[21.342]
Dairy	Cheese, Crescenza	[21.343]
Dairy	Cheese, hard	[21.344]
Dairy	Cheese, Dana-blue	[21.345]
Dairy	Cheese, rind off-notes	[21.346]
Dairy	Cheese, Cheddar	[21.347]
Dairy	Cheese, Swiss, origin	[21.348, 349]
Dairy	Cheese, surface mold	[21.350]
Dairy	Cheese, Tageggio	[21.351]
Dairy	Yogurt	[21.330]
Dairy	Lactic acid bacteria (LAB) cultures	[21.352]
Dairy	Ewe milk, certification	[21.353]
Bakery	Flour, bread-type	[21.354]
Vinegar	Chinese-type, class	[21.355]
Rapid composition determinations		
Antioxidants	Mixed	[21.102]
Antioxidants	Herb extracts	[21.356]
Antioxidants	Infant cereal	[21.357]

Table 21.4 Selected applications of e-nose technology for ingredient testing

Category	Product or application	References
Spices	Spice blends	[21.358, 359]
Grains	Bread flour	[21.354]
Grains	Wheat and barley, QC	[21.360]
Grains	Moldy, musty taints	[21.361]
Grains	Microbial quality	[21.360]
Grains	Rice	[21.362]
Oil	Olive oil	[21.363–365]
Oil	Type discrimination	[21.366]
Oil	Type discrimination	[21.367]
Oil	Olive oil, origin	[21.222]
Oil	Coconut, adulteration	[21.368]
Sweeteners	Natural, high intensity	[21.369]
Sweeteners	Honey, origin	[21.370–372]
Sweeteners	Honey, class	[21.373]
Sweeteners	Honey, Italian	[21.374]
Nuts	Almonds, damaged	[21.375]
Flavors	Blends	[21.376]
Flavors	Spray-dry retention	[21.377]
Vinegar	Type discrimination	[21.378]
Cocoa	Bean quality	[21.379]
Cork	Taint, tricholoranisole	[21.380]

Also, a high degree of rigor is required to obtain consistent results. These make it difficult to perform in many locations where the access to isolated sensory laboratories, panelists and trained sensory scientists is limited. Researchers have attempted to develop electronic sensors that can replace some of the common human sensory testing methods to take the burden off of the other researchers [21.420]. Some of these studies are listed below in Table 21.6.

Food Process Monitoring

Process control systems utilize many types of sensors. Human sensory testing is only typically used after processing on finished food products. Researchers have found many potential opportunities to use e-nose sensors to control and direct processing, and in subsequent post-processing steps. A common area is the tracking of food fermentation and ripening processes. These can take years and it is very expensive to perform repeated sensory testing. Also, the storage costs of these types of processes can be high. Methods achieving early predictions of quality are of great interest. Also, many food products are processed using cooking and baking where the sensory quality of the food changes rapidly as the product moisture drops and as product temperature rises. E-noses have been studied as a way to control these processes to help the product stay in the desirable cook-level window. Several of these studies are listed below in Table 21.7.

Food Shelf Life and Storage Monitoring

Another significant application of e-nose sensor technology is to determine food shelf life, maturity or ripeness and shelf spoilage. Many fresh food products ripen, but then degrade and deteriorate chemically and microbiologically during storage. This can lead to health risks and economic loss. A significant amount of work has been done with the most sensitive foods to determine if e-nose sensors can replace human sensory testing and microbiological testing in the determination of the quality, maturity and safety of fresh food. *Casalinuovo* et al. [21.381] reviewed the use of e-noses in the detection of food spoilage. Additional studies are listed below in Table 21.8.

21.4.2 Environmental Monitoring

Human olfaction enables us to detect potentially harmful gases. For example, we have a reflective apnea response when we smell certain odors, such as ammonia, that provides protection for our airways. As global populations increase, monitoring of the environment is an increasing area of potential importance for many. There is a growing awareness of air quality, both indoor and outdoor, water quality, and other sources of potential contamination. Odors can warn about hazards, but can also cause significant nuisances.

E-nose sensors' cross-reactive abilities, to sense multiple chemicals simultaneously, have led to their increased use in environmental monitoring. *Romain* and *Nicolas* [21.515] reviewed the use of MOS e-noses for environmental monitoring. *Feng* et al. [21.516] evaluated a colorimetric sensor for the detection of toxic industrial gases. The quality of indoor air is increasingly becoming the focus of recent studies. Hospitals especially are concerned with indoor air quality and the use of e-noses to detect air-borne pathogens [21.517]. *Trevathan* et al. [21.518] reported a wireless sensor system for marine environmental monitoring called SEMAT. The use of e-nose sensors for air and water monitoring are discussed further below.

Air

Air quality has been a significant problem for agriculture. Many agricultural practices produce odor and VOCs that need to be controlled and are the subject of regulatory control. *Tsujita* et al. [21.519] reviewed the use of gas sensors for air pollution monitoring. Several different applications of e-nose sensors for the monitoring of air quality are listed below in Table 21.9.

Water

Water quality is related to lack of odor. Thus water with any detectable odor is considered of low quality. Future

Table 21.5 Selected applications of e-nose technology in food safety

Determination	Category	Product or application	References
Toxins, natural	Mycotoxins	Corn, fumonisin	[21.384, 385]
Toxins, natural	Mycotoxins	Corn, aflatoxins	[21.386–388]
Toxins, natural	Mycotoxins	Corn	[21.389]
Toxins, natural	Mycotoxins	Wheat, fumonisin	[21.390–393]
Toxins, natural	Mycotoxins	Wheat, Durum	[21.394]
Toxins, natural	Mycotoxins	Wheat, deoxynivalenol (DON) marker	[21.395]
Toxins, natural	Ochratoxin A	Grapes	[21.396]
Toxins, manmade	Pesticides	Insecticides	[21.397]
Toxins, manmade	Contaminants	Whiskey, methanol	[21.398]
Toxins, manmade	Contaminants	Milk, urea/ melamine	[21.399]
Toxins, manmade	Contaminants	Milk, TMA	[21.400]
Toxins, manmade	Contaminants	Octopus, formaldehyde	[21.401]
Pathogens	Mold/fungus	Mushrooms	[21.402]
Pathogens	Bioterrorism	Mixed toxins	[21.385]
Pathogens	Salmonella	Meat	[21.403]
Pathogens	Salmonella	Beef strips	[21.403]
Pathogens	Salmonella	Alcohol markers	[21.404]
Pathogens	Salmonella	Beef-strips, HS-MS	[21.405]
Pathogens	Salmonella	Beef, MOS	[21.406]
Pathogens	Salmonella	Spouts, MOS	[21.407]
Pathogens	Salmonella	Broth culture, MOS	[21.408, 409]
Pathogens	*E. coli*	Broth culture/MOS	[21.410]
Pathogens	*E. coli*	Broth culture	[21.409]
Pathogens	*E. coli*	Vegetables	[21.411]
Pathogens	*E. coli*	Broth culture	[21.412]
Pathogens	Staphylococcus	Broth culture, HS-MS	[21.413]
Pathogens	Listeria	Light scattering	[21.414]
Pathogens	Fusarium	Barely	[21.415]
Pathogens	Fusarium	Corn	[21.416]
Pathogens	Mold	Corn	[21.417]
Pathogens	Fusarium	Wheat	[21.418]
Pathogens	Fusarium	Grain, discrimination	[21.419]
Pathogens	Fusarium	Wheat/tricycle	[21.392]
Spoilage	Mixed microbial	VOC	[21.115]

Table 21.6 A selection of reports using e-nose sensors to replace traditional human sensory

Method/Correlate	Electronic Surrogate	References
Discrimination, Triangle test	E-nose, MOS	[21.421]
Pleasantness	E-nose	[21.422]
Descriptive analysis, aroma	GC-MS-O	[21.423]
Descriptive analysis, aroma, quality	Gas sensors, e-nose	[21.424]
Descriptive analysis, aroma, quality	E-nose, MOS	[21.425]
Descriptive analysis, aroma, quality	E-nose, mixed array	[21.426]
Descriptive analysis, aroma discrimination	Differential e-nose	[21.427]
Descriptive analysis, aroma, quality	E-nose, CBCP	[21.428]
Descriptive analysis, aroma, quality	E-nose	[21.429]
Descriptive analysis, odor classification		[21.430, 431]
Olfactory perception	E-nose	[21.432]

fresh water supplies will become increasing pressured and quality is sure to suffer as supply becomes limited. There is an expectation that the need for water quality assessment will grow rapidly. Thus the need for rapid instrumental methods to replace human olfaction and sensory testing methods will also grow. Several reports of the use of e-noses to monitor water quality and waste water quality are listed below in Table 21.10.

Table 21.7 Selection of reports on the use of e-nose sensors on food processing

Objective	Product	References
Fermentation monitoring	Tea	[21.433–435]
Fermentation monitoring	Yogurt	[21.436, 437]
Dehydration monitoring	Tomatoes	[21.438]
Dehydration monitoring	Carrots	[21.439]
Dehydration monitoring	Lemon infusion	[21.440]
Process monitoring	Milk, UHT	[21.441, 442]
Rapid sorting	Fruit	[21.443]
Baking/Cooking end point	Bread	[21.444]
Baking/Cooking end point	Coffee, roast level	[21.445]
Baking/Cooking end point	Block milk, flavor	[21.446]

21.4.3 Clinical Diagnostics

All animals, including humans, use their sense of smell to detect sickness, whether consciously or subconsciously. Animals such as dogs have been trained to detect illnesses, such as cancer, with a high degree of accuracy. Volatiles sensors have also been evaluated for this application. A brief review of e-nose technology used in clinical diagnostics is included below. The key applications are the detection and diagnosis of infectious diseases, such as respiratory and urinary tract infection, the detection of cancer and the replacement or support of standard clinical methods. Several researchers have reviewed the medical applications of e-nose technology [21.4, 552, 553].

Infectious Diseases

Turner and *Magan* [21.554] reviewed the use of e-noses in the diagnosis of infectious diseases. *Pavlou* et al. [21.555] reported the use of an e-nose to detect volatiles and odors associated with *Mycobacterium tuberculosis* (TB) in mixed culture with other bacteria. *Sahgal* et al. [21.556] used e-nose sensors to identify odors from fungal infections caused by *Trichophyton* species. *Pavlou* et al. [21.557] used an e-nose to differentiate odors from pathogen anaerobes from *Clostridium* species. And *Parry* et al. [21.558] used an e-nose to detect odors from hemolytic *Streptococcus* in leg ulcers.

Diagnosis of Diseases via the Upper Respiratory Tract Odors

Breath odor analysis has been particularly useful in the diagnosis of a diversity of upper respiratory diseases [21.559], and cancers [21.560]. In fact, breath odor markers also exist for other diseases such as diabetes and potentially many more. Several examples

Table 21.8 A selection of reports using e-nose sensors for shelf life and storage testing

Focus	Product	References
Chemical spoilage	Oxidation, polyunsaturated fatty acids (PUFA)	[21.447]
Chemical spoilage	Oxidation, nuts	[21.448]
Chemical spoilage	Meat, warmed-over flavor	[21.449]
Chemical spoilage	MAP, Apples	[21.450]
Chemical spoilage	MAP, pizza meat	[21.252, 451]
Chemical spoilage	Vegetable oil	[21.452]
Chemical spoilage	Fruit, pineapples, fresh-cut	[21.453]
Chemical spoilage	Apples	[21.454]
Chemical spoilage	Grains, wheat storage age	[21.455, 456]
Microbial spoilage	Oranges, mold	[21.457, 458]
Microbial spoilage	Meat, review	[21.249, 459]
Microbial spoilage	Meat, fresh flavor	[21.460]
Microbial spoilage	Meat, bacteria	[21.461]
Microbial spoilage	Meat, VOC	[21.250, 256, 462, 463]
Microbial spoilage	Meat, chilled pork	[21.464]
Microbial spoilage	Meat, pork	[21.465, 466]
Microbial spoilage	Meat, pork, fish	[21.467]
Microbial spoilage	Meat, pork, CFU	[21.468]
Microbial spoilage	Meat, beef	[21.469–471]
Microbial spoilage	Meat, red	[21.472]
Microbial spoilage	Meat, beef, CFU	[21.473, 474]
Microbial spoilage	Meat, meatloaf	[21.475]
Microbial spoilage	Meat, cured	[21.476]
Microbial spoilage	Meat, MAP ground	[21.477, 478]
Microbial spoilage	Meat, vacuum-packed	[21.479]
Microbial spoilage	Meat, chicken, MAP	[21.251]
Microbial spoilage	Fish	[21.480]
Microbial spoilage	Fish, salmon	[21.481, 482]
Microbial spoilage	Fish, sardines	[21.74]
Microbial spoilage	Fish, sea bass	[21.483]
Microbial spoilage	Eggs	[21.484–487]
Microbial spoilage	Vegetables, fresh	[21.411]
Microbial spoilage	Tomatoes	[21.488]
Microbial spoilage	Bacteria, Micrococcaceae	[21.489]
Microbial spoilage	Juice, Alicyclobacillus	[21.490]
Microbial spoilage	Grain/stored, rice	[21.491]
Microbial spoilage	Grain/stored, wheat	[21.492]
Microbial spoilage	Grain/storage, indicator	[21.493]
Microbial spoilage	Grain/stored, rapeseed	[21.494]
Microbial spoilage	Bakery products	[21.495–497]
Microbial spoilage	Bread, mold	[21.498, 499]
Microbial spoilage	Wine	[21.500]
Microbial spoilage	Apple juice	[21.501]
Maturation/ripening	Sausage, fermentations	[21.502]
Maturation/ripening	Blue cheese, ripening	[21.345, 503]

Table 21.8 (continued)

Focus	Product	References
Maturation/ripening	Roasted coffee, ripening	[21.504]
Maturation/ripening	Wine, red	[21.219]
Maturation/ripening	Wine	[21.505]
Maturation/ripening	Tomato	[21.314, 316, 506, 507]
Maturation/ripening	Fruits, snake fruit	[21.508]
Maturation/ripening	Fruits, apple	[21.509]
Maturation/ripening	Fruit, pre-harvest maturity	[21.510]
Maturation/ripening	Fruits, apple, post-harvest	[21.511]
Maturation/ripening	Fruits, post-harvest	[21.308]
Maturation/ripening	Fruit, mango	[21.512]
Maturation/ripening	Fruit, apricot	[21.513]
Maturation/ripening	Fruit, peach	[21.514]
Maturation/ripening	Fish, cod roe	[21.261]

of these are listed below in Table 21.11. Currently, it has not been clearly established that the underlying molecular principles can be attributed to odorous constituents. However, at least for the animal sniffer, such as dogs, this seems to be valid [21.561]. Greater interest in breath odor analytics is expected for clinical diagnostics. This should also drive the development of machine olfaction devices for this application.

Urinary and Renal Disease

Urinary volatiles and odors have been linked to certain types of disorders. E-noses have been used to determine if they can replace the traditional analytical methods. Several reports are shown in Table 21.12. This is another example of how future advancements in instrumental odor detection and recognition technology can be leveraged in nontraditional applications. However, it can be argued that certain mammals currently use urine and the odors of urine for a variety of common communication applications such as marking territories and others.

Digestion

Fermentation of undigested foods in the large bowel by its resident bacteria results in the creation of several chemicals including volatile gases that influence colonic and metabolic health. *Fermentonomics* describes the investigation and analysis of the fermentome by noninvasive means and may have a potentially wide application in medicine. Digestion disorders have seen significant focus by e-nose clinical researchers. One notable report was by *Arasaradnam* et al. [21.581] who used an e-nose to evaluate the VOCs associated with patients' *fermentome* to diagnose inflammatory bowel disease and diabetes.

Table 21.9 A selection of reports using e-nose sensors for air quality monitoring

Focus	Area	References
Odor nuisance	Poultry production	[21.520, 521]
Odor nuisance	Animal production	[21.522]
Odor nuisance	Swine production	[21.523]
Odor nuisance	Landfill	[21.524]
Odor nuisance	Meat rendering plant	[21.525]
Odor nuisance	Waste water	[21.526]
Odor nuisance	Compost	[21.527, 528]
Odor nuisance	Swine production	[21.529]
Odor nuisance	Environmental odors	[21.530]
Air quality	Space station	[21.531]
Air quality	Indoor air	[21.532]
Air quality	Indoor, fungus	[21.533]
Air quality	General	[21.534]
Air quality	Biofilter odors	[21.535]
Air quality	Indoor, mold	[21.536]
Pollution	Toxic gas	[21.516]
Pollution	Review	[21.537]
Pollution	And malodors	[21.538]
Volatile inorganic compounds	NH_4	[21.539]
Volatile inorganic compounds	SO_2	[21.540]
Volatile inorganic compounds	Mixed gases	[21.541]
Volatile inorganic compounds	CO	[21.542]
VOC	Mixed gases	[21.118]
VOC	Formaldehyde	[21.543]

Table 21.10 A selection of reports using e-nose sensors for water monitoring

Focus	Area	References
Water	Review	[21.544]
Water	River pollution monitoring	[21.545]
Water	Pollution monitoring	[21.546]
Water	Taints	[21.547]
Water	Taints, musty smell producing Streptomyces	[21.548]
Waste water	Sludge odor	[21.524]
Waste water	Odor	[21.549, 550]
Waste water	Quality monitoring	[21.551]

Cancer

As mentioned above, certain odors have been found to be associated with certain diseases and these are being explored for diagnostic potential. Machine olfaction is useful since many of these odorants are below the detection thresholds of humans. While they are not below the detection thresholds of certain animals, machine olfaction devices are more convenient for regular use.

Table 21.11 A selection of reports using e-nose sensors with breath analysis to detect upper respiratory disease, or other diseases being detectable via breath smell changes

Disease Focus	Agent	References
Ulcer	*Helicobacter pylori*	[21.562, 563]
Infection	Aspergillus infection	[21.564, 565]
Infection	*Mycobacterium tuberculosis*	[21.566]
Infection	*Pseudomonas aeruginosa*	[21.567]
Pneumonia		[21.568]
Various	Bacterial pathogens	[21.569]
Diabetes	Undefined	[21.570]
Asthma	Nitric oxide	[21.571]
Malodor		[21.572]
Exposure	COPD	[21.573]
Exposure	Pulmonary sarcoidosis	[21.574]

Table 21.12 A selection of reports using e-nose sensors with breath analysis to detect urinary and renal disease, or other diseases being detectable via urinary smell changes

Disease Focus	Agent	References
Type II diabetes	Urine volatiles	[21.575]
Infections	Urine volatiles	[21.576–579]
Renal dysfunction	Uremia (breath analysis)	[21.580]
Renal dysfunction	Monitoring hemodialysis	[21.567]

E-noses have found increasing use in the detection of cancer, especially lung cancer [21.582]. For example, *Di Natale* et al. [21.583] collected breath samples from patients with lung cancer and analyzed them for alkanes and aromatic compounds using an electronic nose, composed by eight quartz microbalance (QMB) gas sensors, coated with different metalloporphyrins. These sensors show a good sensitivity towards those compounds previously indicated as possible lung cancer markers in breath. The application of a partial least squares-discriminant analysis (PLS-DA) with this system correctly classified 94% of the cancer patients. Other systems for the detection of cancer-related odors have used SPME preconcentration with a capillary column GC and pairs of SAW sensors, one coated with a thin polyisobutylene (PIB) film as detectors [21.582–585]. *Dragonieri* et al. [21.586] used an e-nose with breath analysis to detect pleural mesothelioma. Also, *Li* et al. [21.587] used breath analysis to detect breast cancer.

Rapid Clinical Testing

The use of odor analysis in clinical diagnosis has been expanding to areas outside of cancer diagnosis. E-noses, especially CBCP-type sensor arrays, have found use in replacing the standard clinical testing methods using odor analysis to diagnose bacterial eye infections [21.599], blood culture [21.600], asthma [21.601], cerebrospinal fluid assays [21.602], eye, ear, nose and throat infections [21.603], and poisoning from radon in water [21.604] as examples.

Table 21.13 A selection of reports using e-nose sensors in plant health

Plant	Focus	References
Oranges	*Penicillium digitatum*	[21.588]
Oranges	Liberibacter	[21.589]
Blueberries	Post-harvest	[21.318]
Tomatoes		[21.590]
Melons	Drechslera sp. fungi	[21.591]
Mixed	Erwinia detection	[21.592]
Mixed	Phytopathogens	[21.593]
Fruit trees	Fungus	[21.594]
Grape vines	Crown gall	[21.595]
Mixed	Mixed pathogens	[21.593, 596]
Pest/disease monitoring	Mixed	[21.317]
Disease VOC	Review	[21.597]
Oil palm	Stem rot	[21.598]

21.4.4 Agriculture and Supply Chain

Networking of wireless sensors has the potential to allow access to information never before available with potential impacts to supply chain management. While these are not used in agricultural supply chains yet, several reports have started to appear and they include machine olfaction [21.605]. *Ruiz-Garcia* et al. [21.606] reviewed their applications and trends for use in food and agriculture. They have been used to monitor vineyards [21.607], relative humidity sensing [21.608], environmental monitoring [21.609], and in precision horticulture [21.610].

Plant Pathology

Volatile analysis has been useful in the detection and diagnosis of plant diseases. E-noses have been studied for their efficacy in performing these studies faster with less expensive equipment that requires a lower level of expertise to operate. Several studies using e-nose sensors in plant health are listed below in Table 21.13.

Forest Products

E-noses have found extensive use in monitoring forest products. For example, *Baietto* et al. [21.611] used e-nose sensors to detect wood rot. *Wilson* et al. [21.612] reviewed the use of e-nose sensors for the identification of woody plants.

Stored Grain Quality Management

The quality of crops changes during storage. This has a direct impact on the quality and value of the grain.

Table 21.14 A selection of reports using e-nose sensors for stored grain and food quality management

Area	Focus	Product	References
Pest control	Insects	Wheat	[21.455, 613]
Pest control	Insects	Grain	[21.614]
Pest control	Stink bug	Cotton, soybean	[21.615]
Pest control	Insects	Rice	[21.616, 617]
Age monitoring		Wheat	[21.300, 456, 618]
Spoilage	Fungus detection	Grains	[21.382]
Spoilage	Microbial quality classification	Grains	[21.360]
Spoilage		Onions	[21.619]
Quality management	Quality classification	Rapeseed	[21.494]

This is an important area for global commodity businesses. E-noses and sensors have been used to determine the quality of grain and other food in order to effectively manage these supply chains for maximum value and safety [21.620]. Several of these studies involving the use of e-nose sensors for storage quality management are listed below in Table 21.14.

Supply Chain Monitoring

In addition to storage quality management, e-nose sensors have been used along the supply chain to ensure identity and traceability, quality and value. These types of applications are of growing interest with the continued globalization of food supply, especially in the maintenance of identity preservation systems. Radio frequency identification smart tags transmit sensor information for tracking and confirmation. For example, *Abad* et al. [21.621] used radio frequency identification (RFID) with different sensors to track fish logistic chains. *Amador* et al. [21.622] used RFID to track fresh pineapples and their temperatures during transit. *Ruiz-Garcia* et al. [21.623] discussed the use of wireless networks for real-time monitoring of fruit logistics. Also, *Jedermann* et al. [21.624] discussed a similar system for perishable food transport. While this technology mainly logs temperature and moisture data, it is highly likely that miniature e-nose sensors will soon be added to these systems.

Plant Physiology

There have been limited reports involving the use of e-nose sensors to monitor plant physiology and development; however, this could be an emerging area in the future. In one study in this area, *Komaraiah* et al. [21.625] used gas sensor arrays to determine plant cell culture growth.

Feed Quality

There are not many reports of rapid feed quality determination using machine olfaction instruments such as e-nose sensors. NIR methods have been well established. However, *Campagnoli* [21.626] reported on the potential application of e-noses in processed animal protein (PAP) detection in feed materials.

21.4.5 Further Industrial Applications

Machine olfaction is finding application in many more fields. Just a few examples of these are highlighted below. Many are reported as the cost, selectivity, portability and information processing capabilities continue to advance.

Occupational Health and Safety

E-nose technology has reportedly been used in health and safety to detect toxic gases. One example is given by *Gawas* et al. [21.539] who used an nanostructured ferrite e-nose sensor to detect ammonia gas. Others like this have already been mentioned above in the section on safety applications.

Cosmetics Formulation and Quality Control

E-nose sensors have found some use in the quality control of cosmetics. *Branca* et al. [21.627] used an e-nose to detect certain fragrance compounds. *Hanaki* et al. [21.628] developed a system to deformulate the components of blended fragrances.

Pharmaceutics Research

E-noses have been also used in screening methods used to develop pharmaceuticals. For example *Naraghi* et al. [21.629] used an e-nose to screen volatile fingerprints associated with the efficacy of candidate antifungal agents.

Bioindustrial Process Monitoring

As with food process monitoring, machine olfaction devices such as e-nose sensors have also been used in bioindustrial process monitoring where there is a need for rapid online process sensors. *Rudnitskaya* and *Legin* [21.630] reviewed this area and the potential for using e-noses and e-tongues for this purpose. *Cimander*

and *Mandenius* [21.631] reported the use of multivariate process control of bioprocesses using mixed sensors including an e-nose, mass spectrometers, NIR and standard bioreactor probes.

Rapid Microbiological Testing

Finally, machine olfaction device e-noses have found some use in general microbiological testing methods. For example, e-noses have been used to monitor mold growth in liquid media [21.632] and to discriminate bacteria [21.633, 634].

Rapid, In-field Explosives Detection

The use of e-noses for the detection of explosives, such as land mines has been reviewed by *Gardner* [21.635].

21.5 Conclusions

Current instruments available for machine olfaction are not without problems. Sensitivity, selectivity and reproducibility have always been problematic for electronic sensors. Even with the advancements produced by improved data analytics, biorecognition elements and nanoenabled transduction elements, these devices can require a very large number of samples to train the pattern recognition procedures, and then potentially years of calibration maintenance to tune out all types of interferences and noise associated with the samples of interest. Thus, the economics of these methods can suffer if they include estimates of the training and calibration efforts. Also, biosensors may have limits on their useful service lives. This could also change the economics of their use especially if high levels of expertise are required for their maintenance and use. Gas sensor arrays are inexpensive but can suffer from low selectivity and sensitivity. Mass-spectrometer-based sensors are fairly selective and sensitive with the appropriate sample preparation, but they are expensive and require a high level of expertise to maintain and operate. Also, while recent advances in sensor technology have been significant, it is the opinion of the author that no system currently available can completely produce data equivalent to human olfactory perception. However, this is likely to change as sensor technology and an understanding of human brain science and cognition advance.

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22. High-Throughput Receptor Screening Assay

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The recent technical advances in functional expression of olfactory receptors (ORs) make full deorphanization of human OR repertoire a realistic objective. Such a global knowledge of the precise mechanisms of odorant/receptor pairings will represent a crucial step for the development of an accurate model of how human nose perceives its chemical environment. Beyond its interest for basic science, it will also lead to the development of industrial applications such as receptor-based odorant design, development of selective odor blockers or enhancers and represents therefore an interesting opportunity for players active in the field of flavors and fragrances. Here, we will describe and discuss a high-throughput screening approach that aims at the objective of human OR mass deorphanization. However, the completion of this ambitious task is not a prerequisite to the development of commercial applications. With the expanding number of deorphanized ORs, structure–activity relationship studies on OR responding to an odorant of interest has already started. Likewise, the use of the screening approach to identify either blockers for malodor-responding ORs or positive enhancers of fine fragrance-tuned ORs is underway. These different aspects will also be discussed. Finally, beyond the human ORs, other classes of human chemoreceptors for volatiles as well as animal chemoreceptors may also represent industrial opportunities that will be briefly reviewed.

22.1 Working with ORs

The discovery of olfactory receptors (ORs) by *Buck* and *Axel* [22.1] as well as the experimental confirmation of the combinatory theory of odor coding [22.2], initially proposed by *Polak* [22.3], have paved the way for an accurate understanding of the molecular mechanisms of olfaction. Beyond their fundamental interest, these pioneering researches have also led to consider industrial applications such as the design of antagonists that would selectively block the perception of a given malodor, the setup of positive allosteric modulators (PAMs), molecules with no or only weak intrinsic olfactory properties but that could boost the perception of other odorant, the identification or design of new molecules that would share the same odor characteristics than expensive compounds used in perfumery.

22.1.1 Interest of Massive Deorphanization of ORs

The proper achievement of these applications needs a deep and accurate comprehension of the odor coding, and deciphering this code will require an extensive knowledge of the interactions of each individual odorant with its ORs. The combinatory theory of olfaction predicts that the odor perception elicited by one molecule will rely on the activation of at least one, but most likely several different odorant receptors. Therefore, it is important to know different receptors that are implicated in the detection of the target odor prior to consider modifying the perception by acting on these receptors. So far there are few demonstrations of the involvement of a determined OR in the perception of a given odorant molecule. In a study combining in vitro characterization of the OR7D4 and genotype-phenotype analysis, *Keller* et al. [22.4] were able to demonstrate that this OR plays an important role in the perception of the male sweat malodor component androstenone. Likewise, the hypersensitivity to isovaleric acid has been linked to the presence of the functional haplotype of the segregating pseudogene OR11H7P [22.9]. Along the same line, the variation of detection threshold of *cis*-3-hexen-1-ol among the population can be explained by different sensitivities of the haplotypes of OR2J3 [22.15] while the sensitivities for β-ionone and guaiacol odor intensity are dependent on the presence of the functional allele of OR5A1 [22.17] and OR10G4 [22.5], respectively. These studies have often been benefited by previous reports on the relatively high frequency of specific anosmia, hyposmia, or hyperosmia that have allowed making the link between the perception phenotype of the considered odorants and the genetic defect in the corresponding receptor. Although the identification and frequency reports of anosmia or hyposmia have been performed for several odorants (for example [22.22, 23]), these data are far from being able to cover all the odorants of interest. Moreover, to be of use, the frequency of the phenotype must be relatively high in the population in order to get a sufficient occurrence in a reasonably sized cohort of tested volunteers. This may not be the case for many odorants.

A complementary approach for deciphering the olfactory code consists in a systematic deorphanization of the ORs of the species of interest (human, in our case). It took several years between the first identification of mammalian ORs and the demonstration of the activation of a mouse OR by an odorant. Now, more than 20 years later, the number of OR deorphanizations remains low. Based on a compilation of published results, it appears that one or more ligands have been identified for at least 55 human ORs (Table 22.1). Knowing that the human genome encompasses about 400 sequences that encode a complete and potentially functional OR protein [22.26, 27], it can be considered that about 14% of the human OR repertoire is deorphanized. This per-

Table 22.1 Deorphanized human ORs publicly reported

OR	Agonist	Reference
OR10A6	3-Phenylpropyl propionate	[22.5]
OR10G3	Ethyl vanillin	[22.6]
OR10G7	Eugenol	[22.6]
OR10G9	Ethyl vanillin	[22.6]
OR10J1	PI23412 (Sandal wood)	[22.7]
OR10J5	Lyral	[22.8]
OR11A1	2-Ethylfenchone	[22.6]
OR11H4	Isovaleric acid	[22.9]
OR11H6	Isovaleric acid	[22.9]
OR11H7P	Isovaleric acid	[22.9]
OR1A1	Citronelal	[22.10]
OR1A2	Citronelal	[22.10]
OR1C1	Linalool	[22.6]
OR1D2	Bourgeonal	[22.11]
OR1E3P	Acetophenone	[22.12]
OR1G1	1-Nonanol	[22.13]
OR2A25	Geranyl acetate	[22.6]
OR2AG1	Amyl butyrate	[22.14]
OR2B11	Coumarin	[22.6]
OR2C1	Nonanethiol	[22.8]
OR2J2	1-Octanol	[22.8]
OR2J3	*cis*-3-Hexen-1-ol	[22.15]
OR2M7	Citronellol	[22.8]
OR2W1	1-Octanol	[22.8]
OR3A1	Helional	[22.16]
OR4D6	β-Ionone	[22.17]
OR4D9	β-Ionone	[22.17]
OR4E2	Amyl acetate	[22.5]
OR4Q3	Eugenol	[22.5]
OR51E1	Isovaleric acid	[22.18]
OR51E2	Propionic acid	[22.8]
OR51I2	Isovaleric acid	[22.19]
OR51L1	4-Allylphenylacetate	[22.8]
OR52A5	4-Ethyloctanoic acid	[22.20]
OR52B2	Decanoic acid	[22.20]
OR52D1	Ethyl isobutyrate	[22.13]
OR52E1	Butanoic acid	[22.20]
OR52E8	3-Hydroxy-3-methylhexanoic acid	[22.20]
OR52L1	Pentanoic acid	[22.20]
OR56A1	Decyl aldehyde	[22.6]
OR56A4	Decyl aldehyde	[22.6]
OR56A5	Decyl aldehyde	[22.6]
OR5D3P	Raspberry ketone	[22.21]
OR5A1	β-Ionone	[22.17]
OR5A2	β-Ionone	[22.17]
OR5K1	Eugenol methylether	[22.6]
OR5P3	(+)-Carvone	[22.8]
OR6P1	Anisaldehyde	[22.5]

Table 22.1 (continued)

OR	Agonist	Reference
OR7A1	Myrac	[22.24]
OR7C1	Androstenone	[22.5]
OR7D4	Androstenone	[22.4]
OR8B3	(+)-Carvone	[22.5]
OR8D1	Sotolon	[22.6]
OR8H1	Scatole	[22.25]
OR8K3	Menthol	[22.6]

centage could even be re-evaluated taking into account that a significant portion (32%) of the human OR genes could be functional pseudogenes, despite their complete coding sequence [22.28].

Notwithstanding this rather optimistic view on the progress of human OR deorphanization, a huge effort remains to be done in order to complete the general human olfactory map. This implies a systematic testing of a diversified odorant molecule library, covering an as large as possible diversity of chemical structures and organoleptic properties, on the human orphan ORs. Regarding the number of chemicals that can effectively be recognized by the human nose, which is far over 10 000, it is impossible to cover the whole range of odorants by a single screening campaign. Owing to the large number of (human) ORs, the even higher number of odorant molecules and the combinatorial nature of olfaction coupled to technical difficulties, deciphering the olfactory code and developing applications seem to be long-term goals. Nevertheless, clear progresses have been made in recent years; they will be further presented in this chapter.

22.1.2 Brief Overview on Different Approaches Used to Deorphanize ORs

To get a grip on OR deorphanization, one needs to make use of a robust system that allows to record the activation of an OR by its ligand. Different experimental systems have been successfully used to demonstrate such interactions and have already been reviewed [22.29–31].

Briefly, a first category of systems consists in working directly on a living animal or on ex-vivo samples where the ORs are expressed. For example, using an adenovirus to overexpress the I7 receptor in rat olfactory epithelium, *Zhao* et al. [22.32] were able to identify octanal as an activator of this OR using electro-olfactogram records on prepared olfactory mucosa. The response of isolated olfactory neurons to odorants and the subsequent identification of the implicated receptor by single cell reverse transcription polymerase chain reaction (RT-PCR) also led to successful deorphanization of mouse ORs [22.2, 33]. However, these approaches can hardly be considered for mass deorphanization of human ORs.

The second category of systems refers to the in vitro expression of ORs in cultured cells. This approach has been widely used to deorphanize ORs. The cell type used can be directly derived from the olfactory epithelium [22.34–36], from heterologous mammalian cells such as human embryonic kidney 293 cell line (HEK293) (see among others [22.13, 16, 37–39]) or Hela [22.40]. Other models such as insect cells where the OR gene has been introduced via a baculovirus system [22.41, 42] or *Xenopus* oocytes injected with the complementary ribonucleic acid (cRNA) corresponding to the OR of interest [22.43] have also been described. Yeast cultures have also been considered for ORs expression [22.44–47]. In these systems, yeasts have been engineered in order either to couple the cell growth on deficient media with the activation of the studied OR or to induce the expression of a reporter gene. Finally, this cell model has also been used to develop microsensors based on the measurements of OR activity by microconductimetry or by surface plasmon resonance.

In this chapter, we will describe the screening platform dedicated to OR that has been set up at ChemCom. The application to antagonist and PAM research will also be presented and discussed.

22.2 How to Identify Ligands with in vitro Functional Assays

22.2.1 Deorphanization

Upon the initial discovery, it took several years before the reliable identification of ligands for ORs. The reason for such a delay is to find in the difficulty to express ORs in usual heterologous cell cultures that were shown suitable for identifying activators of nonolfactory G protein coupled receptors (GPCRs). It was observed that ORs expressed in these systems are not or only poorly targeted to the plasma membrane of the cultured cells [22.36, 48–50]. Different strategies have been followed to fix this problem. Some consist in adding the N-terminal sequence tag to the ORs in order to avoid their retention in the endoplasmic reticulum [22.16, 37, 51–53]. Although improving the targeting of several ORs, this approach was not found to be sufficient to allow the cell surface expression of any OR. Another approach has consisted in expressing, along with the

OR, ancillary elements such as the odorant response abnormal protein 4 (ODR-4) [22.36] or receptor transport proteins (RTP) 1 and 2 [22.39]. These chaperone proteins have been shown to enhance the targeting of ORs to the plasma membrane in cultured cell lines. Although their mode of action is not yet fully understood, the expression of the short version of RTP1, RTP1S, and RTP2 was found to be the most efficient system to allow a functional expression of human ORs in our hands. Therefore, we developed a HEK293T cell line that stably expresses both chaperones. This cell line has served as a major tool for deorphanizing of human ORs.

From this cell line, the activity of an OR can be shown using different functional assays (Fig. 22.1) that rely on the modification of a second messenger concentration occurring upon the triggering of the OR by its activator. Dose–response analyses performed on HEK293T cells stably express RTP1S and RTP2 that have been transiently transfected with OR2W1 along with $G_{\alpha 15olf47}$ (Fig. 22.1a), a modified version of cyclic-nucleotide gated chanel subunit 2 A (CNG2A) (Fig. 22.1b), no additional factor (Fig. 22.1c), cyclic adenosine monophosphate (cAMP) response element (CRE)-luciferase (Fig. 22.1d) or CRE-GFP (green fluorescente protein) (Fig. 22.1e). In the olfactory neuron, the activation of the receptor results in a stimulation of the $G_{\alpha olf}$ protein that in turn activates type III adenylate cyclase. The ensuing increase of cAMP concentration opens a cyclic nucleotide gated (CNG) channel that allows an entry of extracellular calcium in the cytoplasm of the cell (for recent reviews, see [22.54, 55]). Different functional assays have been developed based on the element of this olfactory cascade. At ChemCom, we implemented different assays that can be used to deorphanize ORs or that may serve as secondary confirmation assays.

Calcium Imaging-Based Functional Assays

Measurement of variations of cytoplasmic calcium concentration has been widely used to monitor the activity of GPCR and ion channels. It can be readily performed using fluorescent calcium-sensitive dies, such as Fura-2 or Fluo-4 [22.56]. Calcium imaging-based assays have been used to demonstrate the functionality of ORs (among others, see [22.33, 37, 40]). Actually, two types of calcium assays have been described. The first relies on the use of a type *q* G protein that couples the activation of the OR with the release of calcium from intracellular stores to the cytoplasm. The latter is based on the use of either the native form or a modified version of the olfactory CNG channel.

Some of the family I of GPCRs are able to activate a G_q protein that triggers the phospholipase C/inositol triphosphate (PLC/IP3) pathway, leading to a release of calcium from intracellular stores to the cytoplasm. The existence of such a transduction cascade has been suggested in olfactory neuron (reviewed in [22.57]) but, contrasting with the G_{olf}-cAMP-CNG cascade, it does

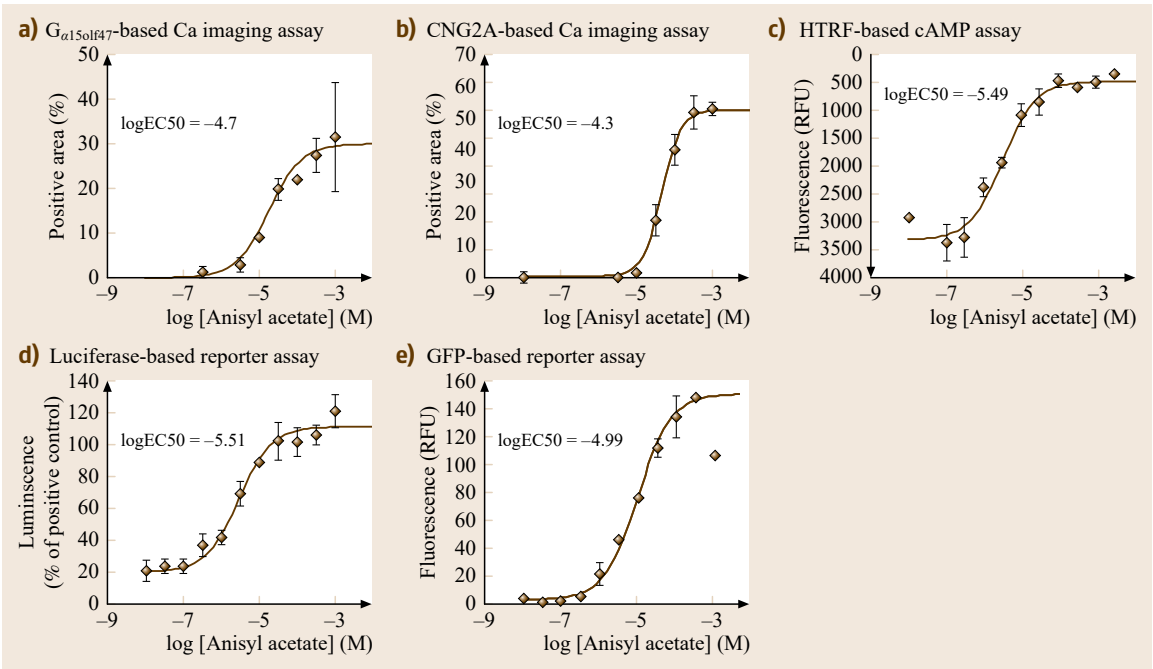

Fig. 22.1 Responses of OR2W1 to anisyl acetate measured with different functional assays

not seem to be mandatory for odor recognition and its physiological significance remains unclear. It has been reported in HEK293 cells that a calcium response occurs upon OR activation without the need of exogenous coupling G protein [22.11, 16]. However, we and others were not able to detect OR activation under this condition in our cell line. Other studies have shown that introduction of an promiscuous G protein such as $G_{\alpha 15}$ or $G_{\alpha 16}$ [22.13, 37, 38] or the chimeric $G_{\alpha qo}$ where the last 5 amino acids of $G_{\alpha q}$ were swapped for the corresponding residues of the $G_{\alpha o}$ [22.52] are required to allow the coupling with the PLC/IP3 pathway. In our hand, the chimeric $G_{\alpha 15olf47}$ was found to be the most efficient G protein for coupling the ORs to the intracellular calcium cascade.

In HEK293 cells that transiently express an OR along with a coupling type *q* G protein, an increase of fluorescence emission of the calcium-sensitive dye reflects the calcium burst that occurs upon activation of the receptor. This fluorescence increase can readily be monitored under a fluorescence microscope. An advantage of this experimental approach relies on its resolution at the cell level. Indeed, even if 1 or 2% of the studied cell population gives a recordable response to the stimulation by an odorant; they can be detected and an analysis can be performed. This may not be the case with conventional fluorometry that averages the response of the studied population. In this case, the response of a small percentage of cells will be too low to be interpreted as specific signal. It is probably the reason why quantification by calcium imaging under a microscope has been widely used to characterize the functional response of ORs. Experiments performed at ChemCom on several tens of receptors indicate that the percentage of responding cell determined by calcium imaging under a microscope upon transient transfection of the receptor may vary from 1% or less to about 50% of the cell population, depending on the OR tested.

The kinetic of calcium release in the cytoplasm of the cell, visualized by the increase of fluorescence emission (Fig. 22.2) can also be determined by microscopy-based calcium imaging. The burst of calcium is relatively rapid and occurs typically a few seconds upon injection of the OR activator. It recovers more slowly and a complete return to the basal level is observed within 2 min after injection (Fig. 22.2a). This characteristic time course of the calcium response has consequences when considering the G-protein-based calcium assay for screening purposes, as discussed in the following.

The time course of calcium response induced after a stimulation of OR2W1 with 1 mM anisyl acetate has

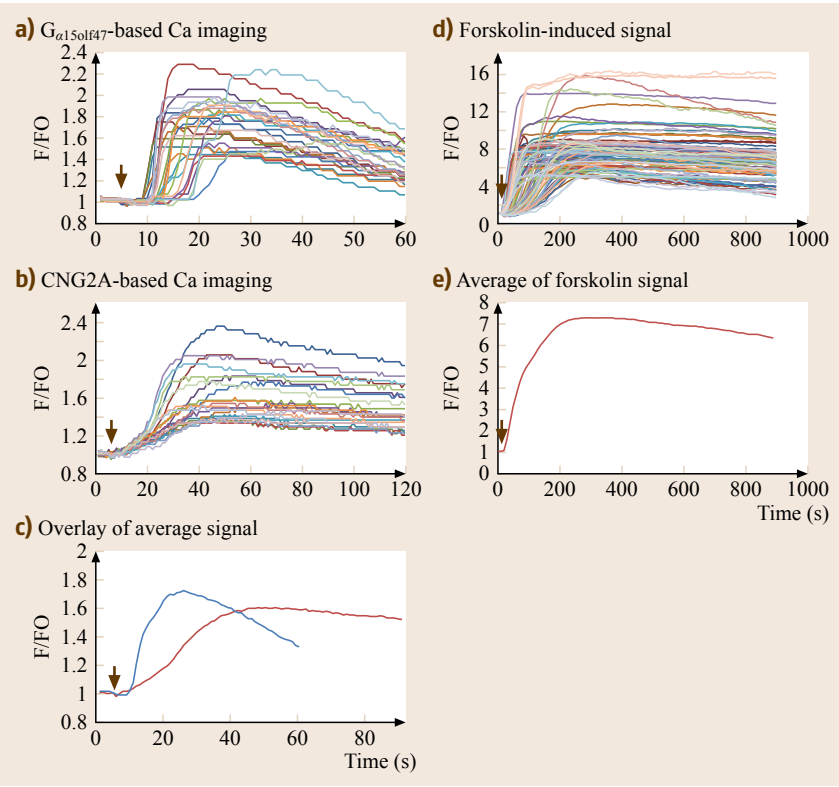

Fig. 22.2 Comparison of calcium responses relying on $G_{\alpha 15olf47}$ *or on CNG2A*

been determined using the $G_{\alpha 15olf47}$-based (Fig. 22.2a) or the CNG2A-based (Fig. 22.2b) calcium imaging assay. Each trace on the graphs in Fig. 22.2a,b represents the calcium fluctuation in a single cell. Arrows indicate the injection of the ligand. Note the difference of time scale between both graphs. The graph in Fig. 22.2c compares the average of traces from the graphs in Fig. 22.2a,b and clearly shows the difference in the response time courses.

The long-lasting signal observed with the CNG2A-based assay is further illustrated in graphs in Fig. 22.2d,e, where cells were stimulated with 10 μM (micromolar (μM)) forskolin in the presence of isobutyl methyl xanthine (IBMX). The signal is still close to its maximum 15 min after stimulation.

The use of CNG as an agent allowing a calcium response to OR stimulation in a heterologous expression system has also been described [22.40, 50]. Under these conditions, the calcium increase follows a slower kinetic. The firing of the cells begins more than 20–30 s after stimulation and a plateau is obtained after about 40 s (Fig. 22.2b). The recovery to the basal level can take several additional minutes (Fig. 22.2d,e). When the olfactory-specific guanosine triphosphate (GTP) exchange factor Ric8b [22.58] is co-expressed, it tends to reduce the latency, whereas the addition of the phosphodiesterase inhibitor IBMX slows down the return to the basal level.

From an experimental point of view, the rapidly fluctuating signal observed when using $G_{\alpha 15olf47}$ leads to take a picture every second during at least 1 min. Thus, the assay is slow and limits the throughput to 400–500 assay points per day. On the other hand, the long lasting and more stable signal observed when using CNG is compatible with the development of an assay based on end-point measurement. It consists in measuring the fluorescence just before the injection of the putative activator and executing one second measurements several minutes (5 min) after the injection. When working with multiwell supports, such as 96 well plates, a whole plate can be read within 5 min on a microscope equipped with a programmable automated stage and with a single injector. Thus, a throughput of 3000 assay points per day is a realistic estimate taking into account the handling of the plates and the setting up at the microscope. It needs, however, to be noted that the system works well for screening of a limited number of compounds to be injected because it limits the handling time. Therefore it is more tailored for the screening of one molecule of interest against a large array of receptors.

Direct Measurement of Cyclic AMP

The increase of intracellular cAMP has also been used to demonstrate the activation of ORs in a heterologous expression system [22.18, 58, 59]. Different cAMP assay kits are commercially available. At ChemCom, we have successfully used the homogenous time resolved fluorescence (HTRF)-based cAMP assay developed by Cisbio [22.60]. It consists in a competitive immunoassay between native cAMP produced by cells and the cAMP labeled with the dye d2. The tracer binding is visualized by a Mab anti-cAMP labeled with Cryptate. When excited by a 337 nm ultraviolet (UV) source, Cryptate normally re-emits a 620 nm wavelength light. Upon binding to labeled cAMP, the energy is transferred to the d2 dye and re-emitted at a 665 nm wavelength. The specific signal (energy transfer) is inversely proportional to the concentration of cAMP in the sample.

This type of assay is relatively simple to put in use and does not require additional elements such as coupling proteins. It can be set up for screening application. However, its cost can be deterrent for large scale screening campaigns and it is therefore better adapted as a secondary assay used for confirmation purposes.

Gene Reporter-Based Assays

Many recent OR deorphanizations have been performed using a reporter-based assay. It consists in introducing into cells, along with the OR of interest, a gene under the control of a cAMP sensitive reporter. Upon the stimulation of the OR and subsequent increase of cAMP, the reporter gene is transcribed and translated into a detectable protein. Several hours (typically, 4 h) after adding the stimulating agent, the reporter can be quantified. One of the advantages of this type of assay relies on its high sensitivity that is explained by the signal amplification that occurs at different steps between the triggering of the receptor and the synthesis of the reporter protein.

The most commonly used reporter corresponds to the firefly luciferase. The enzymatic activity of this protein can readily be quantified by a luminescence-based assay. The Dual-Glo assay provided by Promega allows a stable light emission that lasts during more than 30 min after addition of the reagent buffer. This characteristic is suitable when processing large series of plates and is convenient for high-throughput screening (HTS) purposes. The Dual-Glo assay also relies on the constitutive expression of a second enzyme, the *Renilla* luciferase, that serves for normalization of the expression. This normalization is not mandatory if the transfection of the receptor and the firefly luciferase is well standardized. Under these conditions, transfection of the *Renilla* luciferase gene can be omitted.

Alternatively to luciferase, other reporter genes under the control of cAMP sensitive promoter can be used. The gene of the secreted alkaline phosphatase (SEAP)

has been successfully used to demonstrate the activity of chemoreceptors [22.61–63].

The well-known green fluorescent protein can also serve as reporter [22.64]. It can be revealed by conventional fluorometry or under a fluorescent microscope. In our hands, it is less sensitive than the luciferase-based assay due to the fact that a fluorescence signal is less well detected and has a weaker signal-to-noise ratio than a luminescence-based signal. Therefore, a longer incubation period (8 h or more) is required to obtain a detectable signal with well-working receptors. On another hand, no particular treatment is required to reveal the fluorescence and the cells can be kept alive after measuring the fluorescence. This can allow some interesting applications, such as selection of responding cell populations by flow cytometry-based cell sorting or correlation studies of receptor expression (revealed by immunofluorescence) and functionality.

22.2.2 High-Throughput Screening for ORs

The importance of nonchemosensory GPCRs in physiological and physio-pathological processes has made them a target of choice for new drug discovery [22.65, 66]. In order to find new inhibitors as well as positive or negative modulators for these GPCRs or simply activators in the case of still orphan receptors, a strategy consisting of a systematic testing of large repositories of hundreds of thousands of small molecules was adopted by the drug industry and some large academic labs in the 1990s. This approach, known as HTS has led to some successes in identifying valuable activators for GPCRs.

Setting up the System

For ORs, an HTS approach appears as a pragmatic way to systematically deorphanize these but must be adapted to match its particularities. Contrasting with the random search of agonists among thousands of different chemical structures, libraries used for ORs deorphanization are composed of known odorants, increasing considerably the chances of success. On the other hand, it would not make a lot of sense to focus on the deorphanization of one single OR with an extended library of compounds, as it is done for GPCRs. At the very outset, all orphan ORs are of the same importance in the coding of odors. In fact, the OR-specific HTS must be seen as bi-dimensional by contrast with the classical, monodimensional HTS applied to pharma GPCRs. The first dimension corresponds to the molecules of the library to be tested (as in the case of HTS for pharma GPCRs) and the second dimension corresponds to the number of ORs considered. Therefore, the optimum strategy for the deorphanization of ORs consists in testing midsize libraries of odorants on a large array of ORs.

The construction of an expression library that contains all the purportedly functional human ORs constitutes a mandatory prerequisite to that kind of large-scale deorphanization screening. At ChemCom beginnings in 2000, we dedicated a significant effort in analyzing the weekly releases of the still in progress human genome sequencing project in order to constitute a database of human OR sequences. The same work was also done and published by other labs [22.67–71]. The collected information was used to clone the 388 unaltered human OR genes from genomic deoxyribonucleic acid (DNA) of different individuals into a mammalian cell expression vector.

The design of the odorant library may rely on different criteria. One of them might correspond to the organoleptic properties of the compounds. Ideally, the deorphanization library should contain representatives of all the category of odors. However, there is no general and accepted consensus for the description of odors and many different systems of odor classification have been proposed. Constituting the library on one particular classification might introduce a bias. A second criterion might correspond to the chemical structure of the compound. Although there is no obvious and systematic link between a given chemical function and a particular odor type, it can be assumed that a chemically well-diversified library will increase the chances to hit a large number of ORs. The availability of the compounds is also a nontrivial criterion. Unfortunately, there is no pre-made library dedicated to ORs research such as the chemical libraries that have been designed for the screening of GPCRs. Several hundreds of odor compounds coming from natural products or chemical synthesis are commercially available in flavor and fragrance (F&F) companies (e.g., Sigma Aldrich Fine Chemicals (SAFC) propose more than 1600 aroma raw materials in its catalog) and can be the primary source for deorphanization compounds. Finally, the construction of the screening library must rely on a compromise between these different criteria: the compounds will be chosen among known odorant catalogs and will cover different categories of odors. Since different classifications exist, one may consider compiling odor categories from different classification systems. Among different categories, attention will be paid to select the most diversified chemical structures.

Another important point when setting up a HTS concerns the choice of the functional assay. Again, it will have to fit different criteria that are mainly

1. The sensitivity of the assay
2. Its ability to be easily performed in large scale experiments
3. Its cost effectiveness.

A bunch of different functional assays have been used for HTS on nonsensory GPCRs (reviewed in [22.66]). For ORs, different assays that have been reported in the earlier section can be considered. The assays relying on a transient increase of intracellular calcium concentration imply that the measurement must be performed shortly after injection of the putative activator into the incubation buffer. Therefore the use of these assays for the screening of large libraries of compounds requires devices, such as the Fluorimetric Imaging Plate Reader (FLIPR) system from Molecular Devices or the Functional Drug Screening System (FDSS) from Hamamatsu Photonics, that are able to manage multiple injections and the recordings of several tens of conditions at a same time. Such kind of devices have been used to deorphanize ORs [22.40], but remains expensive. Assays based on the direct measurement of cyclic AMP, such as the HTRF-based cAMP assay, can also be considered for HTS. However, their cost can be deterrent for large-scale screening campaigns. At ChemCom, we have adopted the luciferase-based reporter gene assay as a primary screening assay. In addition to its sensitivity, it is relatively easy to achieve since it just requires a single step of reagent buffer addition prior to the reading. This assay can be fully automated, allowing a reasonable throughput of 5000–6000 measurements per working days, including controls. Typically, a large-scale deorphanization screening may involve a 400-compounds library and 300–400 different ORs that represents 432 000–576 000 measurements (each compound is tested at three different concentrations; control included).

Short Description of a HTS Run

The first step of screening consists in preparing screening libraries to be used during the entire screening campaign. Different compounds are diluted at a fixed concentration in a compatible solvent with the solubility of the compounds and the cellular assays. Usually, dimethyl sulfoxide (DMSO) is used as a solvent since it is relatively well tolerated by the cells and does not influence the functional response when used at a final concentration below 0.3% (v/v). For compounds that do not dissolve into DMSO, ethanol may be used as an alternative. The stock library is formatted in 96 well plates. Eighty wells are dedicated to screening compounds whereas the remaining 16 wells contain controls including blank (solvent alone), the positive activator forskolin that gives a maximum luminescence response and the ligand used to activate a reference OR. The stock library is stored at −20 °C and is used during the whole screening campaign. It is thawed at the day of the experiment to prepare three dilution plates, corresponding to the final concentrations to be tested in the μM range (in the incubation medium EMEM (Eagle's modified essential medium)). This dilution step is performed by the automated pipetting station Sciclone (Fig. 22.3).

The *Sciclone* (a) from Perkin Elmer corresponds to the compound management and liquid handling workstation with a *96 head*. It is linked to a *Twister* (b), a high capacity plate stacker. This stacker is a plug-and-play system offering flexibility. The *Twister* can manage three racks (c) that may each contain thirty 96-well plates. This station is also composed by a thermal plate sealer *PlateLoc* (d) from Velocity 11 that allows the sealing of the compounds plate after addition of argon gaz. This station is used to prepare the odorants plate at different concentrations, to carry out the serial dilutions, and to add the odorants on the assay plate for screening and/or validation experiments. The station is further equipped with a barcode reader (f). Each plate is labeled with a unique barcode in order to be tracked during the whole process. At this stage, the precise injection protocol is linked with the plate.

In a screening run or a validation experiment, after addition of ligands to the cells, the racks containing 96 well-culture plates are transferred to the incubation station corresponding to a CO_2 incubator (g) located close to the Sciclone pipetting station and exclusively dedicated to the incubation of the odorants during the assay.

The *Multidrop* station is composed by a liquid dispenser *Multidrop* (h) from Thermo scientific and a Twister plate stacker (i). This liquid handling device is used to add the lysis buffer at the end of the incubation.

The reading station is composed of the Spectramax M5 microplate reader (j) from Molecular Devices,

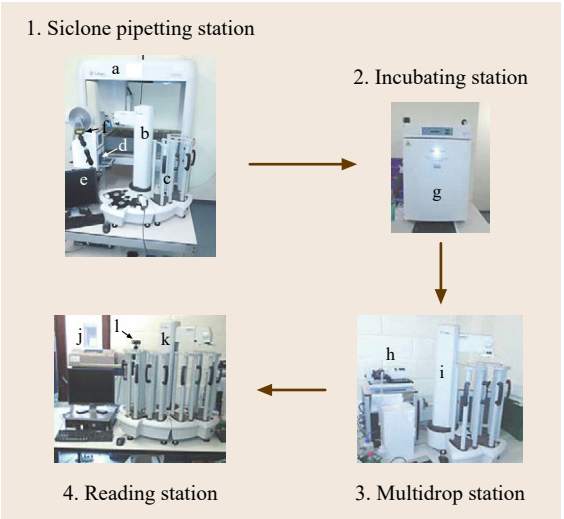

Fig. 22.3 The screening platform developed at ChemCom

a Twister plate stacker (k) and a barcode reader (l). After addition of the buffer, the plates are positioned on the twister, scanned and are red by the M5. The data are transferred along with the injection protocol to the screening database that performs the automated analysis for hit selection.

For each screening run, the cells are seeded in multiwell plates for which each well will represent a defined condition to be tested. Although different types of multiwell plates exist, ranging from 6 to 1536 wells per plate, we selected the 96-well type after a series of set up experiments. Higher formats of 384 or 1536 wells per plate required to be processed by robots for each pipetting operation, including cell seeding that requires to be performed in a sterile environment. The cell line used for screening is a HEK293T cell clone that stably expressed both RTP1S and RTP2 chaperone proteins [22.39, 72] and that was selected based on its ability to give the best functional response when expressing model ORs. For screening, the selected ORs are transfected along with the reporter gene using a standard lipofectant. This step is performed manually one day after the cell seeding. Sixteen hours after transfection, cells are incubated in the presence of the screening compounds. The addition of screening library to the cells is fully automated on the HTS platform (Fig. 22.3). After addition of compounds, the plates are stacked by a robotic arm on racks that can be put directly in a cell culture incubator for the 4 h incubation step. At the end of the incubation, the reaction buffer is added to the cells and after at least 30 min of incubation, the light emission due to the luciferase reaction is read on the Spectramax M5 luminometer. Again, these later steps, consisting of buffer addition and luminescence reading, are automated.

The main step of the screening relies on the determination of potential activators that are called *hits*. At ChemCom, we have developed a hit selection method based on a statistical analysis that is performed by plate. For each plate, the distribution of the responses is determined and the average value of the distribution is calculated. A well that shows a response higher than the value of average +2 standard deviations is considered as positive. The presence of outlier values (responses elicited by strong activators) can make the distribution of experimental data diverge from a normal distribution and can finally result in a skewing of the selection. Therefore, a Shapiro–Wilk test is first used to determine whether the distribution fits the Normal law (Fig. 22.4). If not, extreme points are removed and standard deviation is re-computed. To be granted as a hit, a compound must have produced a luminescence signal that exceeds the average +2 standard deviations value of the plate for at least two out of the three concentrations tested.

The hits are further validated by concentration–response analyses. Four independent tests of hit used at concentrations ranging from 10 nM to 316 µM are performed using the luciferase-based reporter assay. To be validated, the compound must produce a concentration-dependent increase of the luciferase signal that exceeds twice the value obtained for cells incubated with the buffer alone, in at least three out of the four tests. Additional experiments are performed to demonstrate that the activation is well receptor dependent by comparing concentration–response measurements performed in presence or in absence of the receptor. Confirmations

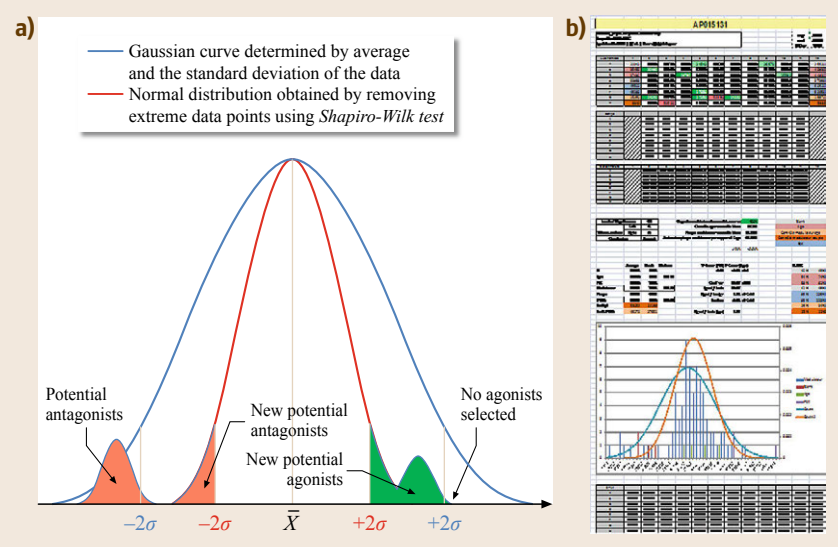

Fig. 22.4a,b Hit selection analysis. **(a)** Schematic representation of the analysis method used to determine hits in the screening procedure. *Blue line*: Gaussian curve determined by average and the standard deviation of the data. *Red line*: Normal distribution obtained by removing extreme data points using *Shapiro–Wilk* test. **(b)** Example of an analysis template of screening

of the specific response of the hits on their respective ORs are also achieved using alternative functional assays, such as calcium imaging-based assays or direct measurement of cAMP, as described earlier.

22.2.3 High-Throughput Screening for Single Compounds

For molecules of interest, it can be worth considering an alternative screening approach that consists in screening the human OR library with the selected compound. This screening of ORs is performed in a similar way than the deorphanization screening with the notable difference of introducing 80 different ORs per 96 well plate. Each receptor is challenged with three different concentrations of the tested odorant (one plate per concentration). Hits are selected by applying the average +2 standard deviations method. However, we have observed that some of the receptors may present a basal activity that can be significant and may introduce a bias into the analysis. Therefore, an additional control, consisting in a plate containing the receptors that are incubated with buffer alone, is systematically introduced during the screening of ORs and serves to normalize the values of OR stimulated by the molecule of interest.

The validation process is achieved by concentration–response analysis as described earlier. Its extent is hardly predictable since it depends on the number of hits that may vary in function of the tested odorant. As an example, if hits are found for 50 ORs during the screening phase, a complete validation process can be performed within a week. Under these conditions, the screening of ORs has been proved to be, in our hands, a valuable and rapid approach to find matching receptors for odorants of interest.

22.3 Applications

22.3.1 Odorant-Receptor Characterization and Design

Once a first activator is identified for an OR, it is of interest to determine the range of selectivity of the receptor. According to the current odor coding theory, an OR may respond to more than one ligand and these activating compounds may present clearly different structures although sharing common points. The way to unravel the receptor selectivity consists in performing structure–activity relationship (SAR) studies that use the first identified activator as a starting reference [22.73]. One example of such SAR approach applied to OR has been provided by *Araneda* et al. [22.74] who showed that the rat I7 receptor was activated by aliphatic aldehydes having a length between ≈8 and ≈12 Å, with octanal as a reference agonist. The list of identified activators includes branched aldehydes such as (among others) 7-methyloctanal or 3,7-dimethyloctanal, and double bound-containing aldehydes such as *trans*-2-octenal or *trans*,*trans*-2,4-octadienal. It was further demonstrated by exploring the activating potential of different octanal analogs that, in addition to their aldehydic moiety, activators must have an optimal length exceeding 6.9 Å, as it is the case for octanal in its extended conformation [22.75]. Aldehydes of at least five carbons with a shorter length, below 6.5–6.9 Å, were found to bind but not to activate I7 and turned out, to be antagonist of the receptor. Another example of the SAR study was performed on the mouse mOREG receptor [22.38, 76, 77]. The 4-allyl-2-methoxyphenol, also known as eugenol, was found as the initial activator of this receptor. Series of molecules where one of the three functional groups linked to the benzene ring of eugenol was replaced by an alternative one were assessed. It allowed determining what kind of functional group can be tolerated in each position. For example, replacing the allyl group by an aldehyde (generating vanillin) or by a ketone does not affect the ability of the molecule to strongly activate mOREG. In contrast, a methyl or an ethyl moiety at this para position reduces the activating potential of the molecule whereas a carboxylic acid or an amine completely kills it. Likewise, the methoxy functionality in the ortho position can be substituted by an ethoxy or a methyl rest but not by a hydroxyl group. The swapping of the hydroxyl moiety of eugenol for an ethoxy or an acetate group results in a decrease of activity. It was later found that mOREG can also be activated by other classes of molecules nonrelated to eugenol, including other benzene-derivatives, polycyclic compounds, and cyclohexane-derivatives [22.63]. At ChemCom, the receptive range of OR51E1 was investigated by testing different series of carboxylic acids, starting with valeric acid as the reference ligand. It was found that the optimal structure, among the 60 different molecules tested, was the 5-norbornene-2-carboxylic acid. This compound possesses a 6-carbon ring where the carbons in positions 2 and 5 relatively to the carboxylic function are bridged by an additional $-CH_2$, conferring a rigid tri-dimensional shape with a calculated volume of 125 $Å^3$. The volume of the 5-norbornene-2-carboxylic acid must fit

the bulk of the receptor binding pocket since additional methylations of the bridged ring completely abolish the activating potential of the molecule.

Identification of the activators of a given OR associated with a 3-D modeling of this receptor can serve to predict the ligand binding pocket and its mechanism of interaction with the agonists. This type of in silico investigations have been performed on different human and murine ORs (see among others, [22.10, 43, 63, 74, 78, 79]; for a review see [22.80]). Based on this, modeling predictions of new activators can be executed and further confirmed experimentally. However, there are very few examples of a successful identification of new activators based on the in silico modeling of OR-agonist interactions and this approach still needs to be fed by experimental data. An alternative approach has also been proposed that exploits the results of large SAR studies to predict the suitable structure a compound must have and what it must avoid to be a bona fides activator of a selected OR [22.8, 81]. It consists in the identification of the chemical determinants that are shared by the identified activating compounds and in the computation of a 3-D model of a generic activator. New molecules that fit the model may be experimentally assessed as potential agonists. It needs to be noted that this data mining approach requires extensive SAR studies in order not to miss activating compounds that are only poorly or even not structurally related to the initial identified agonists. The aforementioned example of mOREG that was initially found to respond to eugenol-related compounds and more recently demonstrated to be activated by other unrelated families of agonists, is well illustrative of this necessity.

For some ORs, the receptive range appears so huge that the deduction of a generic agonist structure seems a hopeless task. Some examples of very broadly tuned receptors have been described [22.10, 82] but in our hand, OR2W1 is viewed as the archetypal example of that kind of OR. About 200 different activating molecules have been identified so far. This receptor also possesses the highest validated hit ratio of all the ORs we have deorphanized. In a screening performed with a chemically well-diversified library of 300 compounds, 9% of the molecules induced a validated activation of the OR. The agonist list includes aliphatic, monocyclic, or polycyclic alcohols, aldehydes, and carboxylic acids. Many aromatic compounds were also found among the activators. However, a more careful analysis of the potency of different agonists reveals some trends from this apparent lack of selectivity. For example, the optimal size of aliphatic activators corresponds to 7–8 carbon-containing chains. Considering other aliphatic chain-related molecules of the same length, *n*-alcohols are stronger agonists than aldehydes that are, in turn, stronger than acids. Likewise, saturated cyclic structures seem to be preferred over aromatic cycles as exemplified by the 1-cycohexylethanol that has a 10 time higher potency than its aromatic counterpart, the 1-phenylethanol. Interestingly, none of the molecule with a bulky moiety such as bridged cycles was found to activate OR2W1. Similarly, none of the tested molecules that contain primary amines are among the long list of agonists. At this stage, the tentative prediction about structural requirements for OR2W1 activators is that they must be more or less flat and must have both a hydrophobic and a hydrophilic moiety.

From different screening campaigns that have been performed at ChemCom, more than 100 ORs have been robustly deorphanized. Albeit not all of these ORs were submitted to extensive SAR studies, a global analysis of our data shows that receptors can be set in different categories regarding their agonist selectivity. Along with OR2W1, a series of ORs can be classified as broadly tuned since they respond to more than 20 different molecules among which obvious structural or organoleptic similarities are hard to identify. The OR1A1 that was previously reported to respond to a large series of activators [22.10] belong to this category. More surprisingly, OR1D4 that was found to respond to bourgeonal and to be expressed in both the olfactory epithelium and in sperm cells [22.11, 83] may also be labeled as broadly tuned. A second category encompasses ORs that respond to a structurally defined family of activators. For example, receptors activated exclusively by carboxylic acids have been identified. In addition to the earlier discussed OR51E1, other members of class 1 ORs present a similar selectivity for molecules with a carboxylic function. Receptors that are selective for agonists that contain a hydroxyl or an aldehyde group have also been discovered as well as ORs that only respond to cyclic nitrogen-containing aromatic substances such as pyrazines. The last category regroups receptors that are defined as narrowly tuned. In some cases, only a very small number of agonists is found for an OR, despite the effort invested in SAR exploration to identify alternative agonists. OR7D4 [22.4] is a good illustrative example of such narrowly tuned OR since it responds only to 5-α-androst-16-ene-3-one, androsta-4,16-dien-3-one, and with a very poor efficacy to androsta-4,16-dien-3β-ol. Likewise, OR8D1 is selectively activated by sotolone (3-hydroxy-4,5-dimethylfuran-2(5*H*)-one) and abhexone (3-hydroxy-4-methyl-5-ethylfuran-2(5*H*)-one), two structurally similar molecules that differ by the elongation of the methyl-function in position 5 of the cyclic lactone structure by one carbon moiety to yield an ethyl function in the latter. Recently, the range of selectivity of OR51E2

that shares a high degree of homology with OR51E1, was found to be restricted to two short chain carboxylic acids: propionic and acetic acids [22.84].

It is also worth exploring and comparing the receptive potential of closely related receptors. Indeed, some ORs corresponding to clearly differentiated genes in a genome may share a high degree of homology in their primary structures. A comparative analysis of the range of selectivity of these so-called paralogs may helps to understand the precise mechanism of interaction of the ORs with their agonists. The human OR subfamily 10G is an interesting example for such a multireceptor SAR study. Four of its members, OR10G3, OR10G4, OR10G7 and OR10G9 have been deorphanized by us (unpublished results) and by others [22.5, 6] with eugenol- and vanillin-related molecules. An SAR with a series of 28 analogous compounds with a structure derived from the 2-methoxyphenol (Fig. 22.5, compound 1) was performed on these four ORs. The trend that emerges from this study is that OR10G7 is more tolerant in term of selectivity, although it has a higher affinity for molecules with three carbon chain substitutions in the para position, relatively to the hydroxyl group on the benzene ring (Fig. 22.5, compounds 3, 4, 5, 14 and 23). OR10G3 showed a narrower selectivity, with a preference for compounds having a short hydrophobic substituent in the para position. OR10G4 and OR10G9, the very closely related paralogs of OR10G7 (the three ORs share up to 95% of homology in the transmembrane domains and loops), both responded to the same compounds. Their selectivity is also narrower than that of OR10G7 and more oriented to compounds with a short substituent in the para position. However, compared to OR10G3, they exclude different compounds from their agonist list. Interestingly, in a recent study [22.5], different haplotypes of OR10G4 were assessed in vitro and the data were correlated to the smelling performances of individuals bearing the different OR10G4 alleles. It turned out that OR10G4 can partly explain the intensity and valence perception of the 2-methoxyphenol (also known as guaiacol) but not of vanillin or ethylvanillin. Since we used a single haplotype of each receptor in our comparative SAR study on OR10G paralogs, it would be worth taking into account the allelic variation for the comparison of paralog's selectivity.

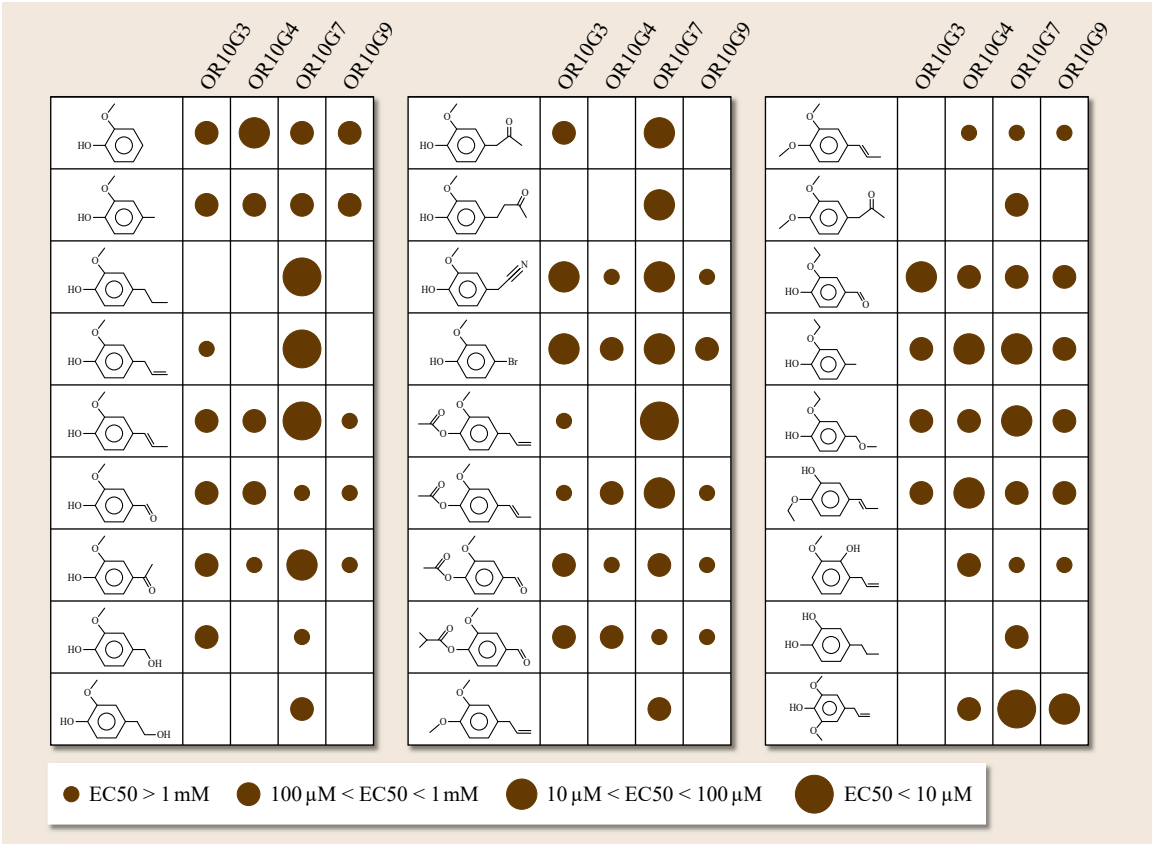

Fig. 22.5 Comparative structure–activity relationship study on OR10G receptors

From simple in vitro analysis based on heterologous expression systems, the interaction of a receptor with an odorant molecule can be considered as a clue but not as an evidence of the implication of this OR in the perception of the odor of its activator. The fact that many ORs are activated by several compounds with clearly different organoleptic properties reinforces the idea that the (cor)relations between the chemical structures and the odor families are not straightforward. However, some ORs are associated with molecules belonging only to well-defined odor families. In this case, dedicated SAR focusing on compounds that are known to possess a particular note may help to identify the corresponding ORs. Musk molecules, whatever their chemical structures: polycyclic, macrocyclic, nitro, and more recently alicyclic, are an example for which ChemCom has found very specific ORs. At ChemCom, different representatives of the musks families have been introduced in a screening library and several human ORs were found to respond to at least one musk molecule. After an extensive musk-oriented SAR, it turned out that some of the receptors are activated by representatives of the four chemical families that possess a musk note while others have a restricted profile of activation. This is reminiscent of mOREG and of the OR10G family described earlier. The association of ORs with different musk families is suggestive of their involvement in the perception of musky odors and makes them serious candidates for screening of additional libraries dedicated to the identification of new musk molecules. A similar example of association of human ORs with molecules sharing an amber note is also under investigation at ChemCom.

22.3.2 OR's Antagonists

Reducing the perception and the impact of bad smells represents an important challenge for the flavor and fragrance industry. Classical approaches for fighting malodors consist in overpowering the undesirable smell with a strong pleasant fragrance or in associating it with a weak but pleasant odor that makes the overall perception shift from unpleasantness to a more acceptable quality. Interfering with the perception of a malodor using an odorless agent that will specifically block the corresponding ORs appears as a new promising strategy. The recent advances in the expression and deorphanization of ORs has allowed to assess this concept experimentally. ChemCom has now evidences for the existence of odorless volatile blockers that counteract some malodors on human panels.

Identifying Antagonists

So far, there are some reports of molecules that were demonstrated to antagonize given ORs. They have been found either more or less serendipitously or by a more rational approach. For example, it was observed that undecanal inhibits the activation of the human OR1D4 by bourgeonal [22.11] and also reduces the perception of bourgeonal as shown by a psychophysiological study [22.83, 85]. In some cases, inhibitors were found among members of the same chemical family to which the agonist belong, as it is the case for methyl isoeugenol and isosafrol that antagonize mOREG [22.86, 87] or small aldehydes that antagonize the rat I7 receptor [22.75]. This makes sense since the mode of action of competitive antagonists consists in occupying the binding pocket and in stabilizing the receptor in an inactive conformation. If the antagonist has a stronger affinity for the receptor compared to the activator, the inhibitory effect is observed. It is therefore not surprising that molecules competing for the same binding site present strong structural similarities. A simple way to identify inhibitors for a receptor consists in checking the antagonist potential of structural analogs of its cognate agonist(s). Such an approach can also be coupled to 3-D-modeling of the receptor. A molecule that is predicted by a docking simulation to bind strongly to an OR but that did not activate the receptor when assayed experimentally has a good chance to be an antagonist. This is well exemplified by the study on the mouse receptor OR42-3 where undecanedioic and dodecanedioic acids were identified as antagonists by this way [22.43]. The data mining approach described by *Sanz* et al. [22.81] is a potential alternative to predict the antagonist nature of molecules. In this case, vanillin that was classified in a subgroup of molecules that contains other inhibitors of the human OR1G1 did well antagonize this OR in an in vitro assay.

It appears that this approach is not a definitive solution for identification of efficient antagonists of ORs. An OR may sometime share strong similarities with an agonist, ChemCom has discovered blockers that are completely different and hardly predictable based on the agonists' structure. As for activators, the systematic way to fish out these antagonists relies on an in vitro screening approach.

High-Throughput Screening of Antagonists

Although the principles that rule the design of an antagonist HTS are similar to those of HTS applied to OR deorphanization, some differences deserve to be pinpointed. First, the selection of the library of compounds relies on a quite different philosophy. Indeed, in contrast to deorphanization compounds that are preferably selected from known odorants, no organoleptic characteristic is predictive of an antagonist potential. Therefore, the paradigm for OR antagonist screening is the same as for other GPCRs: the larger and more chem-

ically diversified the library is, higher are the chances to find an antagonist. Already thinking to concrete applications, one might introduce some constrains such as selecting weak odorants or odorless compounds and molecules that are volatile. It may also be of interest to block pleasant odors. First from a basic view, it could allow the demonstration of the involvement of a given OR in the specific perception of a given odor. In other cases, it might allow the use of materials in applications where their strong bouquet could represent a limiting factor such as thymol for its bactericidal properties.

Contrasting with the deorphanization screening that is preferentially performed on a large array of ORs, the search of antagonists is restricted to OR that responds to malodors. On a technical point of view, an antagonist HTS run is very similar except that the agonist is added to the incubation medium along with the screened antagonist candidate. As usually done for screening of GPCR, the agonist is set at a concentration (efficacy concentration 80 (EC80)) that triggers 80% of the maximal response of the receptor [22.88]. It corresponds to a compromise between obtaining a well detectable signal and avoiding an over-saturation of the receptor that would hamper the identification of antagonists. This means that an accurate characterization of the OR interaction with its reference agonist by concentration–response analyses must have been performed prior to considering it for an antagonist HTS. When different activators of an OR are available, the most potent is often the best choice. As in the case of activator screenings, three concentrations of the putative antagonist are tested in order to limit the occurrence of either false positive or false negative results. The luciferase-based functional assay can also be used as primary assay for antagonist HTS and is performed as described earlier.

The hit selection process is also based on the same statistical method but, for antagonists, hits are defined as compounds that produce a response under the value of the mean minus 2 standard deviations. The selected hits are also validated by concentration–response experiments. A very important step in the validation process consists in assessing the specificity of the hit. Indeed, the tested chemicals might interact negatively with any of the components of the intracellular signal transduction cascade that is required to induce the luciferase synthesis or, more generally, they could produce a toxic effect that would result in a decrease of the measured signal. To identify these false positives, each hit is tested in concentration–response experiments on cells that do not express the receptor but are stimulated by forskolin, a direct activator of adenylate cyclase III that elicits a strong luciferase response. The hits that inhibit the forskolin-induced response and the OR agonist-induced response at the same concentrations are rejected.

Sensory Evaluation of Antagonists

Once confirmed, an OR-antagonist couple is the starting point for further characterization and investigation. When working on human ORs, the first question to answer is: Does the antagonist block or reduce the perception of given odors? This requires an accurate psychophysiological evaluation of the antagonist that must be performed on well-trained panelists able to score precisely the odor intensity in the presence or in the absence of the antagonist. Although there are several reports of antagonist identifications for murine or human ORs [22.11, 40, 43, 81, 86, 87], there is only one example, published so far, of an antagonist that has been found to inhibit a human receptor and that was further demonstrated to reduce the perception of the receptor's agonist [22.83]. The antagonist influence is even more difficult to demonstrate on mice, since animals cannot directly report on their perceptions. Therefore, humans remain the best models for studying OR antagonism. By analogy with drugs that antagonize GPCRs, the discovery of inhibitors for human ORs will provide tools for understanding the sense of smell in humans. OR antagonists will allow making the link between the activity of an odorant molecule on an OR (observed in vitro) and its result on the brain activity measured for example, in functional magnetic resonance imaging (fMRI)-based studies and finally on the perception assessed by a psychophysiological approach. It will be a way to demonstrate the involvement of a given OR in the perception of a given odor. Odorless blockers will be of high value in this respect. ChemCom identified several tens of antagonists of human ORs that are involved in the perception of pleasant odors or malodors such as sweat, bathroom, kitchen, mildew, urine and hair care products. Some were tested on human panels and have confirmed their in vitro activity in human noses.

Antagonist SAR Studies

Following the same philosophy as the one that prevails for activator, it can be worth investigating the structure–activity relationship of an OR-antagonist couple. That kind of studies can be motivated by different reasons such as identifying more appropriate inhibitors for specific applications or alternative blockers with more suitable physicochemical or organoleptic properties.

Exploring implies chemical synthesis which represents significant costs but will allow to identify new chemical structures leading to patent applications and the selection of the most appropriate compounds with respect to their use.

22.3.3 Modulators

The concept of allosteric modulation is emerging in the field of GPCR [22.89–91]. It is defined as the ability of a compound to interact with a region of a receptor that is different from the activator binding pocket and leads to a potentiation of the activation of the receptor by its cognate agonists that interact with the orthosteric binding region of the receptor (i. e., the binding pocket). A PAM may have no intrinsic ability to activate the receptor, although the case of allosteric agonism has been described. For GPCRs of pharmaceutical interest, PAMs represent a promising new approach since they can act more specifically and present reduced side effects.

Such modulators have first been identified for members of the family C GPCR that are characterized by the presence of a large extracellular domain. More recently, PAMs have been found for GPCRs of family A to which ORs belong. Although there is no example of PAMs described for ORs until now, this possibility remains to be investigated.

The screening of allosteric modulators is about the same than for antagonists. A particularity of the PAM screening is that the reference agonist is set at a concentration (efficacy concentration 20 (EC20)) that induces an activation corresponding to 20% of the maximal response of the receptor. The hit selection is performed as described for the agonist and hits are validated by concentration–response experiments where the reference agonist is set at its EC20. To discriminate between a true allosteric modulation and simple additional agonist effect, it is recommended to make a first preliminary agonist screening of the library on the selected OR. Such compounds must then be removed as they might also introduce a bias into the data analysis.

The ORs aimed at by this research are preferably selected within the OR responding to fragrances or flavors of interest. A PAM could boost the perception of a particular compound or even of a more complex bouquet. PAMs represent an important commercial value as some ingredients used in perfumery, such as hedione, have been empirically found to possess that sort of property. It is possible that they act as PAMs but their target receptors remain to be identified. On an economical point of view, PAMs could serve to lower the quantity of expensive odorants that are ingredients of commercial fragrances or that are rarely used because of their production costs. Likewise, reducing the concentration of certain ingredients that have raised concerns about their potential human or ecotoxicity could represent an interesting opportunity for PAMs. If it may be experimentally confirmed, the concept of olfactory PAM will certainly be promising in view of a great commercial success.

22.4 Other Chemosensory Receptors

22.4.1 Deorphanizing Other Receptors Involved in Olfaction

Two other types of chemosensory receptors have been described in the olfactory mucosa. The first one corresponds to trace amine associated receptors (TAARs; [22.61]) and the second to vomeronasal type I receptors (V1Rs ; [22.92]). In mouse, TAARs are expressed in the main olfactory epithelium and in the Grueneberg ganglion [22.93]. At least some of them respond to amine compounds that trigger stereotyped behavioral responses [22.94] or are involved in social interaction [22.95]. Very few is known about the olfactory role of TAARs in human. Of the six full gene sequences identified in the human genome, only TAAR1 and TAAR5 have been detected in the human nose [22.96, 97] and have been deorphanized [22.97–99]. TAAR1 responds to a large array of amine compounds whereas the TAAR5 receptive range is restricted so far to three compounds, among which trimethylamine is the most potent.

Although distantly related to ORs, TAARs trigger the same transduction cascade in the olfactory neuron. This means that functional assays that work with ORs can also be used for TAARs. At ChemCom, we consider TAARs as a putative additional class of odor receptors and we include them in our screening campaigns.

V1Rs refer to a family of putative pheromone receptors that are present in the vomeronasal organ of different species, including mouse and rat [22.100]. In human, the vomeronasal organ may still be observed but seems completely vestigial [22.101, 102]. Only five sequences of full gene remain in the human genome and the expression of four of them have been detected in the olfactory epithelium [22.103, 104]. The coupling cascade of the V1Rs is not yet clear but does not seem to stimulate adenylate cyclase. Only one publication reported the identification of aldehydes and alcohols as activators of human V1Rs using a heterologous expression system [22.105]. So far, only the activation of hV1R1 has been confirmed by an independent team using a purified receptor [22.106]. The role of human V1Rs in percep-

tion of odors or in perception of pheromones remains to be discovered. Nevertheless, it could be worth pursuing the research effort on human V1Rs since they might be involved in human pheromone perception, a concept that is still controversial.

22.4.2 Deorphanizing ORs in Animals

Vertebrates

Interacting with animal olfaction can be the basis of a series of applications, such as pest control, improvement of feeding of pets, or breeding animals. For many vertebrates of commercial interest, olfaction is the main sense used for food seeking, social interactions, or alarming to danger. In terms of mammal pest control, identifying receptors that mediate the alarm signals induced by a predator-emitted molecule may lead to the development of highly efficient repellents. In contrast, blocking the perception of molecules that play a role in mating can be a specific and environment friendly way to limit the proliferation of undesirable species in a particular area. Since the principles of odorant detection have been well conserved throughout the evolution of the vertebrates, consequently, the receptors that mediate olfaction are also concerned by this conservation. Therefore, the screening system set up for human ORs can be applied to deorphanize ORs of any species of interest belonging to this phylum. However, it is important to note that the number of ORs predicted to be functional vary from one species to the other but reach close to 1000 or more in mouse or dog. Therefore, the approach of OR screening, as described for human ORs seems a better option. It implies some knowledge of the physiological and behavioral aspects linked to the olfaction of the targeted animal and requires a prior identification of odorant (an individual molecule or a blend) of interest that can be either natural attractants or repellents. The receptor screening approach coupled to an SAR study may help in the design of new, more efficient or cheaper to produce compounds acting on the behavior. Seeking antagonists follows the same logic as described for human ORs. First, receptors for attracting molecules are sought in a receptor screening and, once identified, are submitted to an antagonist screening using a dedicated library of volatiles.

If nothing is known about the number of genomic sequences of the animal's ORs, the sequencing of its whole genome or its olfactory transcriptome may reasonably be considered. Various companies proposed an à la carte whole genome or transcriptome sequencing for an affordable price that does not exceed 10 000 dollars. Likewise, the physical obtaining and cloning of the OR open reading frames into expression vectors can be outsourced to specialized companies for about 400 dollars per sequence and requires 6 months. Assuming a target animal that would harbor 1000 ORs in its genome, a scale time of 1 year and budget of ≈400 000 dollars are realistic estimates for constituting a usable library of ORs.

Animal TAARs and vomeronasal receptors are targets of potential interest as they were demonstrated to play a role in avoidance behavior of mice [22.94] and at least one of the mouse TAARs (TAAR5) is involved in social interaction [22.95]. Similarly, vomeronasal receptors from type 1 and type 2 seem to play a role in rodent social interactions and are considered as pheromone receptors (reviewed in [22.107]). If the TAAR repertoire remains limited to less than 20 members in the studied mammal species and can be easily managed as additional ORs in screening, both vomeronasal receptor families own more than hundred members in rodents. They represent potentially interesting targets for pest control programs but robust screening systems allowing a systematic identification of V1R and V2R activators or inhibitors are still to be developed.

Insects

Some insects are well-known pests for agriculture or represent vectors for various infectious diseases that affect humans. The discovery of insect ORs [22.108–110] has paved the way for the elucidation of the molecular mechanisms of that chemical perception. Although the general functioning of the insect and vertebrate olfaction relies globally on the same principles, the receptors involved are different. In insects, ORs correspond to a heterodimeric complex where one of the subunits, known as OR83b or ORCO (olfactory receptor co-receptor), is common to all the ORs whereas the second subunit that is variable, defines the selectivity for the activator. Both subunits are related to the GPCR family but present an inverted membrane topology compared to classical GPCR members [22.111]. It has been shown that insect ORs behave as ion channels that promote entry of calcium in the olfactory neuron upon activation by its agonist. In addition to this fast ionotropic response, activation of the dimer also results in the production of cyclic AMP [22.112, 113].

Different functional expression systems have been assessed for insect ORs. The Xenopus oocyte-based expression system coupled to an electrophysiology-based assay has led to the deorphanization of multiple ORs [22.114–117]. Likewise, a calcium-based screening platform relying on HEK293 expressing mosquito ORs has been described and led to the identification of conventional *Aedes aegyptis* OR ligands and less conventional ORCO agonists [22.118]. Because of their higher throughput capacities, it is probable that these

heterologous cell-based systems will outperform the more conventional approach based on antennography or single sensillum recording, although these later techniques present the advantage of not requiring a prior identification and cloning of ORs for detecting olfactory active molecules.

Additional proteins such as odorant binding proteins (OBPs) or sensory neuron membrane proteins (SNMPs) have been proposed as possible partners in the activation of insect ORs (reviewed in [22.119, 120]). OBPs are present in the lymph that surrounds the neurons in the hairs of the sensory organs. They serve as solubilizer for odorant and pheromonal compounds. OBPs could also be involved in transporting the ligand to the receptor, although a direct interaction of OBPs with the ORs remains to be demonstrated. The fact that functional expression of insect ORs in heterologous systems does not require OBPs shows that these latter are not mandatory for the activation of the receptors. The number of OBP genes varies from one insect species to another, but can encompass several tens of members. This is suggestive of a ligand selectivity for OBPs that has been confirmed experimentally. Along with ORs, OBPs constitute potential targets for the identification of compounds that would interfere with the chemical perception of insect pests. Protocols that allow the in vitro quantification and monitoring of the binding of volatile compounds with insect OBPs have been described [22.121] and can be up-scaled to develop HTS for OBPs.

As noticed for mammals, the affordability of genome sequencing will certainly promote the extension of HTS to ORs and OBPs of insects. Despite the high variability from one species to another, the known insect olfactomes seem more limited than in mammals [22.122], a point that should certainly facilitate the screening effort. Regarding the number of pest-related issues and the growing need of ecologically safe alternatives to classical insecticides, the development of a new generation of species-specific attractants or repellants may represent a business opportunity for more than one industrial player. It is to bet that the first molecules of this type that will reach the market will represent the necessary proof of concept for fostering the interest for HTS approach in the programs aiming to manage insect populations.

22.5 Concluding Remarks

Progresses achieved in the functional expression of human ORs have paved the way for a systematic deorphanization of these chemoreceptors by an HTS approach. Owing the rapid progress in deorphanization in hORs and in their functional expression, it can be predicted that this project will be completed soon, leading to the constitution of a first olfactory map that will index the activators for all functional human ORs. With an increased number of deorphanized and characterized ORs, it will be easy to determine the profile of ORs responding to any single molecule and to more complex mixtures, in terms of OR activation and/or OR inhibition. This powerful tool will facilitate new applications such as identifying or designing new odorants or blends that will create new sensations or will mimic complex or expensive aromas or fragrances. The accumulation of data from the deorphanization project will also help the development of more accurate in silico modeling of OR-ligand interactions that could ultimately replace the cell-based experimental approach. As there are many more odorant materials (single compounds or blends) than ORs, an important challenge will consist in understanding the interaction of several molecules (including nonodorant compounds in the case of complex extracts) at the level of the receptor. Any compound could be either an agonist, an antagonist, or a positive or a negative modulator; several compounds could act at the receptor level leading to a degree of activation that cannot be anticipated in sensory studies. This might come out onto significant changes in the composition leading to costs saving in commercial formulations or replacement of harmful compounds.

The identification of specific antagonists for ORs activated by malodors that both inhibit the OR response in vitro and block or reduce the perception of the malodor by human, represents a key step towards commercial applications. Therefore HTS-based antagonist search will represent a major part of the industrial activity on human ORs. The concept of antagonists applied to ORs is likely to open new opportunities and additional values as antagonists will not induce habituation in contrast to well-known covering or masking formulae. Therefore, those blockers can be incorporated in consumer products (e.g., perfumes) allowing their users to profit themselves of a protection against external offensive odors.

Many ORs are expressed in nonolfactory tissues [22.123] where they may play a physiological role (reviewed in [22.124]) and in the following chapter of this book). Therefore, deorphanization of the ORs, establishing SAR for agonists and identifying antagonists may raise interest for the drug industry. In a recent re-

port that describes the implication of the mouse Olfr78 in blood pressure regulation, ectopically expressed ORs have been viewed as chemosensors [22.84]. Thus, deorphanization of the human ortholog, and identification of the natural endogenous activators for these ORs allows us to investigate its function. Likewise, antagonists of ectopically expressed ORs can constitute valuable tools to demonstrate the implication of these receptors in physiological processes. For example, two antagonists of OR51E2, the human ortholog of Olfr78, have been fished out from an antagonist screening performed at ChemCom and might be of interest to assess the involvement of this receptor in blood pressure regulation.

As this chapter intended to show, the HTS approach applied to human and animal ORs is one way for enhancing our detailed knowledge of the early stage of olfactory perception, but also represents an opportunity in the biotechnology sector, including the drug industry, for the development of original industrial applications.

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23. Psychophysical Testing of Human Olfactory Function

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This chapter is an up-to-date review of psychophysical means for testing the human sense of smell. Strengths and weaknesses of major psychophysical paradigms are discussed, including ones associated with the measurement of odor detection, discrimination, identification, memory and both suprathreshold intensity and pleasantness assessment. Factors that influence olfactory test results are discussed in detail, including the influences of test parameters, such as test length, on test reliability. It is pointed out that non-forced-choice tests, unlike forced-choice tests, are incapable of discerning subject biases and malingering. Issues related to the comparison and interpretation of test results from nominally disparate tests that differ in sensitivity, reliability, operational demands, and other factors are discussed.

The goal of this chapter is to provide an overview and discussion of the major psychophysical methods available for assessing human smell perception. In general, psychophysical tests are those that quantify perception on the basis of verbal or other overt conscious responses on the part of a subject. Psychophysical measurement is historically rooted in concepts developed in the 19th century by such notables as *Weber* [23.1] and *Fechner* [23.2], and in the early to mid-20th century by *Thurstone* [23.3], *Stevens* [23.4], and others (*Peryam* [23.5]). Detailed information regarding the history, development, and mathematical foundations of psychophysical methods, including ones directly associated with olfaction, are available elsewhere [23.4, 6–18].

23.1 Stimulus Presentation Procedures

As illustrated in Fig. 23.1, numerous devices have been used to present odorants to humans for psychophysical testing. These include:

1. The draw tube olfactometer of *Zwaardemaker* [23.17, 19]
2. Glass sniff bottles and specialized cannisters [23.20–26]
3. Odorized glass rods, wooden sticks, felt-tipped pens, alcohol pads, plastics embedded with odorants, or strips of blotter or tissue paper [23.27–33]
4. Plastic squeeze bottles [23.34–38]
5. Bottles from which blasts of odorized air are presented [23.39, 40]
6. Microencapsulated *scratch and sniff* odorized strips [23.41–44]
7. Laboratory-based air-dilution olfactometers [23.23, 45–54]
8. Exposure chambers or hoods, including mobile units with analytical equipment and subject waiting rooms [23.46, 55, 56].

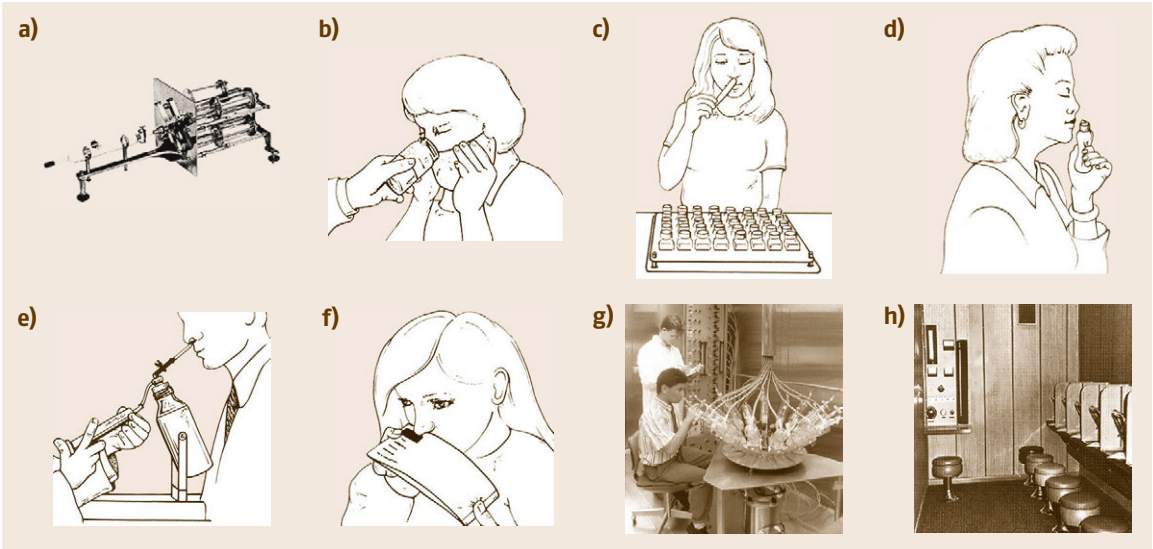

Fig. 23.1a–h Procedures for presenting odorants to subjects for assessment. **(a)** Early draw-tube olfactometer of Zwaardemaker. **(b)** Sniff bottle. **(c)** Perfumer's strip. **(d)** Squeeze bottle. **(e)** Blast injection device. **(f)** Microencapsulated *scratch and sniff* test. **(g)** Sniff ports on a rotating table connected to a dynamic air-dilution olfactometer. **(h)** Odor evaluation room of a mobile odor evaluation laboratory designed to evaluate responses of panel members to diesel exhaust (courtesy of Richard L. Doty, 2006)

For most clinical assessments, the only requirement for stimulus presentations is that they are reliable and that the stimuli reflect the involved odor qualities. For detailed structure-activity studies, air-dilution olfactometry is usually required. Regardless of the equipment involved in presenting odorants, the psychophysical paradigms are generally the same.

23.2 Measurement of Basal Olfactory Sensitivity

23.2.1 Detection and Recognition Thresholds

Paralleling to some extent the footsteps of the sensory evaluation of other sensory systems, most notably vision and hearing, the most popular modern procedures for quantifying olfactory function have included ones that assess the lowest level of stimulus that can be detected (detection threshold) or recognized (recognition threshold). The absolute or detection threshold is defined operationally as the lowest concentration where the presence of some type of subtle odor sensation that lacks a traditional odor quality is reliably detected, whereas the recognition threshold is defined as the lowest concentration where odor quality can be reliably discerned (e.g., *rose-like*). In most modern threshold tests, forced-choice procedures are employed; a subject must indicate, on a given trial, which of two or more stimuli (e.g., a low concentration odorant and one or more nonodorous blanks) is perceived as smelling stronger, rather than to simply report whether or not an odor is perceived. Such procedures minimize contamination by response biases (the conservatism or liberalism in reporting the presence of an odor under uncertain conditions) and typically provide lower and more reliable threshold values [23.57, 58].

Estimates of threshold values can be determined using numerous procedures, including:

1. The method of constant stimuli, where different concentrations of an odorant that span the threshold region are presented in random order to the subject
2. The method of limits procedure, where the stimuli are presented stepwise in either ascending or descending concentrations until the stimulus is reliably detected or recognized
3. The method of adjustment, where the subject adjusts, employing a specialized olfactometer, a stimulus to match another stimulus or to meet some criterion of perception, such as a perceived difference

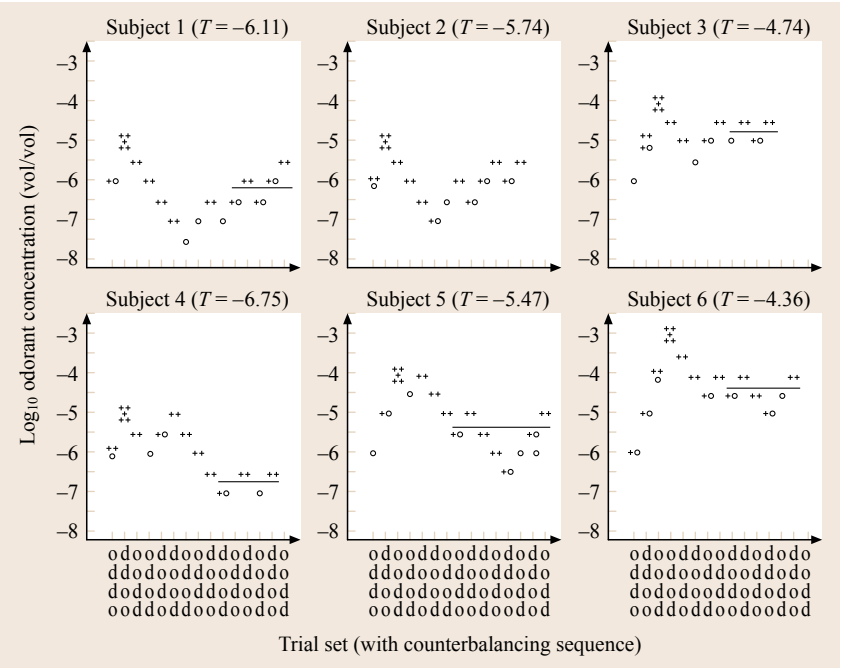

Fig. 23.2 Data illustrating single-staircase detection threshold determinations. Each plus (+) indicates a correct detection when an odorant versus a blank is presented. Each minus (−) indicates an incorrect report of an odorant. Threshold value (T; phenyl ethyl alcohol vol/vol in light USP grade mineral oil) is calculated as the mean of the last four of seven staircase reversals. The o's and d's on the abscissa indicate the counterbalancing order of the presentation sequences for each trial and are read downward (o: odorant presented first, then diluent; d: diluent presented first, then odorant)

4. Staircase procedures, in which the presented odorant concentration depends on the subject's performance on prior presentations or trials.

The staircase procedure, which was first used in the chemical senses in the mid-1970s [23.59], is a variant of the method of limits technique, but requires far fewer trials to obtain a reliable threshold [23.60]. In both the method of limits and staircase procedures, the direction of initial stimulus presentations is usually made from weak to strong in an effort to reduce adaptation effects of prior stimulation [23.61], although ascending and descending procedures generally provide similar detection threshold values [23.62].

Staircase procedures are most commonly employed in clinical situations, since they are reliable and economic of time. In the staircase procedure we developed for testing in the chemical senses [23.59], a trial consists of the presentation of two stimuli in rapid succession, one containing a concentration of odorant and one a blank comprising of either plain air or the material in which the odorant is dissolved. The subject's task is to report which of the two stimuli smells strongest. The employed concentration series extends below and above the perithreshold region of most persons in half-logarithmic dilution steps. Correct responses are required on five consecutive trials at a beginning concentration level before the next lower concentration is presented. If an incorrect response is made before five correct trials are attained, the process is repeated at one log concentration step higher. Once five consecutive correct trials occur at a given concentration level, the staircase is "reversed" and the next pair of trials is presented at a 0.5 log concentration step lower. From this point on, only one or two trials are presented at each step. If the first trial is missed, the staircase is moved to the next higher 0.5 log step concentration. When both trials are correctly performed, the next trial is given at one half-log unit concentration step lower. The threshold estimate is calculated as the mean of the last four of seven staircase reversal points. The general procedure is illustrated in Fig. 23.2, and is described in detail in a video [23.63].

As with all olfactory test measures, threshold values are relative and depend on such factors as the method of stimulus dilution, concentration step sizes, stimulus duration, species of molecule, type of psychophysical task, time between stimulus presentations, and the number of test trials [23.64, 65]. Some threshold measures, such as ones based on single ascending series presentations, exhibit considerable intra- and inter-subject variability [23.66–68]. Forced-choice paradigms and algorithms that incorporate more trials within the perithreshold region, such as staircase procedures, produce less variable measures and can be quite reliable. Within limits, reliability is systematically related to the number of trials within a test procedure [23.69]. For example, in the test procedure shown in Fig. 23.2, test–retest reliability is strongly related to the number of staircase reversals ($R^2 = 0.984$).

23.2.2 Difference Thresholds

In classical psychophysics, the smallest amount by which a stimulus must be changed to make it perceptibly stronger or weaker is considered a *just noticeable difference* or JND. This difference or differential threshold is, in effect, a measure of resolving power of the sensory system. In general, the size of the increment in odorant concentration (ΔI) required to produce a JND increases as the comparison concentration (I) increases, with the ratio approximating a constant (K); i. e., $\Delta I/I = K$ (Weber's law; [23.1]). The smaller the K value, the more sensitive the system is to fine changes in stimulation.

However, it is well known that K is not always a constant across the entire stimulus concentration continuum, being influenced at the extremes of the sensory continuum by the size of I [23.70]. Moreover, K can vary considerably among studies, depending on stimuli and test procedures. For example, using the Zwaardemaker olfactometer, *Gamble* [23.71] and *Hemandes* [23.72] found K values around 0.33 for a number of odorants. Using the same test procedure, *Zigler* and *Holoway* [23.73] reported, for the odor of Indian rubber, that K followed a hyperbolic function with a value of 0.17 at the upper end of the stimulus series and 0.99 at the lower end of the series. Using more modern procedures, *Wenzel* [23.54] found an average K of 0.15 for phenyl ethyl alcohol, whereas *Stone* and *Bolsey* [23.74] noted an average K of 0.28 for acetic and propionic acid. *Slotnick* and *Ptak* [23.75] reported an average K value of 0.32 for pentyl (amyl) acetate, whereas *Cain* [23.76, 77] found, in two subjects, K values for ethyl *n*-butyrate, *n*-amyl alcohol, *n*-butyl alcohol, and *n*-amyl butyrate of 0.30, 0.19, 0.07, and 0.09, respectively.

23.2.3 Signal Detection Measures

Signal detection theory (SDT) rejects the concept of a threshold as such and focuses on the roles of signal and noise as the milieu of the detection situation and subject factors, such as expectancies and rewards, in influencing the detection decision. SDT provides both a measure of sensory sensitivity and the subject's response criterion or bias [23.15]. The response criterion is the liberalism or conservatism of a subject in reporting a sensation under uncertain circumstances. For example, while two persons may experience the same subtle degree of sensation from a very weak stimulus, one may report that no sensation is perceived (e.g., because of lack of self-confidence) and the other may report that a sensation is clearly perceived. In this example, the stimulus was perceived to the same degree

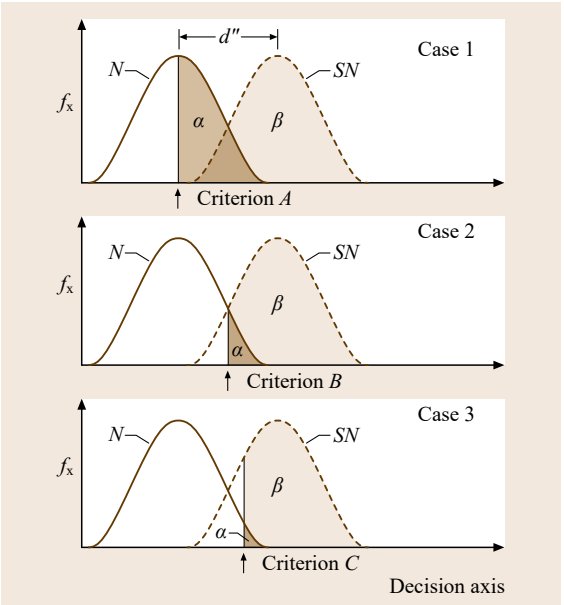

Fig. 23.3 Hypothetical distributions of signal plus noise (*SN*) and noise alone (*N*) plotted on the same axes (after [23.78], courtesy of *Doty*, Academic Press, 1976)

but the two subjects had different criteria for reporting its presence. In a classical non-forced-choice detection threshold paradigm, the investigator would conclude that these two subjects differed in sensitivity to the stimulus, when, in fact, they only differed in regards to their response biases.

In SDT, it is assumed that a stimulus is imbedded within a background of noise and that both can fluctuate over time. Noise can reflect a number of factors, such as variations in attention, stimulus fidelity, neural firing unrelated to the stimulus, and fluctuations in distracting physiological processes. When a signal is added to the *noise* (*N*) distribution, a *signal plus noise* (*SN*) distribution is in evidence. In most cases, the *N* and *SN* distributions are assumed to be normally distributed and can be represented on the same set of *z*-score axes, as shown in Fig. 23.3. The distance between the means of these distributions is the measure of the subject's sensitivity, d'.

The concept of the response criterion is also illustrated in Fig. 23.3. In this hypothetical example, d' (sensitivity) is held constant. The subject's task is to report whether or not an odor is perceived. Reports of *no* are represented by the areas under the *N* and *SN* curves to the left of the vertical line depicting the subject's response criterion, whereas reports of *yes* are indicated by the areas to the right of this line. In case 1, a very liberal criterion is depicted, with the subject reporting the presence of an odor on all of the SN trials (β) and

on half of the N trials (α). Thus, while correct detection of the odorant occurred all of the time (β), a large number of false alarms (α) occurred. This could reflect an instance where the subject was rewarded for reporting the detection of an odor and not admonished for making false alarms. In case 2, a less liberal response criterion is depicted, reflecting fewer correct detections (β) and fewer false alarms (α). In case 3, the observer had a very conservative response criterion, reflecting few false alarms but also somewhat fewer correct detections. This pattern of responses would result, for example, if a subject was penalized for making false positives and given few rewards for successfully detecting the odor. An important point is that, in all three of these hypothetical cases, sensitivity (d') was equivalent, as indicated by the same distance between the N and SN distributions.

In a typical SDT odor experiment, the subject is presented with a large number of odorant trials (*SN*) interspersed with nonodorant (*N*) trials. The odorant is usually a single concentration chosen to be detectable approximately half of the time [23.29, 79]. Commonly, an equal number of blank and odorant trials are presented. The proportion of the total odor trials (*SN*) on which a subject reports detecting an odor (the hit rate) is calculated, as is the proportion of blank trials (*N*) on which an odor is reported (the false alarm rate). When data meet the requirements for parametric statistics (normal distributed data with homogeneous variances), d' can be computed by converting these proportions to normal distribution standard deviation values (z-scores). d' equals the z-score for hits minus the z-score for false alarms [23.10]. The classical measure of response bias is the ratio, at the criterion point, of the ordinate of the SN distribution to that of the N distribution. Procedures for calculating d' for frequency distributions that are not normally distributed are available, including a number of nonparametric signal detection measures (see, [23.80–85]).

Although hundreds of trials are typically employed in signal detection studies, fewer trials have been used. *Potter* and *Butters* [23.86] and *Eichenbaum* et al. [23.87], for example, computed d' in olfactory studies using only 30 test trials. Such estimates are somewhat unstable, although *O'Mahony* et al. [23.88], in a taste study using sodium chloride, found that, after 40 trials, *Brown*'s nonparametric R index [23.80] fell within 5% of the values obtained after 200 trials in a little more than half the subjects tested. Ideally, however, all of the subjects should evidence such response stability, so as a general rule one should employ as many trials as practically possible.

23.3 Measurement of Suprathreshold Odor Perception

Numerous psychological attributes of odors can be quantified, including odor strength, hedonics (pleasantness/unpleasantness), and quality. These attributes change as a function of odorant concentration and species. Suprathreshold measures of intensity have been generally found to be less sensitive to olfactory dysfunction than a number of other measures. In a classic case, for example, a suprathreshold measure completely missed the marked influence of age on olfactory function [23.89]. Factor analysis studies suggest that suprathreshold measures may tap, at least to some extent, underling physiological processes that are distinct from those measured by threshold measures and some suprathreshold tests [23.90].

23.3.1 Rating Scales

Most persons in industrial cultures have used rating scales at one time or another. Such scales date back to antiquity [23.91]. For example, Galen employed a rudimentary hot–cold scale that was the basis for hot and cold body temperature scales used by physicians until thermometers were developed in the 19th century. In olfaction, rating scales of odor pleasantness/unpleasantness were used at least as early as 1923, when Young employed a 7-point scale ranging from −3 (very unpleasant) to +3 very pleasant to compare over time the consistency of hedonic responses [23.92].

In olfactory assessment, two types of scales are most popular today: category scales, where the relative amount of a sensation is signified by indicating which of a series of discrete categories best describes the sensation, such as was done by Young above, and line scales (also termed visual analog or graphic scales), where the subject or patient indicates the strength of the sensation by placing a mark along a line that has descriptors (termed anchors) located at its extremes (very weak–very strong). Because of the tendency of subjects to group responses at the extreme end of intensity scales, particularly for prothetic continua, a number of test developers have made an effort to incorporate ratio-like or logarithmic properties into the design of their scales to more closely mimic results obtained from magnitude estimation (Sect. 23.3.2) [23.93–96]. Responses on rating scales are influenced by a number of factors, including intent (trying to make the intervals on the scale equal in width), discrimination (use of categories narrows when discrimination is good and widens

when discrimination is poor), and expectation (ideas regarding the number of times each category should be used) [23.97]. For discussions of the general properties of rating scales, including the influences of category number on their psychometric properties, the reader is referred elsewhere [23.11, 97–100].

23.3.2 Magnitude Estimation and Matching Procedures

In magnitude matching, the relative magnitudes of members of a stimulus set are estimated on another continuum, with the goal of determining the ratio relations among the intensities (or other attributes) of the set. Unlike rating scales, the subject's responses are not confined to categories or a response line. In magnitude estimation (ME), the most common of such matching procedures, numbers are assigned to the relative magnitude of the sensations. For example, if the intensity of one stimulus is assigned the number of 100, a stimulus perceived half as strong would be given the number 50. If the intensity was perceived as four times as strong as the initial stimulus, then the number 400 would be assigned. In the *free-modulus* version of this procedure, the subject can choose any size or range of numbers he or she wishes, so long as they reflect the relative magnitudes of the perceived intensities. In the assigned modulus version of this test, a preassigned number is provided to the subject (often one from the middle stimulus of the series) in an effort to make his or her responses more reliable. The key element of interest in magnitude estimation is the relationships between the numbers, not their absolute values.

ME has been used to evaluate the relationship between odor and intensity for a number of compounds. In one procedure, negative numbers are used to denote unpleasant values and positive numbers pleasant values, with 0 signifying neutrality. As with other ME applications, the subject is instructed to denote the relative degrees of pleasantness and unpleasantness using ratio estimates. As shown in Fig. 23.4, while the intensity of an odor increases monotonically as a function of its concentration, this is not the case for hedonic responses which can be much more idiosynchratic [23.101].

Unlike the manner in which the data are presented in Fig. 23.3, ME intensity data are most commonly plotted on log–log coordinates (log odorant concentrations on the abscissa and log MEs on the ordinate) and the resulting functions are typically linear. Regression is used to determine the function, $\log P = n \log \Phi + \log k$, where P = perceived intensity, k = the Y intercept, Φ = stimulus concentration, and n = the slope. This function can be represented in its exponential form, $P = k\,\Phi^n$, where the exponent n is the slope of the function on the log–log plot. The Y intercept is frequently ignored, since it depends on the number system chosen by the subject or the experimenter. In olfaction, n varies in magnitude from odor to odor, but is generally less than 1, reflecting a negatively accelerated function on linear–linear coordinates. Some investigators have altered the power function equation in efforts to take into account such factors as differences in thresholds and vapor pressures [23.58, 103, 104].

Like most other sensory tasks, ME is influenced by procedural and subject factors [23.13, 58] and there is evidence that, for untrained or mathematically unsophisticated subjects, category scales and line scales may be superior to magnitude estimation when such factors as variability, reliability, and ease of use are considered [23.105, 106]. Since accurate responses to stimuli in an ME paradigm require a good memory for the prior stimulus, fidelity can be compromised if too much time lapses between stimulus presentations. On the other hand, if the trials occur too quickly, adaptation can distort the relationship. Some subjects fail to consistently provide ratio estimates of stimuli, and some fail to understand the concept of producing ratios [23.107, 108]. A contrast phenomenon can intervene; for example, a moderately intense odor is reported to be more intense when presented with weak comparison stimuli than with strong comparison stimuli, making it imperative that counterbalancing of stimulus presentations is made [23.109, 110]. The standard assigned to the subject, as well as units in which the stimulus concentration is expressed, can affect the exponent [23.111], and stimuli spanning a restricted range generally produce larger exponents than stimuli having a more expansive range [23.112]. Despite such influences – a number of which are shared by other psychophysical measures – such problems can be minimized by careful standardization and monitoring of the instructions, test procedures, and test stimuli.

Traditionally, only the slope (exponent) of the ME function has been used as the index of sensory function. The intercept or the distance of the function from the X-axis has been generally neglected, since its values are influenced by idiosyncratic differences in the use of numbers and by the type of ME paradigm employed (fixed versus free modulus). To gain information regarding the function's ordinate position, procedures have been developed that provide such information and, in addition, correct for differences among subjects in number usage and subject factors [23.113]. In one paradigm, judgments of the intensity of sensations from two modalities (broad band low frequency noise and smell) are made at the same time and placed on a common scale [23.114]. Under the assumption that subjects

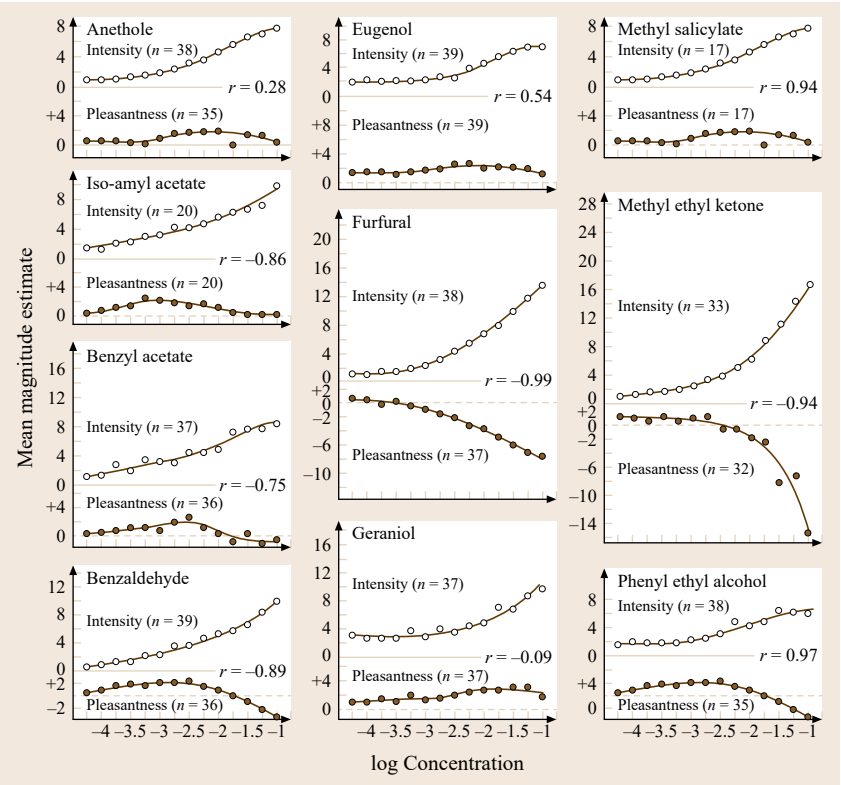

Fig. 23.4 Relationship of pleasantness and intensity magnitude estimates to odorant concentration in propylene glycol for 10 odorants (after [23.102] courtesy of *Doty*, The Psychonomic Society, 1975)

experience stimuli on the loudness continuum in the similar manner, differences among subjects' loudness ratings can be ascertained and the odor intensity continuum adjusted accordingly. Such normalization allows, theoretically, for a direct comparison of scale values across subjects. Thus, if the adjusted odor intensity magnitude value for one subject is 10 and for another subject is 20 at the same concentration level, the second subject is presumed to experience twice the odor intensity as the first subject.

Contrasts between the scaling parameters of ME procedures and rating scales have led to classification systems of the underlying sensory continua which may have some physiological merit. In 1957, *Stevens* and *Galanter* [23.97] classified perceptual continua that are related to stimulus magnitude by power functions, such as brightness and loudness, as *prothetic* (derived from the Greek prostithenai, which means *to add*). This was assumed to reflect their association with additive physiological processes such as the recruitment of more neurons and greater rates of firing in response to increases in stimulus intensity. When category ratings are plotted against magnitude estimates of the same stimuli, a downward curvilinear function is typically produced. Other continua, such as pitch, do not conform to a power function association and were classified by these authors as *metathetic*, which in Greek derives from a word meaning *to change or substitute*. According to Stevens and Galanter, metathetic continua exhibit uniformity of discrimination over the involved stimulus range and to a large extent are made of stimuli that reflect *what and where* rather than *how much*. When magnitude estimates and category scale values of metathetic stimuli are plotted against one another, linear or near-linear functions are found. The underlying changes across metathetic continua are assumed to reflect activation of more discrete physiological spatial elements as the magnitude of the stimulus property increases. For example, in the case of pitch, increases in frequency are associated with substantive recruitment of spatially distinct regions of the basilar membrane, rather than the additive recruitment of more neurons within the region.

The question arises as to whether the hedonic properties of equivalently intense and hedonically disparate odors reflect prothetic or metathetic continua. Using a procedure developed by *Eisler* [23.115] that expanded the prothetic/metathetic dichotomy into a continuous *protheticity* measure, *Doty* [23.116] addressed this issue. Power functions were fitted to the relationships between (a) magnitude estimation pleasantness values and (b) pleasantness values obtained from category

ratings and rank orderings of a diverse set of 20 relatively isointensive odorants. The functions relating the pleasantness of the diverse stimuli were nearly linear and only slightly prothetic, i. e., essentially metathetic (respective category scaling and rank order/magnitude estimation exponents = 0.60 and 0.63). Conceivably this could represent, as occurs with the case of pitch, recruitment of discrete sensory channels in a substitutive, rather than an additive, manner as pleasantness increases. In a second component of this study, analogous exponents were obtained from pleasantness data derived from quarter-log-step volume dilution series of two hedonically disparate odorants, the pleasant smelling odorant methyl salicylate and the unpleasant smelling odorant furfural. Methyl salicylate was found to scale in a more metathetic-like manner and furfural in a more prothetic-like manner (respective exponents = 0.68 and 0.20; respective rank order/magnitude exponents = 0.69 and 0.21. Thus, for olfaction, the degree of hedonic protheticity appears to depend upon the stimulus. It would be of interest to know whether, as odorant concentrations increase, metathetic-like odorants such as methyl salicylate recruit more spatially segregated elements of the olfactory receptor sheet than prothetic odorants like furfural, in a fashion analogous to pitch's association with the recruitment of substitutive activity from differing segments of the basilar membrane.

23.3.3 Odor Discrimination Tests

Odor discrimination tests seek to determine the degree to which a subject can tell the difference between odorants having different qualities (smells). The simplest of such tests are ones in which a subject is simply required to report whether two odorants smell differently. In one type of discrimination test, a series of same-odorant and different-odorant pairs is presented. The proportion of pairs that are correctly differentiated is taken as the measure of discrimination [23.82, 86, 88]. Numerous variations on this theme exist, including picking the *odd* stimulus from a set from which only the *odd* stimulus differs, either at a constant delay interval [23.32, 117] or at varying delay intervals [23.118]. The latter approach provides a measure of short-term memory as well as a measure of basic discrimination. Recently a discrimination test that employs a set of 15 stimuli representing pairs of different ratios of two odorants from a set of six odorants has been described [23.119].

Multidimensional scaling (MDS) is a very sophisticated means of establishing discrimination ability. In one MDS paradigm, subjects are asked to rate all possible pairs of a relatively large number of odorants on a visual analog scale depicting their similarity (e.g., a scale with anchors ranging from *completely different* to *exactly the same*). The correlations among these ratings are then subjected to an MDS algorithm that places the stimuli in two-dimensional (2-D) or three-dimensional (3-D) dimensional space relative to their perceived similarities [23.120]. Persons with poor discrimination tend to have spaces that have no distinct or reliable groupings, whereas those with good discrimination exhibit groups largely based on quality and pleasantness attributes. Although MDS clearly detects discrimination deficits, it is time consuming and statistical procedures for comparing one person's MDS space to normative data are not available. In persons with normal smell function, MDS stimulus spaces derived from the ratings of the names of odorants overlap with those derived from the actual smelling of odorants, signifying the presence of well-defined conceptual representations of odors [23.121, 122].

23.3.4 Odor Recognition Tests

Odor recognition tests are closely related to odor discrimination tests and, in some cases, overlap with them. Several types of quality recognition tests have been used. In the simplest type, a subject is asked whether each of a presented set of odorants is recognized. Identification is not required. This procedure is relatively crude, lacks normative referents, and is easy to malinger. A more quantitative odor recognition paradigm is the stimulus matching task. In one such test, a set of stimuli are provided and the subject is required to match the stimuli to those of a set of identical stimuli. An example of such a test is provided by *Abraham* and *Mathai* [23.123]. These investigators presented patients with eight vials that contained four odorants (two vials per odor). The patients were required to pair up the equivalent two-vial containers. The test score was the number of pairs that were correctly matched on each of two test administrations.

23.3.5 Odor Identification Tests

Tests that require a subject to identify an odorant are among the most popular procedures for assessing smell function. Such tests can be classified into three groups: naming tests, yes/no identification tests, and multiple-choice identification tests. The respective responses required, on a given trial, in these three types of tests are:

1. To name the stimulus
2. To report whether the stimulus smells like an object named by the examiner (does this smell like a rose?)

3. To identify the stimulus from a list of names or pictures.

Odor naming tests in which no response alternatives are provided are commonly used by neurologists to measure olfactory function [23.124, 125]. Unfortunately, like simple recognition tests, their value is limited since:

1. It is difficult even for most normal persons to name even familiar odors without cues
2. They lack normative referents
3. They are easy to malinger.

Yes/no identification tests are much more useful, since they require a patient to report whether or not each of a set of stimuli smells like a particular substance named by the experimenter and malingering can be detected when performance significantly falls below chance. Two trials with each stimulus are usually given, with the correct alternative provided on one trial and an incorrect one on the other (e.g., lemon odor is presented and the subject is asked on one trial whether the odor smells like lemon and on another trial whether the odor smells like smoke). Although yes/no identification tests require that the subject keeps the percept in memory long enough to compare it with the target word (which, of course, must also be recalled from memory), it has been argued that it is less influenced by cognitive demands than multiple-choice identification tests (see below). Since the chance performance of a yes/no identification test is 50% compared to 25% on a four-alternative multiple-choice identification test (see below), its range of discriminability is lower, and therefore more trials are needed to obtain equivalent statistical power.

A number of multiple-choice odor identification tests have been described in the clinical literature [23.22, 32, 33, 42, 125–130]. These tests are conceptually similar and, in the few cases that have been examined, strongly correlated with one another [23.90, 130, 131]. The most widely used odor identification test – the University of Pennsylvania Smell Identification Test (UPSIT) – became commercially available in the early 1980s [23.42]. This microencapsulated odor identification test has been administered to over a million persons worldwide and has been translated into over 20 different languages. On a given forced-choice trial, subjects are required to identify each odorant from a set of four descriptors [23.42, 43] (Fig. 23.1). The number of correct items serves as the test measure. Clinically, a patient's test score can be compared to norms and a percentile rank determined relative to one's age and sex [23.43].

Another odor identification test that has been used in some smell and taste clinics employs what is called a confusion matrix [23.12, 130]. In one version of this test, 10 suprathreshold stimuli are presented to a patient in counterbalanced order 10 times (100 total trials) [23.130]. The subject is required, on a given trial, to indicate which one of a set of 10 written response alternatives smells most like the odorant – response alternatives that actually reflect the stimuli that are presented, i. e., ammonia, chlorine bleach, licorice, mothballs, peppermint, roses, turpentine, vanilla, Vicks vapor rub, and vinegar. The subject must provide an answer even if no smell is perceived. The percentage of responses given to each alternative for each odorant is determined and displayed in a rectangular matrix (stimuli making up rows and response alternatives making up equivalently ordered columns). Responses along the negative diagonal represent correct responses, whereas those that fall away from the diagonal potentially represent *confusions*. The percentage of correct responses is used as the main test measure, although some of its proponents argue that the confusions (off-diagonal responses) may provide meaningful clinical information. The latter has yet to be clearly demonstrated. This test requires 45–60 min to administer, making it impractical in most clinical settings.

23.3.6 Odor Memory Tests

There are numerous means for assessing odor memory. For practical reasons, explicit, rather than implicit, memory tasks are most commonly performed – tasks where odors are presented which the subject must recall after various delay intervals. It is important to note, however, that odor memory itself is a complicated construct that is intimately interwoven with emotion, prior experiences, and semantic processes [23.132]. Moreover, deficits on a normal odor memory test could reflect problems with encoding (because of decreased sensory input), retrieval (inability to recognize the stimulus), or connections with higher brain centers associated with experiences with the odor (degree of familiarity). Despite attempts to minimize labeling of the inspection odor with a familiar word or item on the part of a subject, such labeling often occurs and, thus, the memory of the label, not the memory of the odor percept per se, which is often being measured across delay intervals. In other words, once an odor is recognized as that of an orange, for example, all that has to be remembered over time is the concept *orange*, not the specific smell of the orange. Later, when given stimuli from which to select the earlier perceived odor, the subject simply looks for the smell of an orange (which has been known and stored in long-term memory for much of his or her life). In effect, the odor is not what is being uniquely remembered over the retention interval,

only its name or concept and remembrance of having smelled the substance.

Although some investigators have attempted to employ novel, nondescript, and unfamiliar odorants in such tasks, it is difficult to find target odors that are not labeled by subjects as pleasant or unpleasant, fruity or non-fruity, medicine-like or nonmedicine-like, chemical-like or nonchemical-like, etc. although some odorants have been identified that are difficult to name [23.133]. Importantly, it is critical to recognize that performance across the delay intervals is what is classically assessing the *memory* component of the task, not necessarily the overall test score. As noted below, a number of odor memory tests are, in fact, essentially odor discrimination tests with varying inspection (delay) intervals. When scores on a (nominal) odor memory task differ between two groups (as evidenced by a main group effect in an analysis of variance), then a significant interaction term between the delay interval and the group would indicate an effect on memory, per se. Without an interaction with delay interval, the difference could simply reflect discrimination, not memory, although admittedly memory is a component of nearly all olfactory tasks, including those of detection, identification, and discrimination.

In one popular odor memory test paradigm, which can be labeled the *single-target match-to-sample test* (STT), a subject is required to smell a series of odorants, one at a time, and to select, after intervals that can range from less than a minute to a few hours, that odorant from an array of several odorants (distractor odors) [23.118, 134]. Alternatively, the recall period can consist of a single odor which the subject indicates is either the same or different from the target [23.135]. In clinical versions of the STT, repeated trials are typically performed at one or more retention intervals for each of several target stimuli [23.118]. A second popular test paradigm, the *multiple-target match-to-sample test* (MTT) [23.134] is similar to the ST except that initially a set of odorants is presented and one or more of the set of odorants is subsequently identified in either a yes/no paradigm or by selection from a larger set of stimuli into which they are embedded. A less common, but potentially useful, odor memory paradigm employs *single-probe serial position recall* [23.136]. In this task, several odors are presented in succession to a subject. After a brief period, one of the odors is presented again and the task of the subject is to recall the serial position of the previous presentation of the odor, for example whether it was the first, second, third, fourth, fifth, or sixth odor that had been presented in a series of six odor presentations. In addition to examining overall percent correct, the number of correct identifications at each serial position can be calculated. Thus, measures of primacy and recency can be assessed. Like findings using this paradigm with visual and auditory stimuli, erroneous responses for olfactory stimuli are more frequently attributed to serial positions adjacent to the probed position [23.136].

A number of examples of studies that have employed match-to-sample odor memory tests in clinical settings are available. *Jones* et al. [23.137] presented 20 pairs of odorants at 0 and 30 s delay intervals to 14 alcoholic Korsakoff psychosis patients, 14 alcoholic controls, and 14 nonalcoholic controls. The subject's task, on a given trial, was to indicate whether the second stimulus was the same or different from the first. At the 30 s delay interval, the subjects counted backward by threes in an effort to minimize semantic labelling. The Korsakoff psychosis patients performed more poorly than the controls at both the 0 and 30 s retention intervals. It is not clear, however, whether this effect was due to deficits in odor memory, discrimination, or both. Interestingly, acute alcohol intake has been shown to have similar adverse effects on a 12-item single target match-to-sample odor memory discrimination test in young nonalcoholic subjects [23.138]. In this double-blind study, ingestion of alcohol designed to reach the legal level of intoxication significantly depressed the test scores although, as with the Jones et al. study, no delay interval effects were observed at the short delay intervals employed (10, 30, and 60 s).

Jones–Gotman and Zatorre [23.139] reported that odor memory deficits were evident in patients who had undergone surgical cerebral extirpation for control of epilepsy. The memory task consisted of the presentation of eight target odors and eight new foils, and the yes–no recognition testing was performed twice after the initial testing – 20 m later and 24 h later. Relative to controls, impairment was noted in those patients who had received excision from the right temporal or the right orbitofrontal cortices.

Gregson and his colleagues have employed a single-probe serial position recall paradigm to demonstrate odor memory deficits in patients with schizophrenia [23.140], Kallmann's syndrome [23.125], and Korsakoff psychosis [23.141]. In their paradigm, three odorants are presented in a row to the patient. A fourth odorant, which is the same as one of the three odorants, is then presented and the patient's task is to report which of the three prior odors it represents. Seven three-odor combinations of 12 inspection stimuli were administered. Patients who had difficulty with this initial task were subsequently given two-odor combinations. The test score was the number of odors that were consistently recognized.

23.4 Issues in Olfactory Psychophysical Measurement

It is important to keep in mind that each approach to olfactory measurement has strengths and weaknesses, and that comparisons among the results obtained from nominally distinct test measures, such as those of odor identification, detection, discrimination, memory, and suprathreshold changes in odor intensity, can be problematic. This is because such measures typically differ on a range of both olfactory and nonolfactory factors that confound the comparisons and associated interpretations. Among such factors are odorant species, the time required for test administration, the number of trials involved, and abilities to perform nonolfactory elements of the testing. A key issue relates to differences in test reliabilities which, in turn, influence their relative sensitivities in detecting subject differences due to age, sex, personal habits, and disease. As shown in Fig. 23.5, the test–retest reliability of most olfactory tests is a function of the number of involved trials. However, very short tests (three items) may exhibit spuriously high reliability coefficients, since smell abilities are forced into a few broad categories. Spuriously high reliability coefficients also occur when reliabilities are based on samples that contain persons with smell dysfunction, since correlation coefficients are influenced by the range of values on which they are based. It should be pointed out that while a high reliability coefficient indicates that a group of individuals scored similarly *relative to one another* on a test from one test occasion to another, all of the individual's test scores still may be lower (or higher) on the second than on the first test occasion. In other words, systematic changes in the test values can occur for a number of reasons that are not necessarily reflected in the reliability coefficient.

An important consideration in olfactory testing is that despite differing names, most olfactory tests are not mutually exclusive and are usually correlated with one another [23.90]. This likely reflects the dependence upon common psychological and physiological mechanisms, not the least of which is the olfactory neuroepithelium that undergoes a considerable change with age and environmental exposures [23.142–145]. Functionally, a test of odor identification requires the ability to detect an odor, as well as to discriminate among odors and to remember them. A forced-choice threshold test requires a comparison of an odor or a weak sensation with alternatives, a task that again requires memory as well as discrimination. Differing degrees of semantic labeling may occur in odor discrimination tasks, just as occurs in odor identification tasks, again pointing to communalities among such procedures. To what degree different tests differentially tap higher brain structures or networks is debatable in light of the fact that such tests are not equated for reliability or discriminating power. This problem is not specific to olfaction, as it has long been apparent in psychological measures of cognition [23.146].

In an ideal world, olfactory tests would be matched on the basis of sensitivity, reliability, discriminability, and a range of nonolfactory controls (e.g., tests in another modality that require the same operational tasks) so as to eliminate, or at least understand, nonolfactory factors that may be contributing to the results. This is particularly true when mentation may be altered, as in dementia or head trauma. It is for this reason that we often employ the Picture Identification Test, a test essentially identical to the UPSIT but which uses pictures rather than odors as test stimuli [23.147]. If persons do well on this test, we know that the putative olfactory deficits are not due to lack of understanding the concepts involved in the test or to the operational procedures involved in taking the test.

From a practical clinical perspective, a number of basic issues arise that require notice. One common question is whether testing should be performed bilaterally (both sides of the nose at the same time) or unilaterally (i. e., each side of the nose separately). While most individuals with chemosensory dysfunction have bilateral dysfunction [23.131, 148, 149], unilateral dysfunction is not uncommon and often goes unnoticed [23.150]. As with hearing or vision, it is important to know whether one or the other side of the olfactory system is dysfunctional, and clinically unilateral testing can detect disorders that otherwise would go unrecognized (early stage tumors such as olfactory groove meningiomas). Interestingly, odor recognition memory is better under bilateral than unilateral test conditions [23.134] and, in the menopause, estrogen replacement therapy appears to influence the asymmetry noted on such memory tasks [23.151]. When performing unilateral testing, it is prudent to close the contralateral naris without distorting the septum by using a piece of Microfoam tape (3M Corporation, Minneapolis, MN) cut to fit tightly over the borders of the naris and have the patient exhale through the mouth after inhaling through the nose [23.43]. As in the case when both nares are blocked, this precaution decreases the likelihood for air to enter the blocked nasal chamber via the retronasal route.

Prior experience with odors, particularly that obtained on taste and smell organoleptic panels, needs to be taken into consideration in olfactory studies, as such experience can alter a range of olfactory test measures. For example, repeated testing within the perithreshold odorant concentration range results in decreased

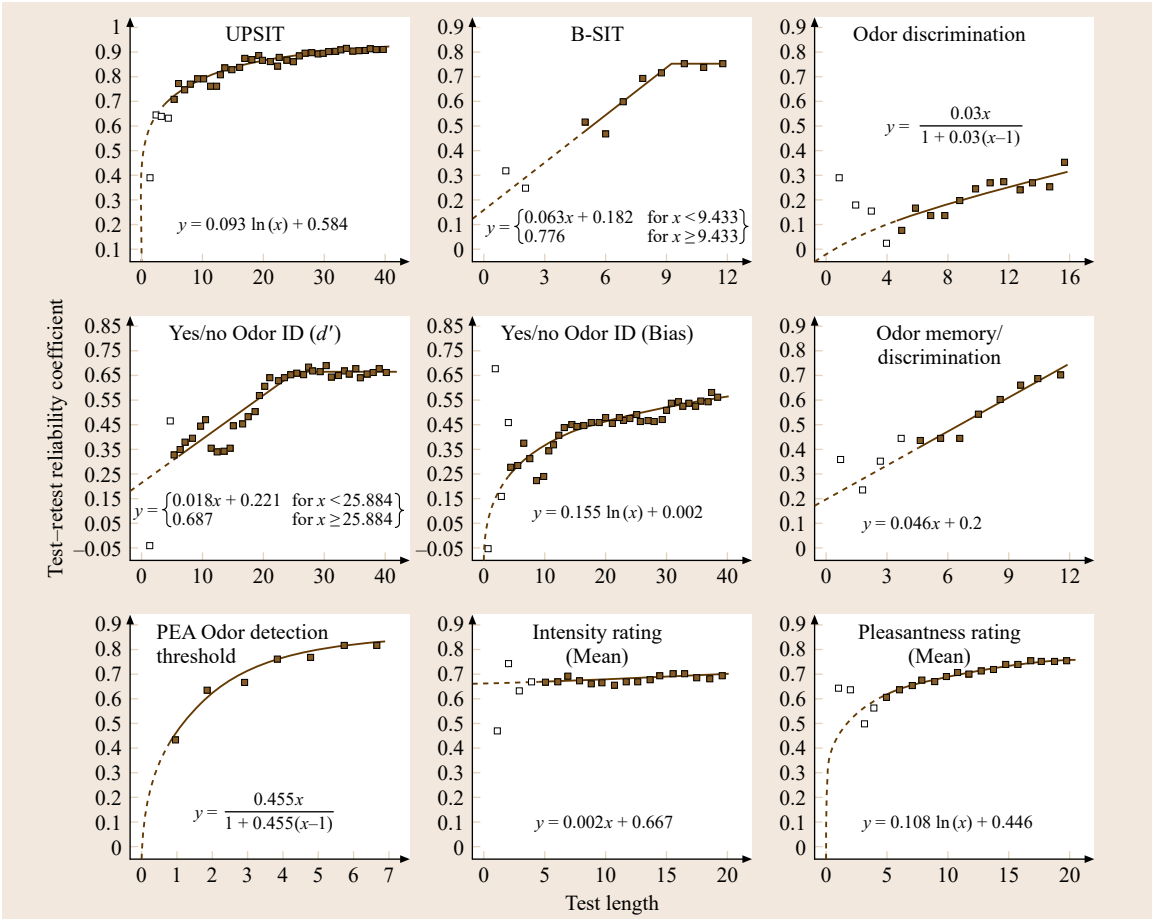

Fig. 23.5 Relationship of reliability to cumulative test length for test measures amenable to such an evaluation (after [23.69], courtesy of *Doty* et al., Oxford University, 1995)

thresholds or enhancement of signal detection sensitivity measures [23.79, 152–154]; practice with feedback influences the ability to name odors [23.155, 156]. Interestingly, the hedonic quality of odorants can be influenced by repeated exposure, making unpleasant odors less unpleasant and pleasant odors less pleasant [23.157]. Assuming that adaptation is not the primary basis for this phenomenon, affective components of odors appear to habituate somewhat independently of odor intensity.

One factor that is often overlooked in the administration of olfactory tests is the influence of adaptation or habituation on the olfactory test measure, a temporary decrease in smell function such as reflected, for example, by heightened detection threshold values or decreased intensity ratings (for a review, see [23.158]). Some chemicals produce a decrement in the perception of other chemicals (termed cross-adaptation). For these reasons, it is critical to use and maintain as optimal as possible inter-trial intervals that minimize confounding by such factors. The UPSIT, for example, was designed to minimize adaptation by:

1. Employing largely multicomponent "natural" odorants
2. Requiring minimal sampling of each odorant
3. Having verbal, rather than odorous, response alternatives
4. Ordering the presentation of odorants such that dissimilar odorants follow one another (thereby minimizing cross-adaptation)
5. Allowing adequate time between the smelling of each odorant item [23.42]

Another important issue relates to non-forced-choice tests that are susceptible to subject exaggeration and malingering. Recently, *Karnekull* et al. [23.159] demonstrated, in a nonclinical population, a positive

correlation between neuroticism and negative responses to both environmental odors and noise. Those highest on the measure of neuroticism rated odors as being more intense. Other work suggests that beliefs about odors can determine the amount of attention paid to them and how strongly they are rated. For example, when subjects are told they are being exposed to an unhealthy odor, their ratings of its intensity are greater, and the time required for adaptation is longer, than if they are told the odor is healthy [23.160–162]. However, forced-choice threshold tests do not detect threshold differences between persons who complain of chemical hypersensitivity to odors and those who do not [23.45, 163–165]. It is of interest that, unlike psychiatric malingerers, olfactory malingerers tend to downplay health problems that might influence the putative basis of their claimed disorder (smoking, dental problems, medication usage) [23.166]. On the other hand, they exaggerate symptoms congruent with their supposed problem, such as its severity, weight loss, appetite change, and interference with everyday activities.

23.5 Concluding Remarks

It is apparent from this chapter that a wide range of procedures are available for assessing olfactory function. However, much more work is needed to determine the relative value of such procedures in specific clinical and experimental settings. A challenge for the future is to determine the common and unique conceptual and physiological factors that are measured by such procedures.

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24. Olfactometers According to EN 13725

Dietmar Mannebeck

In 1995 the technical committee TC264 air quality of the European Committee for Standardization convened the working group 2, which developed an odor testing standard. It was released in 2003 as EN 13725 air quality – measurement of odor concentration using dynamic olfactometry. As the methodology of dynamic olfactometry according this European Standard is approved among others in Australia as AS 4323.1 and in Chile as NCh 3190, the EN 13725 today is the most applied standard worldwide.

Other used methods for determination of odor thresholds are ASTM 679 as well as Japanese and Chinese standards. Compared with the EN 13725 these standards are working on a lower detail level concerning technical requirements and execution description.

In 2012 the TC264 took a resolution to review the standard. A revised draft standard is expected to become available in 2017.

The revised standard will contain comprehensive guidance on reference material, measurement uncertainty, health and safety issues, materials for olfactometry, sample storage and the sampling of different odor sources. Once the endpoint of the revision has been reached a release of an ISO standard based on EN 13725 is envisaged.

Like the measurement of noise, olfactometry is an effect-based measurement method to determine the effect of an odorant on perception in humans. The most common purpose of odor measurements in general is to estimate the degree of nuisance due to odor impact.

The sense of smell reacts extremely sensitively and differently to a large number of chemical substances. The odor threshold, i. e., the minimum gas-phase concentration of an odorant that triggers a perception response, is substance-specific and varies over an extensive range. The odor threshold for butanoic acid, for example, is around $6 \times 10^{-5}\,\mathrm{ppm_v}$ ($0.00016\,\mathrm{mg\,m^{-3}}$), while that of benzene is $300\,\mathrm{ppm_v}$ ($1000\,\mathrm{mg\,m^{-3}}$). The odor threshold detectable by humans is for many compounds below the analytical detection limit of the most sophisticated instrumentation. Unlike in olfaction, the detection and assessment of noise involves a straightforward measurement of primarily just a single variable (sound pressure) with its different frequencies using physical sensors. Odor measurement, on the other hand, often calls for the measurement of up to several hundred components through the analysis of the odor's constituents using technical sensors and subsequently the evaluation of their accumulated effect on odor perception. In other words, the effect of a mixture of odorants on odor perception cannot be commonly derived from the concentrations of its individual components. A universal technical solution to this enormous challenge is unlikely to be found in the foreseeable future.

At present, no general relationship has been established between the results of measurements using technical sensors and human odor perception. Moreover, current sensors are mostly too insensitive for

many applications, thus the human nose is indispensable as a sensor for olfactometry for the foreseeable future. Instruments that use technical sensors (electronic noses) therefore do not qualify as olfactometers, as specified according to the definitions of EN 13725 and discussed in the following.

24.1 General Characteristics

24.1.1 The Process for Determining Odor Concentration

According to the definition given in DIN EN 13725:2003, an olfactometer is a dilution system in which the odor sample, diluted or administered within a neutral carrier gas is presented to a panel for assessment. Presentation of the odor to the panelists can either start below the odor threshold and continue in an ascending sequence or take place in a random sequence below and above the threshold level.

The responses of the panel members can be gathered in two ways. In a *yes/no* inquiry, the panelist must choose between *yes, there is an odor* and *no, there is no odor* for each presentation of the smell. The alternative procedure is a forced choice inquiry. Here, the panel member must identify an odor sample among several simultaneously presented samples and choose from one of three different responses, namely *certain*, *inkling* or *guess*. Even if panelists are unable to detect a difference, they are forced to respond. The results are then evaluated by means of a so-called probit analysis or, as described in EN 13725, through an evaluation of the *certain* responses.

Since only the *certain* and *yes* responses are used in the calculation of the results, both methods yield comparable results and are accorded equal status in EN 13725. However, the more economical nature of the *yes/no* inquiry method in terms of the lower quantities of sample and dilution gas required, as well as its faster analysis time, make this method the most frequently used to date worldwide.

Figure 24.1 illustrates the general working principle of a computer-controlled olfactometer. The sample air from a sample bag is mixed with nonsmelling clean air in a dilution unit. To present the diluted sample air (purple) via a sniffing cone to the panelists (P 1–4), the dilution unit, a switch valve as well as the panel response are operated by a microcontroller.

Typically in odor assessments via olfactometry the device supplies the odor at a threshold concentration at which it is detected by just 50% of the population. This concentration is generally quantified in odor units (European) per volume, typically per cubic meter (ou_E/m^3), whereby the E suffix indicates that the value is measured according to EN 13725.

One ou_E/m^3 is defined as the quantity of an odorant distributed in $1\,m^3$ of odorless gas that just fulfills the condition of 50% prevalence of detection.

Features of the Measuring Method and Factors Affecting the Measurement Results

Many factors can affect the results of olfactometry measurements. The relevant parameters and their most important factors are as follows:

- *Dilution units* (olfactometer): Accuracy and stability of the dilution steps, flow rates, suitability of the materials used
- *Reference* (odor) *gases*: Traceability, stability during storage and transport
- *Panel members*: Mean value and standard deviation of the reference substance measurements
- *Assessment room*: Air quality, temperature, quietness.

In addition, the trueness of an olfactometric test in relation to the source depends decisively on sampling and sample handling.

24.1.2 Dilution Units (Olfactometer)

An olfactometer is an instrument used in the detection and measurement of an odor dilution. Olfactometers are

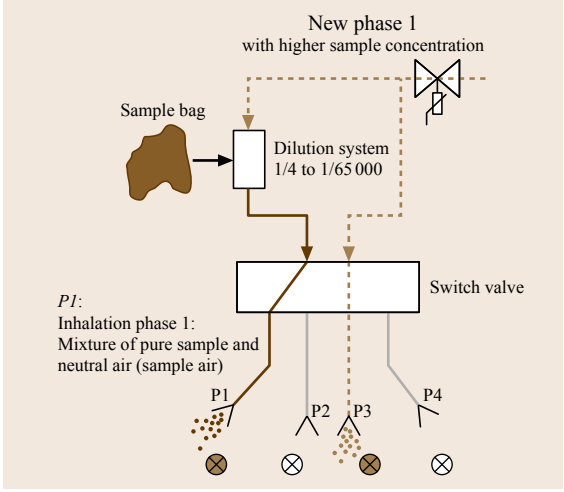

Fig. 24.1 Working principle of an olfactometer

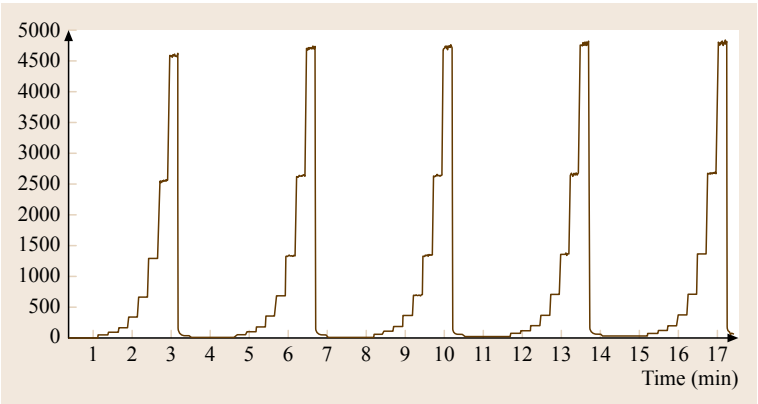

Fig. 24.2 Diagram of calibration with five repetitions per dilution step (example with propane tracer gas concentration in ppm)

used in conjunction with human subjects – more specifically, the human sense of smell – in laboratory settings to quantify and qualify human olfaction.

Requirements of an Olfactometer

The dilution apparatus of an olfactometer must satisfy a number of requirements and quality criteria. The use of inert (odorless) materials such as glass, stainless steel or polytetrafluoroethylene (PTFE) for all odorant-carrying parts of the olfactometer must be used in order to prevent corrosion, contamination and odor modification. It is known that a coating of stainless steel and glass such as silicone-carbon (SiC) improves the chemical resistance of an olfactometric dilution unit.

Furthermore, the device must meet certain standards of accuracy of dilution, repeatability and trueness. It should be noted here again that the odor perception of individual panelists is logarithmic, i. e., they are, depending on the concentration range of the respective odorant, only just about able to reliably distinguish a halving or doubling of the odor concentration. Such an extremely high accuracy of the dilution ratios is not absolutely necessary since the requirements on accuracy dictate that they must be in an appropriate ratio to other error sources, mainly the human as a sensor.

Requirements of a Dilution System Conforming to DIN EN 13725:2003.

- Dilution range from less than 2^7 to at least 2^{14}, with a range of at least 2^{13} between the maximum and minimum dilutions.
- Predilution is permitted and can be applied to reduce the concentration of a sample to within the measurement range of the instrument.
- The step factor between consecutive steps must be greater than 1.4 but less than 2.4.
- The evaluation process must enable panelists to sample easily without distraction throughout the assessment of the odor.
- The air flow delivering the odor to the panelist must be at least 20 l/min.
- The port must be shaped in such a way that the air velocity in the port is at least 0.2 m/s.

Calibration of an Olfactometer According to DIN EN 13725:2003.

- Each step should be run at least five times with the recording of at least 10 single values in each case (Fig. 24.2).
- A full calibration of the performance of the dilution unit must be made at least once per year.
- The accuracy A_d of a set dilution step must be $\leq \pm 20\%$ (Fig. 24.3).
- The instability I_d of a given stimulus must be greater than 5% (Fig. 24.3).
- The reference material (analysis gas) used must have an accuracy higher than $\pm$ 3%.

Fig. 24.3 Mobile measurement laboratory (odor room) in use on site

24.1.3 Panel Members

The results of an assessment using an olfactometer depend quite decisively on the individual panel members and in particular on their olfactory acuity, their ability to reproduce odor threshold detection, their mental concentration, and their tendency to guess.

Panel Selection and Recurrent Screening

Panel members are typically selected according to a set of criteria to ensure homogeneity in terms of their average sensitivity and a good reproducibility of odor threshold detection. The requirements that panel members must satisfy and their behavior during assessments is covered by a code of conduct in DIN EN 13725, which states among other things that:

- Panel members shall be at least 16 years of age and willing and able to follow instructions
- The panel member shall be motivated to carry out his/her job conscientiously
- The panel member shall be available for a complete measurement session; preferably for a sufficient period to build up and monitor a history of measurement
- From 30 min before and during olfactometric measurement, panel members shall not be allowed to smoke, eat, drink (except water) or use chewing gum or sweets
- Panel members shall take great care not to cause any interference with their own perception or that of others in the odor rooms by lack of personal hygiene or the use of perfumes, deodorants, body lotions or cosmetics
- Panel members suffering of a cold or any other ailment affecting their perception of smell (e.g., allergic fits, sinusitis) shall be excluded from participating in measurements
- Panel members shall be present in the odor room or in a room with comparable conditions 15 min before the measurements start in order to get adapted to the actual odor environment of the measuring room
- During measurements panel members shall not communicate with each other about the results of their choices. Informing them of the correctness of their choices after the measurement can enhance the motivation of the assessors during the measurements.

Example of the Selection of a New Panel Member

Although it is well known that the range of sensitivity to individual odorants is much wider than for many multisubstance mixtures, sensitivity is currently determined exclusively with *n*-butanol as the reference substance. For *n*-butanol, the odor threshold concentration requirements of a sample group is a mean of 0.040 µmol and within the limits of 0.020 and 0.080 µmol/mol, which corresponds to 63 to 246 $\mu g\,m^{-3}$, and the standard deviation of the individual threshold estimate (ITE) should be less than 2.3.

The measurements should take place on three nonconsecutive days with a total of at least 10 and at most 20 single threshold estimates per panel member. In general, gathering roughly 20 ITE on *n*-butanol per panel member on these three selection days has proven effective, as the associated improvement in statistical certainty has a positive effect and more panel members achieve compliance with the demanded values.

Above and beyond the criteria given in DIN EN 13725:2003 relating to the mean and standard deviation of reference substance measurement, the following points also have a large bearing on the quality of an olfactometric assessment:

- Panel member motivation (working atmosphere, payment)
- Identification with the work (main task as a panel member in a company)
- Information on the measurement (awareness of the importance of the test)
- Discipline (compliance with the code of conduct for panel members)
- Length of panel membership (experience of different odors)
- Frequency of panel member work (panel's state of training)
- Panel member stress (number of samples and breaks).

Limits to Panel Member Performance

Panel member performance is the limiting factor for the number of samples measurable by a panel in a single day. The work of a panel member demands a high degree of concentration and is therefore much more stressful than initially supposed. The methods therefore must be optimized so that panel members are exposed to as little stress as possible without the quality of assessments declining, thereby ensuring that as large a number of samples as possible can be processed in a day. The intervals between the dilution steps should not be smaller than necessary, with a step factor of two being considered optimal in most cases. Closer gradations expose panel members to more stress without achieving a significant improvement in results.

The method of limits with ascending odor concentration operates largely in the range below the threshold and with very low odor concentrations in the proximity

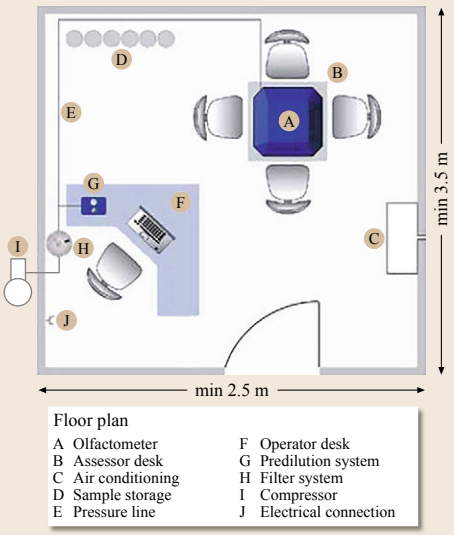

Fig. 24.4 Laboratory odor room, permanently installed

of the threshold concentration. The stressing of panel members via this method is much lower than with the method of constant stimuli.

If reference air and the diluted sample are presented alternately to panel members with each breath (inhalation), then adaptation is reliably prevented and panel members can compare each inhalatory breath with the next. The reproducibility of odor threshold identification is thus improved and the stressing of panel members is lower. Only given these preconditions can an average panel reliably measure about 16 samples per day if every four to six samples are followed by a break of at least 30 min. If a measurement project calls for a higher number of samples per day, several panels have to be employed.

24.1.4 Requirements of an Odor Room According to DIN EN 13725:2003

The European standard describes three types of possible odor rooms, each of which must be agreeable and odor-neutral:

- Stationary, permanent laboratory
- Mobile units, purpose built into a truck, van or container (Fig. 24.4)
- Specially adapted rooms.

Further requirements on the environment of the odor room are as follows:

- The room shall be kept well aired
- The maximum temperature in the room should be 25 °C
- Temperature fluctuations during the measuring process must not exceed ± 3 °C
- The room must not be exposed to direct sunlight
- No noise or light sources that can affect measurement are permitted
- The room must be hygienically clean
- It must be possible to force-ventilate the room via active carbon filters
- The CO_2 volume fraction in the room shall be less than 0.15%.

During prolonged sample transport to a permanently installed laboratory at the headquarters of the measurement institute, validation of storage stability is advisable. To this end, a single measurement should be performed on site after brief storage and an identical set of samples investigated again after a time equal to the planned storage time (maximum 30 h according to DIN EN 13725:2003). Only when storage stability has been confirmed should the maximum permissible storage time according to European standards be exploited in subsequent tests.

Sample preparation by dilution with nitrogen during sampling can improve a sample's storage properties. In all cases, temperature and exposure to sunlight during storage and transport must be controlled and documented to avoid thermal degradation or oxidation of the odorant molecules in the sample. One or more *reserve samples* for subsequent measurements should be planned if the samples are not to be immediately evaluated.

During measurements on site (at the location of the odor), the possibility of storing samples in an air-

Table 24.1 The table below lists the details that the assessment record or report must contain

Area	Required details
Olfactometer	Last calibration (with instability and accuracy)
Method	EN 13725
Measurement room	Temperature Max. 25 °C Delta max. 3 °C
Panel	Abbreviation, position Number and position of responses and errors on blanks Reference values min. 10, max. 20 ITEs History of reference values
Laboratory	Reference values; 20 laboratory values Precision, accuracy (A and r) History of laboratory values
Project	Project name Operator Place of measurement
Measurement	Date, time Dilution range Number of rounds Subsequent panel selection delta Z; zero sample errors Remarks on measurement
Sample	Designation Date, starting time of sampling Sampling time Predilution for sampling

Example of a measurement record conforming to DIN EN 13725:2003

conditioned odor room ensures optimum storage conditions. Storage time should be kept as short as possible in all cases. During measurements on site, sampling and measurement time can be mutually adapted to permit greater flexibility, for instance during facility setup and optimization and in the event of changes in operational processes or disturbances to facility operation.

24.1.5 Ongoing Quality Control, Repeatability and Accuracy of the Laboratory

To ensure reproducible results and minimal measurement uncertainties of the olfactometric method, DIN EN 13725:2003 demands certain standards of repeatability and accuracy of laboratories. These refer to a reference concentration (EROM) of the standard reference material *n*-butanol at a concentration of 123 $\mu g/m^3$ or 0.040 $\mu mol/mol$.

As a result, over and above checks of the individual panel members, the overall olfactometric system comprising the odor room, olfactometer and panel are tested with the aid of *n*-butanol. DIN EN 13725 demands compliance with the following values in this scenario

$$\text{Required repeatability } r \leq 0.477\,,$$
$$\text{Required accuracy } A \leq 0.217\,.$$

These requirements are calculated with the aid of at least 10 and at most 20 values of the geometric mean of all panel members Z_{ITE}. When a sufficient number of threshold estimates is available, an evaluation based on 20 values is better, as the scatter is thus improved (lower).

Even if the requirements of laboratory values r and A are still satisfied, there is a risk of the demanded accuracy no longer being achieved in the event of constantly high or low laboratory values (sensitivity), as the systematic deviation from the EROM becomes too large. In practice, when selecting the panel for a measurement, it must be ensured that *sensitive* and *insensitive* panel members are uniformly distributed in a panel in accordance with EN provisions in order to keep the panel average as constant as possible.

The ongoing control of laboratory values against the standard reference material *n*-butanol must be repeated in accordance with DIN EN 13725:2003 after a maximum of 12 samples in each case. An assessment at the start of each measurement of the overall measurement system comprising panel members, olfactometer and odor room using *n*-butanol as the reference material has therefore become established practice in olfactometry laboratories.

24.1.6 Assessment Record or Report

Dynamic Olfactometry as the method to determine environmental odor concentration is standardized in Europe for more than 30 years.

State of the art today are fully automated olfactometers. Due to the fact that odors are always mixtures of several chemical components, standard industrial mass flow controllers, calibrated on single components as used for olfactometry for many years are not suitable for olfactometry. Modern olfactometers are based on the proven principle of combining gas jet pumps and fixed sapphire orifices. Today, dedicated laboratory information management systems (LIMS) for odor labs are used, allowing the laboratory to perform on a much higher quality level than in the past. This LIMS guides and assists the lab to qualify the sensory panel, keeping track of lab performance as well as managing reference gases and projects. All this information has to be recorded according to EN 13725:2003 (Table 24.1). The improvement of the

quality of the olfactometric method over the last 15 years has been demonstrated in several international lab comparison tests with more than 40 participating laboratories.

Although sensor technology has seen major achievements and breakthroughs in recent decades [24.1], the human panelist is still the sensor of choice when it comes to the question of ambient odor rating, and derived strategies related to controlling the quality of ambient air. Considering this fact, panelists play the central role in the quality of the olfactometrical results.

However, further developments and optimizations are required such as ergonomic design of olfactometers, thereby offering the panelists a distraction-free environment, as well as improvement of the materials used for the sample dilution to limit the distracting impact on olfactory perception to a minimum.

24.2 Evaluation and Presentation of Measurement Results

The calculation of olfactometric analyses starting with the presented dilutions via a panel member's ITE through to the measured value in ou_E/m^3 is described in the following.

A complete set of panel member responses consists of at least eight ITEs from at least four panel members. An ITE is counted as a panel member result if the latter gives a true, positive response for two successive dilution steps. The geometric mean from the last not-identified dilution step and the first correctly identified step forms the individual result (ITE). From the responses from all panel members, the mean value is calculated as the Z_{ITE}. Panel members whose individual sensitivity deviates by more than a factor of five ($\Delta ITE \geq 5$) from the mean value of all panel members are excluded from the calculation of the odor concentration. The same applies to panel members who supply more than 20% false responses to the presented neutral gas (presentations without odor). The software of most olfactometers excludes panel members automatically.

References

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24.2 British Standard Institution: EN 13725: Air Quality–Determination of Odour Concentration by Dynamic Olfactometry (BSI, London 2003), German version EN 13725:2003

25. Assessment of Environmental Odor Impacts

Bettina Mannebeck, Heike Hauschildt

The field of environment odor assessment involves evaluating odors in ambient air, focussing primarily on offensive odors. Olfactometry is an indispensable tool used in this field to assess odors and establish their concentration at the source. This area of application incorporates regulated methods for sampling and measurement, and includes field inspections that aim to evaluate odors at or in close proximity to the problematic neighborhood. The human sense of smell is the primary tool for such analyses, representing an ideal proxy for the local population affected by the offending odor.

Various odor emissions are encountered in modern-day environments, many of which are increasingly relevant in terms of odor nuisance and are thus subject to legislations by local and national authorities. The past 20 years have seen a substantial increase in the number of regulations on environmental odors, both at the European Union (EU) level and at a national level by its member countries. This development has been concurrent with a growing common understanding and acceptance of annoyance and nuisance related to such odor emissions. Certainly, the effects of man-made environmental odors must be considered in the context of health and wellbeing, especially given the well-accepted holistic nature of health, which is defined by the World Health Organization as *not only the absence of disease, but also a state of complete physical, mental, and social well-being* [25.1].

However, the potential of odors to cause disease-like conditions is not fully understood, to date. Although symptoms of illness are often related to situations of extreme exposure, it is generally accepted that many somatic symptoms can be attributed to the exposure, rather than being caused by it per se. In other words, it appears that the psychological impact of odorant exposure has perhaps a more significant impact on wellbeing than the direct pharmacological or toxicological effects, at least in view of the low concentration levels often encountered, which potentially exhibit high odor potency and are thereby perceived as being at high levels. Further systematic studies would be required to comprehensively rule out the potential of actual physiological effects. Nevertheless, even if specific odorants are not of toxicological concern, the persistent exposure to unpleasant odors can dramatically reduce the quality of life of those affected. This might lead to source-directed, media-supported activities and/or lawsuits by the exposed community, resulting in an altered behavior by the perpetrator of the odor, by introducing abatement and/or avoidance strategies [25.1].

While odor regulations in the EU still vary greatly between its member states, methods for environmental odor assessment have been formalized at the EU level, with European standards as well as national, regional, and local guidelines available.

Given the fact that environmental odor is primarily concerned with its effects on human individuals or populations, it is reasonable that odor impact is evaluated by human assessors, both directly at the odor source and in the areas affected. To fulfill the required criteria for this important task, the assessors are carefully selected and tested using a defined routine to ensure that they can serve as a proxy for the broader population; in particular, this ensures that assessors

are not overly sensitive or conversely insensitive (or perhaps anosmic) to the target odorants. In addition to the requirements related to the olfactory sensitivity of individual assessors to specific reference materials, the necessary skills required by the panelists are also defined.

25.1 Odor Measurement at the Source

Olfactometry for investigating and evaluating odor emissions from sources is carried out according to the European standard EN 13725 [25.2]. Although this standard defines the method of odor assessment, there are currently no standardized practices for the method of sampling, neither at a national level nor from the EU. The annex of EN 13725 contains some details on sampling, with further details on measurement and sampling of stationary source emissions available in EN 15259 [25.3]. National guidelines in some instances, for example guidelines issued by Verein Deutscher Ingenieure (VDI), provide additional information on sampling and assessment.

25.1.1 Sampling

Planning and conducting sampling requires knowledge on the objectives of the measurement, the nature of the odor and its mass transport, and the targeted production processes that require monitoring. According to EN 13725, a sampling strategy should take into account the following aspects, which can be reviewed during a preliminary visit to the site [25.2]:

- Relevant odor-producing processes(s) to be identified
- Assessment of the toxicity and potential risk to the sampling team and the panel members of any emissions
- Location(s) of odor emission points
- Likely fluctuations in odor emissions with time (these can be estimated by using a continuous monitor like a flame ionization detector (FID))
- Odor sampling point locations
- Conditions affecting the odor emission:
  - Uncontrolled conditions, for example, the weather
  - Controlled or controllable conditions [25.2].

EN 15259 defines requirements for measurement sections and sites at waste gas ducts, and provides guidelines on the measurement objectives, plan and assessment report [25.3]. These considerations provide further steps towards gathering representative samples, conducting reliable assessments, and achieving comparable results.

Further questions that need to be addressed when setting up the sampling and measurement strategy relate to in situ assessment versus on-site sampling with off-site assessment, the storage period and conditions of samples for the latter scenario, and the sampling duration and the number of samples to be taken at each location. Some proposals for answering these questions are given in the following section.

Whether the analyses of samples are performed in situ or off-site after sampling depends on the specific goals of the measurement. In scenarios for which informative statements about emission behavior must be made available at short notice, for example as is necessary during the initial setup and optimization phase of an assessment task, in situ evaluation is helpful and often essential.

According to EN 13725 [25.2], the period between sampling and measurement, including sample transport, should not exceed 30 h. In Germany, VDI 3880 [25.4] dictates that for storage periods of longer than 6 h, proof must provided that the odor concentration in the samples has not changed from its original concentration at source. This check must be performed for each source and process condition, although a further examination may be omitted if the stability of odor samples has been proven at sources with identical waste gas compositions.

A frequently used sampling time is 30 min, as this is often required by law, in Germany by environmental laws. However, since a mean concentration value is often uninformative in cases of neighborhood nuisance caused by brief peak concentrations, shorter sampling times adapted to facility processes are advisable in some cases. This can be the case for sampled facilities with batch operation and/or discontinuous emission behavior.

A further sampling requirement is that the number of samples collected and analyzed must suit the task. To determine compliance with emission values, sampling is usually performed threefold. Facilities with consistent operating conditions that usually do not change over time require at least three single measurements with the highest odor flow rate during undisturbed continuous operation and at least one further measurement for each regularly occurring condition with fluctuating

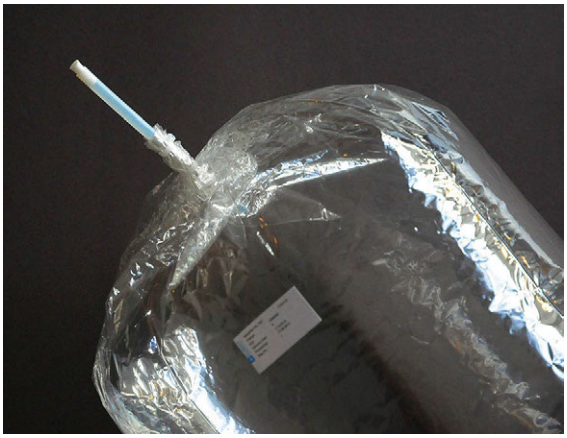

Fig. 25.1 Sample bag for odor samples made from Nalophan NA conform to EN 13725 (after [25.2])

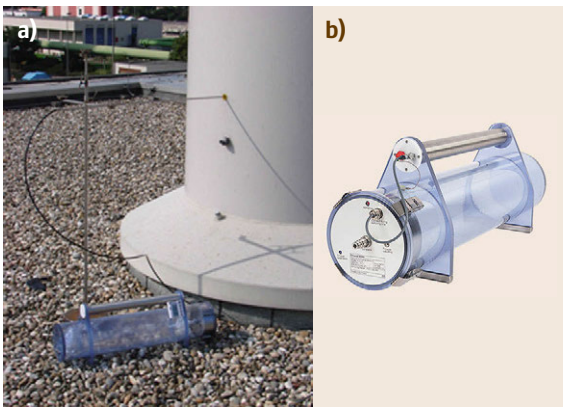

Fig. 25.2a,b Sample collection system consisting of a stainless steel probe, delivery pipe, and sampling device with sample bag at a duct source during sampling

emission behavior. Facilities with operating conditions that change over time require a higher number of measurements and at least six during the operating conditions that cause the highest emissions.

The demands on the material of all sampling materials (Fig. 25.1) are precisely defined in EN 13725 [25.2].

Different Types of Sources

Odor emission sources vary, and three different types of emission sources have been defined according to the shape of the emission source and to the presence of a volume flow. The three types of sources are: point sources, diffuse sources, and aerated diffuse sources.

Point Sources. Point sources or active duct sources, are waste gas ducts or ventilation outlets with a defined area and air flow rate. These sources are primarily industrial, controlled waste air sources such as stacks

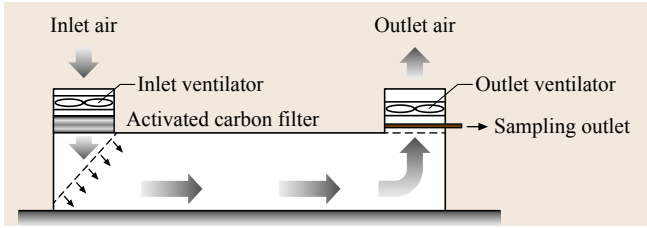

Fig. 25.3 Schematic diagram of an aerated sampling hood for diffuse or passive area sources: The inlet air is cleaned via the activated carbon filter and then directed over the open surface (liquid or solid) where it is loaded with the source-specific odor. The sampling outlet is used to collect the sample over an adequate period of time

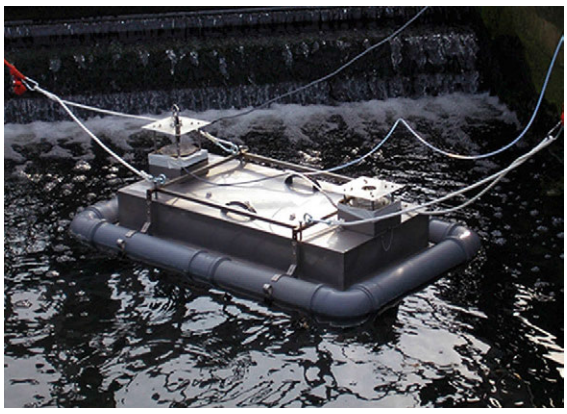

Fig. 25.4 An aerated sampling hood with floaters for liquid surfaces

and chimneys. In EN 15259 [25.3], these are termed stationary odor sources. A source is defined as active when the area-related waste air flow rate is greater than $30\,m^3/(m^2h)$ [25.4]. The complete measurement of active duct sources, in addition to sampling for olfactometry, includes the measurement of the waste air parameters such as flow rate, temperature, and humidity. The odor mass flow is calculated as the product of the measured odor concentration and the measured air flow rate. Indeed, a detailed knowledge of the facility at which the sample is taken, as well as its operating processes, is indispensable in allowing traceable statements that are representative of the state of the source to be made. Sampling at point sources can be done using a sampling chain consisting of a probe, a delivery pipe and a sample collection system [25.2], which usually consists of a sampling device and a sample bag. A typical sampling chain for duct sources is show in Fig. 25.2.

Diffuse Sources. Diffuse or passive sources are defined by convention to have a flow velocity of less than $30\,m\,h^{-1}$ based on the arithmetic mean over the entire surface [25.4]. Such sources are area sources (non-

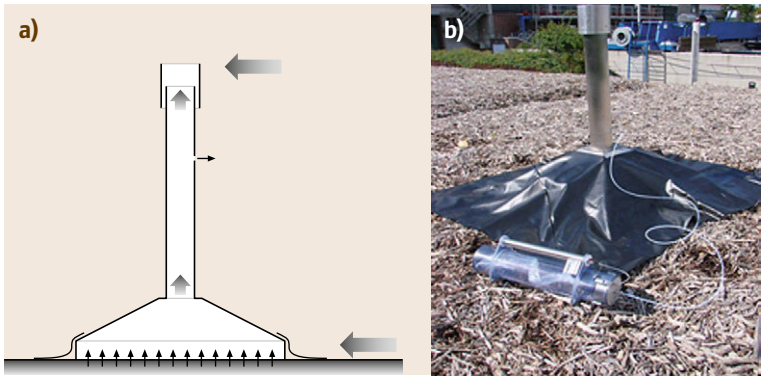

Fig. 25.5a,b Schematic diagram and picture of a sampling hood for active area sources. The sampling hood is placed on the surface of the aerated source. A sheeting (continuous apron) which overlaps the base of the sampling hood with a width of 0.2 m and a chimney collar prevents the dilution of the collected outlet gas by wind. The sampling/measurement orifice is used for sampling and concurrently for the corresponding velocity and temperature measurement. Schematic diagram **(a)** and picture **(b)** for active area sources.

aerated compost piles, waste dumps), passive liquid area sources (waste water tanks or basins), or volume sources (buildings with open windows or doors, leakages, ventilation through joints and windows, via wells or roof pipes). No standardized European regulations on sampling diffuse or passive sources are available at present. VDI guideline 3880 [25.4] describes a sampling strategy to ascertain the odor flow rate using specific measuring equipment, as shown in Figs. 25.3 and 25.4. As diffusion can be expected to have at least a noticeable effect on the overall emission flow, aerated sampling hoods are typically used. Calculation of the odor mass flow requires not only the odor concentration but also a corresponding volumetric flow rate per unit time. Thus the sampling methodology aims to extract a known quantity of air from a box covering a defined area of the source and to supply neutral gas to replace the extracted air with odor-free air. Sampling at diffuse sources can be done using a sampling chain consisting of an aerated sampling hood, a probe, a delivery pipe, and a sample collection system [25.2], which again typically consists of a sample bag and corresponding sampling device.

Aerated Diffuse Sources are diffuse sources with an air flow velocity of at least $30\,\mathrm{m\,h^{-1}}$. Such sources are usually area sources such as biofilters or aerated liquids. EN 13725 outlines two methods for sampling aerated diffuse sources:

- Via sampling hoods (Fig. 25.5)
- By covering the total source (or a large part of it) with foil to allow sampling of the mixed emission.

The flow rate of active area sources is measured in the crude gas ducts, with the sampling points defined according to EN 15259 [25.3]. Subsequent calculation of the clean gas flow rate requires the implementation of a humidity correction factor between the crude and clean gas [25.4] This approach relates to the fact that the measurement of the flow rate by means of a sampling hood placed over the surface of an active area source cannot be used directly for determining the waste air flow rate due to the influence of the wind and the pressure loss associated with mounting the sampling hood and leading to imprecise air flow rates. Still, the distribution of the air flow over the surface of the area source must be determined in addition to the flow rate, which is achieved by dividing the surface of the source into parts and measuring the individual air flows in each of these smaller areas. The amount of parts depends on the size of the source and is defined according to VDI guideline 3880. As long as the air flow rates of the parts differ by less than a factor of two, the source can be considered as homogenous [25.4]. Sampling at aerated diffuse sources can be done using a sampling chain consisting of a sampling hood, a probe, a delivery pipe and a sample collection system comprising the appropriate sampling bags and device [25.2].

25.1.2 Transport and Storage of Samples

Certain conditions must be met during transportation and storage of samples. On the one hand, the temperature should be controlled: the temperature in the sample bag should not exceed 25 °C and it should be kept above the dew point to prevent condensation in the sampling bag. On the other hand, exposure of the sample to sunlight must be avoided during storage and transportation to avoid photooxidation of odorants in the sample. Further, any damage or contamination of the bags, also from the ambient environment, must be prevented [25.2].

25.1.3 Measurement

Due to the nature of human olfaction in terms of its diversity in the perception of odor notes and the extensive dynamic range of sensitivity depending on the chemical structure of the odorant, there are currently no technical

sensors that offer an established relationship between the measurement detection and human odor perception, and most systems are too insensitive for many applications. As such, the human nose is indispensable in olfactometry.

Odor Concentration According to EN 13725

The determination of the odor concentration of a gaseous sample of odorants is determined by presenting a sample to a panel of selected and screened human subjects, varying the concentration by diluting with neutral gas, in order to determine the dilution factor at the 50% detection threshold.

More details concerning the determination of the odor concentration can be found in Chap. 24.

Descriptions of Environmental Odor

It is often helpful or even necessary to describe an odor in more detail than providing just its concentration. Especially in cases of odor annoyance, or when evaluating the efficacy of an odor mitigation technology, it is necessary to describe the odor in more detail.

Helpful parameters can be:

- Intensity – how strong do I perceive the odor?
- Hedonic tone – how pleasant do I perceive the odor?
- Odor character – how does it smell?
- Annoyance level – how disturbing is the odor?

These parameters are usually not part of a standardized evaluation under the current legislation. In some cases, however, for instance in Germany, the evaluation of the function of odor abatement technologies in which a comparison is made between the crude gas and clean gas is part of a license. In these cases, the license states that no crude gas character should be detectable in the clean gas.

Evaluating the intensity of an odor can be performed using an olfactometer according to VDI guideline 3882, sheet 1 [25.5], and likewise the evaluation of the hedonic odor tone can be performed according to sheet 2 of this guideline [25.6]; however, these are rare aspects of commercial projects involving odor measurements due to the required effort (and associated costs) and the absence of legal regulations.

Assessment of samples in a direct evaluation by a trained panel that takes into account the scales of the intensity or hedonic tone levels according to VDI guideline 3882 [25.5, 6] delivers useful additional information at manageable effort. Further, a direct evaluation can be used to gather information on verbal descriptions of the odor character or the annoyance level [25.7].

25.2 Measurement of Odor Impact by Field Measurement

The legislation of several European countries primarily focusses on protecting the population. In Germany, for example, the Federal Pollution Control Act (Bundes-Immissionsschutzgesetz – BImSchG) defines that harmful environmental effects must be avoided. It defines harmful environmental effects as those, which according to their nature, scale (concentration) or duration (persistence), are suited to bring about serious disadvantages or considerable nuisances for the public or neighborhood. In particular, the Act defines that an environmental odor is not a considerable nuisance as long as the human impact of an odor from production facilities is not more frequent than 10% of the yearly hours in inhabited areas or 15% of the yearly hours in commercial areas – provided the odor is not disgusting or nauseating.

The assessment of environmental odors in ambient air in several cases is only possible via the direct evaluation by a human panel. Dynamic olfactometry has a high detection limit, is generally only suitable for measuring odors of at least $10\,\mathrm{OU\,m^{-3}}$, and is thus not applicable for odors that can only just be perceived.

The draft of the European standard prEN 16841 [25.8, 9] presents methods that make direct use of the effects of odorants on the human sense of smell. This standard involves the use of qualified human panel members in the field to directly assess the presence of recognizable odors in ambient air according to a defined measurement plan and provides data that can be used to characterize odor exposure within a defined assessment area. So far the methods for field measurements are described in the guidelines VDI 3940 sheet 1 and 2 [25.10, 11].

The standard presents the following two key approaches:

- Part 1 describes a grid method that uses direct assessment of ambient air by panel members to characterize odor exposure within a defined assessment area.
- Part 2 describes a plume method for determining the extent of the downwind odor plume of a source.

The sensory methods described are only suitable for the assessment of odor in ambient air and are neither suitable for the occurrence of odor annoyance nor for the assessment of substances.

25.2.1 Grid Inspection (VDI 3940, Sheet 1)

Performing grid inspections to determine odor emissions in the vicinity of plants is covered by VDI Guideline 3940 Part 1 [25.10]. Trained odor inspectors are deployed to record odor detection at different monitoring points at different times of the day and night over a period of 10 min each. About 60 samples are recorded at each inspection point at 10 s intervals. The frequencies of the detected odors are then determined according to the concept of the *odor hour* and are statistically analyzed. A single measurement counts as an odor hour when the period for which an odor is present reaches or exceeds 10%. This implies that a recognizable type of odor is observed in at least 6 out of 60 observations, made at 10 s intervals within a 10 min measurement cycle.

The resulting odor hour frequency for each site is then plotted on a map as the number of positive assessments divided by the total number of single measurements, and are often color coded to indicate the severity of odor hours. Depending on the measurement task, this odor hour frequency map is generated for all installation-related odor types (to show the total odor impact) or for one or more specific installation or source odor types. The results of a grid inspection over half a year are shown in Fig. 25.6 for waste water related odors in the Rhine valley (cities of Mainz and Wiesbaden).

Grid inspections pose many challenges for the bodies responsible for monitoring odor emissions. Such inspections begin with a time-consuming and laborious phase of planning, made in coordination with the plant owners and the respective regulatory authorities before the project commences. In addition, a large amount of coordination and administrative work is required throughout the assessment period by the person responsible for the project (the project manager) to ensure that the inspections are performed in conformity with the guidelines.

The still widespread practice of paper inspection records for gathering the data has two serious inherent disadvantages. First, this method represents a high risk to the integrity of the data. Information from lost or incomplete records cannot be restored due to lack of redundancy, i. e., the absence of duplicate datasets. The second disadvantage relates to the fact that a considerable amount of time is lost between sending the records to the monitoring body and their corresponding non-availability of the information to the project manager. Consequently, this leads to a delay in the evaluation and delivery of informative results to the client.

In the current age of Internet applications and an increasing spread of powerful mobile terminal devices, the question perhaps arises as to why these grid inspections are still primarily carried out using paper records and why no satisfactory technical solution has been established, to date. To answer this question, it is necessary to take a closer look at the workflows involved in a grid inspection.

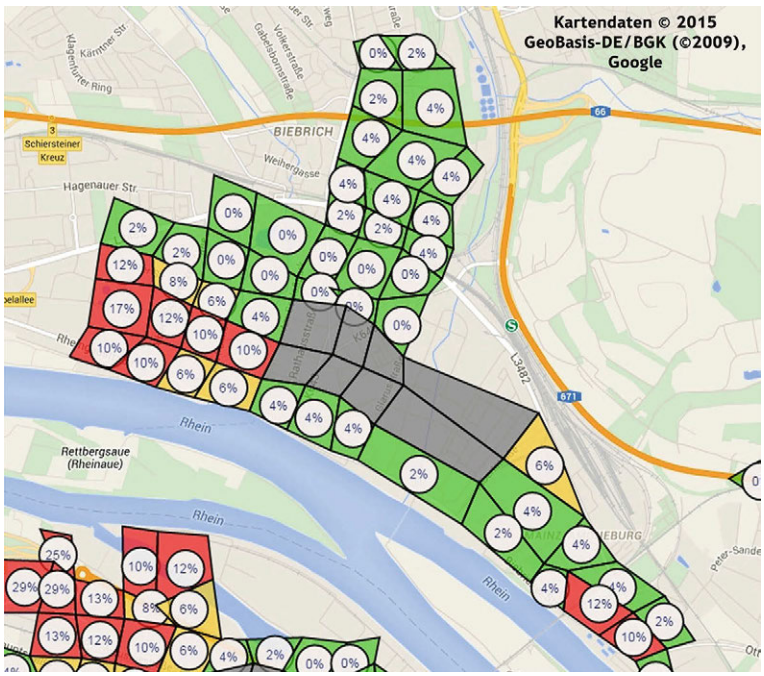

Fig. 25.6 An odor hour frequency map for installation-related waste water odors in the Rhine valley from 13/07/2011 to 27/01/2012. During this half-year, a total of 103 assessment squares (157 measurement points) were recorded. Each assessment square was measured 52 times, with 13 measurements for each measurement point (after [25.12])

Workflows for Grid Inspections

A grid inspection can be divided into three phases: planning, implementation, and evaluation. During the planning phase, site plans and map materials are used to define the inspection points and grid geometry for the evaluation area. The odor characters present in the evaluation area are determined with the help of an emissions register. The data collected during the grid inspection must be representative of the entire year, according to the requirements of VDI Guideline 3940 [25.10]. Attention must thereby be paid to the time and organizational planning to ensure a statistical spread of the scheduled sampling times during the day and night and on all weekdays. Before the project begins, the odor inspectors deployed for the project must be selected, trained, and familiarized with the work according to the requirements of EN 13725 [25.2]. The panel must comprise at least eight members (prEN 16841 [25.8]).

The implementation phase includes assigning and coordinating the odor inspectors and ensuring that the scheduled inspections are performed in conformity with the guidelines. As soon as the data collected during an inspection is passed on to the project manager, they must undergo a plausibility check to ensure that only validated data are incorporated in the evaluation.

The results are generally evaluated by transferring the data from the inspection records into prepared tables, where they can be analyzed and statistically evaluated. The results are usually delivered to the client in the form of a monitoring report that is sent after the end of the inspection period, although interim reports might be provided in some cases.

Optimization Potential of the Workflows

A large amount of work and time is required during the planning phase in order to collate the necessary data from various information sources and media for preparation of an appropriate schedule.

The deployment and organization of trained odor inspectors, most of whom do this work as a part-time side job, present additional challenges for the monitoring bodies. Inspections must be scheduled, adjusted and statistically spread uniformly among the odor inspectors for the entire project period. The monitoring body is also obliged to ensure that the inspectors perform their inspections properly by performing regular checks.

Therefore, organization and coordination of the inspections require fast and reliable communication between the monitoring body and the deployed inspectors. It must also be possible to respond at short notice to changes in inspection dates or times, or canceled or missed inspections, in order to minimize alterations in the inspection schedule. The organization of replacement personnel is an additional task for the project manager.

As is evident from the aforementioned issues, the administrative aspects of projects involve a large amount of work. Following an inspection by an odor inspector, the records must first be sent to the monitoring body, typically by post or e-mail. On arrival, the inspection records are then manually transferred into digital documents to enable a statistical evaluation. This human factor, especially where there are a large number of records, is an additional potential source of errors that can have negative effects on the quality of the results.

The digital documents form the basis for the preparation of interim and final reports, from which odor problems can be identified and possible measures can be derived. The time required for manual evaluation is therefore a decisive factor, which significantly limits the possibility of providing prompt results to the client.

Also the delivery of a final report to the client, from which the results of the inspection can be seen, always represents only a consideration of the past. In several cases an interim report is supplied in order to disseminate the findings on the initial odor emissions situation. According to this delayed availability of information (results from the field inspections) it is in generally not easy to directly trace odor emissions to emission-critical plant processes. Thus the plant owner is not enabled to quickly initiate corrective actions to improve the emissions situation. For a direct traceability, an immediate availability of information on the odor impact situation is indispensable.

The problems outlined above were identified early on in environmental odor emissions assessments and soon led to the development of rudimentary solutions.

The increasing spread of broadband Internet connectivity and efficient web applications in recent years has led to new options that are now available for dealing with these known problems. This development also benefits from the broad availability of powerful and cost-effective mobile terminal devices with Internet connectivity, which provide the basis for the development of comprehensive and efficient online systems that enable interaction between web and mobile applications.

State-of-the-Art Grid Inspection Tools

An online system for grid inspections is currently the most advanced method available for environmental odor emissions assessments. This online odor field inspection manager (OFIM) system combines the available Web 2.0 technologies to create a solution that

supports monitoring bodies from the initial planning steps through to delivery of the final results to the client. OFIM is based on a central online platform that is accessed by a web browser and enables users to log into the system from anywhere and to manage their own grid inspection projects. When new projects are created, the user can carry out all the necessary planning steps via this system, including creating and grouping various odor characters, defining inspection points and grid areas on the basis of digital map material, and defining inspection routes.

If grid points that are defined during the planning are not accessible for inspection, they can be relocated online within a very short time. Individual monitoring schedules that conform to VDI 3940 [25.10] can then be created automatically and the organization of personnel for the inspections can also be dealt with centrally via the online platform.

The inspectors can examine the planned inspection dates via Internet access to the system and can register for participation. Nevertheless, the final assignment of the inspection dates rests with the project manager to ensure the necessary statistical spread of the deployed inspectors.

The monitoring schedule includes information on the availability of inspectors, and with the assignment of an inspection date the system automatically sends a confirmation to the respective inspectors. This substantially reduces the administrative work required for assigning inspection dates and personnel coordination, which until now traditionally proceeded by phone calls and e-mails.

A mobile application for smart phones with the Google Android operating system is used by the odor inspectors to electronically record data during their inspections. Thanks to mobile Internet connections, smart phones using this system can be managed remotely, which is a major advantage compared to the earlier inspection system using pocket PCs. Moreover, this means that the mobile terminal devices can be kept by the inspectors for the entire inspection period, without the need for collection from and/or distribution to the inspectors before and after their use.

A user-friendly interface enables the inspectors to easily download the necessary project data on the day of the inspection, which is updated daily. GPS positioning is used during the inspection to ensure they are at the correct target coordinates. This is used by the monitoring body as an additional option for regular inspector control, as required in VDI 3940 [25.10]. The inspection results are subsequently transmitted to the online platform via the smart phone's Internet connection. This means that the results are imported into the system and are available to the project manager immediately after the inspection, allowing the results to be evaluated promptly. Further, the inspection results are evaluated fully automatically, thereby obviating the need for manual evaluation (and reducing potential human error) by the project manager. The system not only provides a detailed view of individual inspection results for the user, but also a comprehensive overview for a defined viewing period. With the selection of the required period, the results are made available to the users in various views, in tabular or graphical forms. The graphical representations of the results, for example, allow the emissions frequencies of the grid areas for the selected period to be examined at a glance. This allows the project manager to validate the results after a brief plausibility check.

An additional advantage of this online system is that access can also be made available to the client, which enables them to examine validated results for their project at any time. This might be of interest, for example, if certain plant processes are recorded at the same time, thereby making it possible to quickly draw conclusions regarding emission-critical processes and facilitate prompt initiation of measures to improve the emissions situation. If direct examination by the client is not required or not wanted by the authorities, the system can export the data for the preparation of reports.

25.3 Examples of the Evaluation of Odor and Emissions in Ambient Air

25.3.1 Case Studies

The application of grid inspections in real scenarios is explained in the following three case studies. The objectives and tasks in the application of grid inspection were different in each case, thereby illustrating the breadth of the system's use.

Case 1: Complex Plant Situation and Long-Term Inspection Monitoring

A waste treatment facility consisting of many individual plant parts caused marked complaints of odor nuisance among residents living downwind. The authorities deemed that the plant was suitable for approval. Despite the complaints, the plant owners planned to expand the facility.

Olfasense (former Odournet) was engaged to determine the overall emissions situation caused by the facility and its individual plant parts and to thereby participate in providing a solution to the problem in an advisory capacity. The assignment of the odors to specific parts of the plant and the measurement of the emissions of these individual parts were essential for achieving this. Assessment of the approved emissions of the individual plant parts showed that the limit values were exceeded in some locations and therefore the complaints of the residents were plausible. These inspections proceeded for over 4 years and were accepted by the plant operator and the residents as a control instrument.

Over a long monitoring period, it was possible to determine the emissions performance of the individual plant parts and to identify the critical processes. By optimizing the facility, the odor nuisance was reduced to below the limits defined by the authorities. The complaints situation has since been on a downward trend and continues to be monitored by means of inspections using the VDI 3940 method.

Case 2: Large-Scale Urban Area Inspections

Two adjacent German towns with diverse industrial areas and sewage treatment plants were subject to complaints of odors by the local populations. It was previously not possible to clearly assign these to individual plants. Olfasense (former Odournet) was therefore contracted by a governmental body to prepare extensive mapping of the odors in these urban areas.

The inspections were carried out over a 6-month period and included 170 inspection points. Inspections were carried out, recorded, and evaluated on a daily basis. Due to the transparency of the results and the determination of plausibility, it was possible to reliably assign the odors to the offending plants and thereby inform the plant owners of the critical odor situation of their plants.

Case 3: Correlating Grid Inspection Results in a Complaints Management System

A new waste treatment facility was set up with a complete complaint management system.

This grid inspection data system was integrated into an Internet communication platform that generated an online propagation modelling of the odor plume and a complaints recording tool and allowed for information to be transparently provided to the plant operator and local residents.

The system made it possible to integrate the grid inspection data into a complaints management system in order to compare the residents' complaints with objective data and to take appropriate action to control and improve the prevailing mood in the neighborhood. This precautionary measure is a good way of using open communication to prevent a tense neighborhood situation from developing.

25.4 Conclusion

This chapter presents a brief overview of the odor measurement methods used in the field of environmental odor assessment, either at the source or in affected areas. In the coming years, odor measurement will require the assignment of human assessors to capture the exposure-related effects on humans. It becomes clear that with the increasing demand for fast and transparent information state-of-the-art technologies will help to serve affected stakeholders in existing odor conflicts.

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26. Material Odor Emissions and Indoor Air Quality

Andrea Burdack-Freitag, Anja Heinlein, Florian Mayer

Indoor air is a complex and dynamic mixture of a huge variety of volatiles and particulate matter. Some of the constituents are odorous and originate from various sources such as construction materials, furniture, cleaning products, inhabitants and many more. Therefore, every indoor environment has a unique chemical composition in its air space. Volatile organic compounds and odorants in indoor air may cause psychological and/or physiological discomfort in humans. To reduce unwanted indoor air pollutants, it is of great interest to evaluate their sources and chemical structures. This chapter will provide an overview of methods used to evaluate indoor air and material emissions as well as current knowledge of odorants emitted by selected and common sources of indoor odors and in addition human bio-effluents. Measures to avoid and reduce odors as well as health concerns associated with indoor odorants will be discussed. Importantly, this chapter focuses on odorous organic volatiles present in indoor environments – non-odorous volatile organic compounds that can also affect indoor air quality will be mentioned only in passing.

Nowadays, people spend large parts of their lives indoors. Next to noise level and thermal comfort, the quality of air is essential for wellbeing inside homes, offices, schools and other private and public buildings. Nonspecific complaints like eye and airway irritation, dry skin together with general symptoms of headache and lethargy that occur whilst people are sojourning in a specific room or building are attributed to the *sick building syndrome* [26.1]. The cause of the symptoms is not conclusively clarified, yet it is often attributed to the presence of volatile organic compounds (VOCs). In total, several hundred different compounds have been identified in indoor environments [26.2]. Since most indoor materials emit VOCs, efforts are continuously made to reduce the total amount of VOCs (TVOC), semivolatile organic compounds (SVOCs) and carcinogenic substances emitted from buildings, indoor materials and products by complying with maximum emission levels. A number of national and international guidelines and standards for the monitoring and minimization of emissions are available for building products, for example issued by the German Federal Environment Agency (Umweltbundesamt UBA), Committee for Health-Related Evaluation of Building Products (Ausschuss zur gesundheitlichen Bewertung von Bauprodukten AgBB), by DIBt (Deutsches Institut für Bautechnik, German Institute for Building Technology) or by the German Blue Angel Labeling System (Blauer Engel) [26.3–5]. It is nonetheless difficult to document if the use of low-emitting construction materials actually results in fewer complaints, since the symptoms are often complex [26.6]. Additionally, there is a possibility of confusion of odor with sensory irritation [26.6]. For this reason and the fact that consumers refuse products with a strong smell, manufacturers are challenged to reduce not only TVOC emissions but also emissions of odorants. Specialized methods for the evaluation of indoor odors, odorant analysis and identification of odor emission sources have to be employed to tackle these problems.

26.1 Effects on Indoor Air Quality

One aspect influencing indoor air quality is the presence of volatile organic compounds. As a subgroup of VOCs, odorants can affect the perceived air quality in particular when people are consciously aware of their presence. Numerous sources of indoor VOCs and odorants are known, such as construction materials, furniture, plants, air conditioning systems, electronic devices, cleaning agents and humans themselves. Thereby, most odors in indoor as well as outdoor environments are not perceived consciously, either because of their low intensity or because the odors match personal experiences and expectations and are therefore unconsciously integrated into an olfactory background. Through alignment of this background to a present situation the environment is usually perceived as odorless or neutral. The presence of an unusual or particularly intense odor leads to a deviation from the background and hence attracts attention. Thereby, one and the same odorant can affect people in different ways, depending on its origin. For example, a smoky odor in paint is considered offensive, whereas its origin from a fireplace might be acceptable [26.7], from a piece of cured ham even pleasing. Additionally, unpleasant odors are often associated with health risks, which may cause psychological stress as well as physiological symptoms such as respiratory problems, nausea and headache [26.8].

For a long time, efforts have been made to reduce TVOC emissions in construction materials and recommendations for maximum emission levels are available [26.3]. Yet with a reduction in total emissions, the problem of odorous annoyances is not simultaneously solved, since TVOC values and odor intensity are not directly correlated as could be shown for several materials [26.9–11]. Figure 26.1 shows the noncorrelation of TVOC emissions and the perceived odor intensity of an oriented strand board sample. Odorants with low odor thresholds often appear only in trace amounts and are not detectable in routine emission measurements, yet they may strongly affect the sensory impact of the source material.

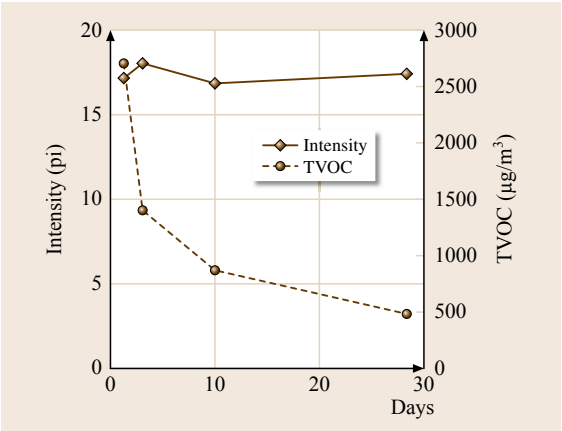

Fig. 26.1 Intensity and TVOC values of an oriented strand board sample (after [26.3])

26.2 Odor Evaluation Methods

Common techniques for odor analysis such as one- and multidimensional gas chromatography (GC) coupled with olfactometry (GC-O) and mass spectrometry (GC-MS), fractionation techniques and dilution methods such as aroma extract dilution analysis have been successfully applied to the analysis of indoor materials as well as air samples. Also, different sampling methods such as headspace and thermal desorption have proven suitable [26.8, 12, 13]. In recent years, online measurements based on mass-spectrometric (MS) detection, such as proton-transfer-reaction [time of flight] MS (PTR-[TOF]-MS), membrane inlet MS (MIMS) and selected ion flow tube MS (SIFT-MS) have been used for the assessment of environmental odorants [26.14]. As the methods mentioned are already well established in odor analysis and are thoroughly addressed in other chapters (Chaps. 17 and 18), they will not be enlarged upon in this chapter. Instead, panels, scales and methods used for the evaluation of indoor odors and odorous emissions from materials will be detailed.

26.2.1 Panels

A sensory panel should be considered as a true *measuring instrument*, and care should be taken in the recruitment of participants. Generally, it is possible to use either an untrained, naïve panel or a trained panel. Naïve panels should represent the average society regarding age, gender, social status and have a normal sense of smell. They should have no or little experience in olfactory testing and assess the presented odor unbiased. The rating of odor intensities and odor qualities by naïve panels should be interpreted with care since the perceptions are usually rather subjective. Yet hedonic tone such as pleasant/unpleasant or nauseous/appetizing from naïve panels are very valuable

since they represent the average perception of society. In untrained panels a number of 20–40 members is recommended to participate in the evaluation while in case of a trained panel 4–12 panelists are, depending on the specifications in various standards, considered as sufficient [26.13]. The members of trained panels have to be checked for olfactory dysfunctions and tested for their sensitivity to certain stimuli regularly. Guidelines for the selection, training and monitoring of panelists are available in ISO 8586:2012 and DIN EN 13725:2003 [26.15, 16]. Hence, trained assessors should be able to rate odors objectively and unbiased by personal preferences or aversions.

Next to the assessment of an odor in terms of its intensity, pleasantness or acceptability (see next sections), the odor quality can also be evaluated by descriptive sensory analysis. This should preferably be done by a trained panel with a well-developed flavor language. The application of standard lists or flavor wheels with fixed odor categories (e.g., fruity) subdivided by specific descriptors (e.g., apple, banana, strawberry and so on) can thereby facilitate the evaluation.

26.2.2 Scales

In order to evaluate different odor characteristics, diverse scales can be utilized, depending on the specific question and on the training status of the panel. Thereby, even for the same odor characteristic, such as its intensity, different designs of scales are in practice – for example category and comparative scales.

Odor Intensity

The odor intensity describes the relative strength of the odor above the recognition threshold (supra threshold). For the evaluation, many descriptive word category scales are in use, for example a seven-point scale with the descriptors *not perceptible, very weak, weak , distinct, strong, very strong* and *extremely strong* used by the German Guideline VDI 3882 Part 1 or a six-point scale with the descriptors *no odor, slight odor, moderate odor, strong odor, very strong odor* and *overwhelming odor* used by the Danish Society of Indoor Climate, based on the scale by *Yaglou* et al. (Fig. 26.2) [26.17–19].

Apart from that, comparative scales to assess odor intensity are widely used in order to measure the intensity more objectively and for a better interlaboratory comparability. Thereby, the intensity of the evaluated air is compared to the odor intensity of a series of concentrations of a reference compound in air. In the guideline DIN ISO 16000-28 [26.20] as well as German guideline VDI 4302, trained panelists rate the perceived intensity Π (PI) in comparison to ascending levels of acetone. The lowest reference point is 20 mg/m^3 (0 pi), where only 50% of the assessors can still perceive acetone (odor threshold), and is raised by another 20 mg/m^3 for each further reference point (e.g., 6 pi = 140 mg/m^3). Special equipment to present these different concentrations to the panelists is available [26.21–24] (Fig. 26.3). They consist of between one and eight funnels to present the different acetone concentrations including blank air. Systems with six to eight funnels present selected defined acetone concentrations while one- and two-funnel systems allow the individual adjustment of many different defined acetone concentrations. Similar to the perceived intensity scale, odor intensity referencing scales (OIRSs) used in the ASTM International Standard E544-99 and DIN EN

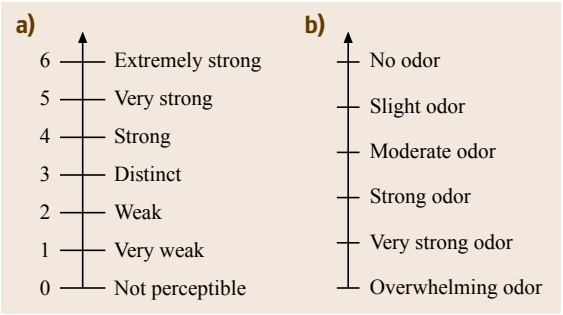

Fig. 26.2a,b Category scales for the evaluation of odor intensity according to **(a)** VDI 3882-1 (after [26.17]) and **(b)** Danish Society of Indoor Climate (after [26.18])

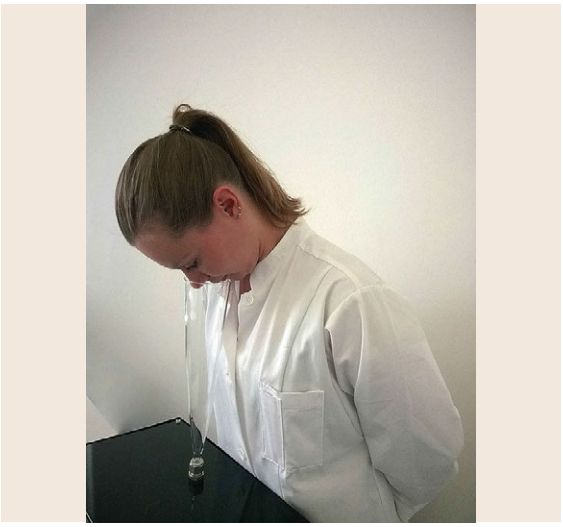

Fig. 26.3 A panelist is sniffing different concentrations of acetone in air that are being presented through special equipment with one sniffing funnel (Acetone Reference Standard ARS 2.0 developed by Fraunhofer IBP) (courtesy of Fraunhofer IBP)

13725:2003 employ a reference odorant, in this case n-butanol. Thereby, the reference odorant can either be presented in a static way using a set of bottles with fixed concentrations of the standard or dynamically using an olfactometer [26.16, 25].

Another approach to evaluating odor intensity is magnitude estimation, which puts the intensity of the assessed odor in relation to another odor. First, the assessor is asked to assign an arbitrary number to the intensity of a first odor (e.g., 50). The second odor is then rated in relation to the first (e.g., 25 if the odor is perceived as half as intense). This approach works best for similar odors [26.26].

Hedonic Odor Tone

Whilst odor intensity is a rather objective descriptor, the description of the hedonic odor tone is of a more subjective nature. It describes the subjective pleasantness or unpleasantness of an odor. Therefore, different category scales are in use, for example a nine-point scale ranging from extremely unpleasant (−4) to extremely pleasant (+4) (Fig. 26.4) or a 21-point scale ranging from unpleasant (−10) to pleasant (+10) [26.27, 28].

Acceptability

Like the hedonic odor tone, the rating of acceptability is of subjective nature. Acceptability or perceived air quality scales are widely used to measure the comfort in indoor environments [26.29]. Acceptability can be rated by a simple yes/no assessment or on a category scale. Panelists are thereby asked to imagine that they were exposed to the tested air for several hours daily. The scale can for example consist of two separate ranges, one reaching from *clearly not acceptable* (-1) to *just not acceptable* (0) and the other reaching from *just acceptable* (0) to *clearly acceptable* (+1) (Fig. 26.5) [26.10, 18, 30–33]. The discontinuity of this scale also forces the panelists to choose whether they perceive the air as generally acceptable or unacceptable. Acceptability votes are a combined impression of odor intensity and hedonic odor tone, as could be shown in experiments [26.29].

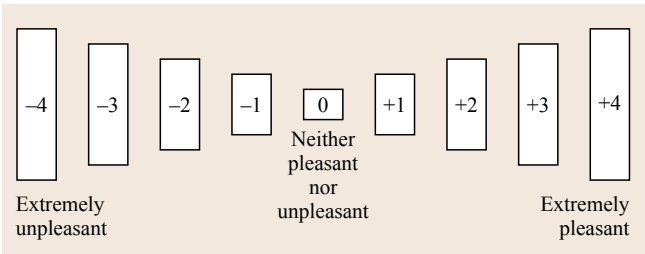

Fig. 26.4 Category scale for the evaluation of hedonic odor tone (VDI 3882-2, after [26.27])

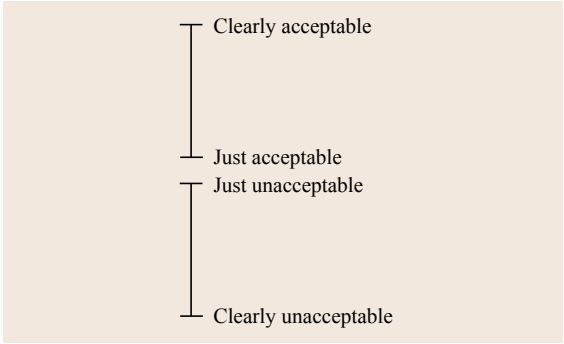

Fig. 26.5 Category scale for the evaluation of acceptability (the Danish Society of Indoor Climate, after [26.18])

26.2.3 Odor Evaluation of Indoor Air and Materials

The odor of a room or material can either be evaluated directly or after sampling. Different sampling techniques, depending on the type of source, and their subsequent analysis will be described in the following.

Evaluation and Sampling of Indoor Air

Ideally, the evaluation of indoor air should be performed directly on-site with a small trained or a huge untrained panel using intensity or acceptability scales as mentioned earlier [26.13]. Thereby, adaption effects have to be considered, which may vary between different odor sources. It could for example be shown that after a few minutes of exposure an adaption to human bioeffluents improves the acceptability of air considerably, adaption to tobacco smoke improves acceptability moderately and an adaption to emissions from construction materials improves acceptability only slightly [26.34]. To avoid adaption effects, dynamic sampling methods duct the air directly into a measurement device in close proximity to the odor source, either in situ or in a mobile laboratory, where the panelists can be exposed to the air in a more reproducible and timely controlled manner.

Due to cost and time constraints, these on-site evaluations are not always feasible. An alternative and widely used approach involves sampling, where an air sample of the investigated room is taken and transported to a test facility. The most crucial point in the course of this is to preserve the integrity of the sampled air. The quality of the sampling bag or container is essential in order to avoid contaminations or chemical-physical interactions between odorants and the sampling vessel such as adsorption processes and a permeation of compounds through the walls from the inside to the outside and vice versa. For this purpose, various gas sampling vessels such as polymer bags, glass bulbs or

stainless steel canisters are available that are considered to be impermeable, inert and odorless. Nonetheless, they should be checked for background emissions regularly [26.35, 36]. Additionally, condensation effects or further possible changes to the sampled air, for example due to degradation of compounds, must be avoided by temperature-controlled storage and transport or dilution of the sample. After transport to the lab, the air sample should be analyzed as quickly as possible, according to the European Standards EN 13725:2003 and ISO/DIN 16000-30 within 30 h from sampling, and according to German VDI 3880 within 6 h [26.16, 37, 38].

Another important and convenient approach to collecting an air sample is by sampling on adsorbent materials (Fig. 26.6). The most critical part of this approach is the choice of adsorbent, since discrimination processes are common. Three types of adsorbent materials are common: inorganic sorbents (e.g., silica gel), carbon-based porous materials (e.g., Carbotrap, charcoal) and porous organic polymers (e.g., Tenax). A combination of different adsorbent materials may help to recover a representative air sample. Hereby, it is important to ensure that no background odors are present on the adsorbent materials. Further ways to trap volatiles from air include liquid adsorbents, which often simultaneously function as derivatizing agents for specific target compounds, and cryo-trapping, which is often used in combination with solid sorbents. A subsequent desorption and analysis, by gas chromatography-mass spectrometry or elution and analysis, for example by high performance liquid chromatography, is then possible from adsorbent materials [26.2].

Fig. 26.6 Sampling of emissions from polymer granules for GC-O analysis. The sample is placed in a gas-tight headspace vessel. A pump sucks air through a small tube filled with Tenax TA adsorbent material (courtesy of Fraunhofer IBP)

Sampling and Evaluation of Materials

To evaluate emissions from materials, several standardized methods are in use, ISO 16000-6/-9/-10/-11/-25 and ASTM D-5516/6670/7143 [26.39]. The tested materials are incubated in emission chambers with stainless steel or glass surfaces that are sealed gas-tight against the outdoor atmosphere, in which temperature, relative humidity, air exchange rate and air velocity can be controlled and adjusted to reflect realistic indoor conditions. Depending on the investigated material, several chamber sizes can be used, ranging from large walk-in test chambers with a volume of several cubic meters, through test chambers with 200–1000 l of volume (Fig. 26.7), CLIMPAQs (Chamber for Laboratory Investigations of Materials, Pollution and Air Quality) with 50 or 225 l of volume, to micro-chambers or Field and Laboratory Emission Cells (FLECs) with only few cubic centimeters of volume allowing quick tests or on-site measurements (Fig. 26.8). After incubation, volatiles can either be trapped on adsorbents for further analysis, the air can be directly evaluated at an outlet of a large emission chamber or whole air samples can be taken in sampling bags (Fig. 26.9), as described above.

Besides to emission chambers, passive flux samplers (PFSs) can also be used to trap emissions of flat surfaces. These are small, transportable devices that consist only of a small petri dish and an adsorption disc. Carbotrap B or a filter coated with 2,4-dinitrophenylhydrazine (DNPH) have been successfully used to trap emissions using passive flux samplers [26.40].

Evaluation of Air Samples

For a standardized presentation of the undiluted air in sampling bags to all panelists, direct sniffing sys-

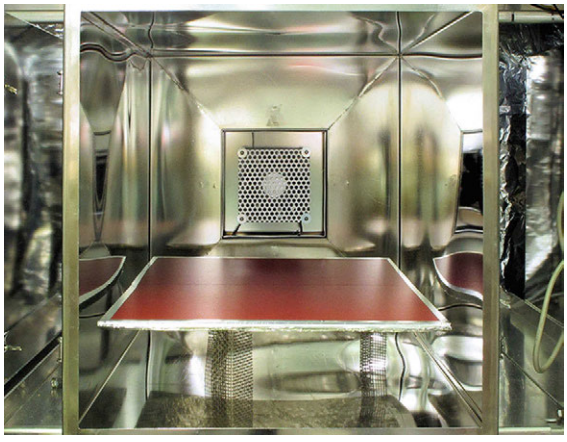

Fig. 26.7 Floor sample in a 200 l emission test chamber (courtesy of Fraunhofer IBP)

Fig. 26.8 Sampling of air using adsorbent material from a micro-chamber (courtesy of Fraunhofer IBP)

Fig. 26.9 Sampling of air from an emission test chamber into a sampling bag for subsequent odor evaluation (courtesy of Fraunhofer IBP)

tems are valuable. Thereby, the air sampling bags are introduced into the device, and the operator triggers a volume flow that causes the sampled air to stream through a funnel to the nose for odor evaluation (Fig. 26.10) [26.20]. Panelists can then rate odor characteristics as described above.

Another, widely used approach to evaluate air samples is dynamic olfactometry, using an olfactometer.

Fig. 26.10 A panelist evaluates an air sample provided by controlled deflation of an air sampling bag using a direct sniffing system (PureSniff) (courtesy of Fraunhofer IBP)

To assess the odor threshold, a diluted air sample can be presented to the panelists in ascending, randomized or descending concentration order. In ascending order the air sample is first diluted by a very large air volume, resulting in a sample with no perceptible odor. For subsequent samples, the volume of diluent is continuously decreased. The detection thresholds can then be determined through the first concentration in which each panelist has detected an odor and can be expressed as odor units per cubic meter (ou/m^3). In descending order, the air sample is continuously diluted with odor-free air by a factor of two. The minimum concentration perceived by 50% of the panel members results in the odor unit value per cubic meter. This method is only suitable for highly odorous samples since it does not allow small differences in the odor intensities of two weakly smelling odors to be distinguished [26.13]. Another disadvantage of the descending approach is that it could provoke adaption in the panelists as well as adsorption and carry-over effects. Similar problems could occur in a randomized order.

As an alternative to odor assessments by humans, *electronic noses* that mimic human olfaction have been developed (Chap. 21). They consist of an array of electrochemical sensors in conjunction with pattern recognition systems. The sensors need to possess partial sensitivity so that they do not only respond to a specific molecule but to a broad range or class of

volatiles [26.41]. In the field of indoor air quality control, specific electronic noses have been frequently used to monitor levels of harmful compounds such as formaldehyde, benzene, CO, NO_2 and toluene [26.42, 43], yet further applications to asses specific indoor odors are conceivable.

26.3 Indoor Odorants – Selected Sources

Some common indoor sources that have been investigated for odorous emissions will be discussed in the following. Table 26.1 gives an overview on potential odorants that have been identified as primary and secondary emissions from different materials. Interestingly, oxidized substance groups such as aldehydes, ketones and acids could be identified frequently and independently in several different sources.

26.3.1 Materials

Different types of construction materials possess their own specific emission patterns, depending on used raw materials, additives, coatings and processing methods. Amongst these emissions, several odor-active substances can impart a typical odor to a material or generate an unwanted off-odor. Secondary emissions, resulting from degradation processes of the materials such as oxidation and hydrolysis can thereby modify or generate odors over time (Fig. 26.11) [26.44].

Many studies use extraction techniques to isolate volatiles and odorants from materials, especially plastics, giving valuable information on material safety issues for example for food packaging or toys. These will not be mentioned here, since the liberation of odorants into air is considerably different than into fluids. Thereby, it should be kept in mind that the presence of a potential odorant in a given material does not necessarily cause the material to emit the typical odor. First of all, the liberation is dependent on physical factors such as humidity and temperature. Secondly, odor thresholds as well as synergistic effects among odorants have to be considered.

Plaster and Gypsum

Plaster is a very common and appreciated building product due to its flexible processing and wide field of application, for example as drywall, stucco or mortar for the coating of walls and ceilings. It consists of small-sized construction aggregate such as sand and binders such as lime. It is traded as solid powder and mixed with water to form a paste before use.

Plasters with organic binders have been investigated for their odor quality by means of gas chromatography-olfactometry/mass spectrometry. The odor of all plaster materials was mainly caused by organic acids such as acetic acid, pentanoic acid and hexanoic acid, which are responsible for acidic-sweaty and musty odor impressions. Furthermore, different samples emitted (*E*)-2-butenal, 2-ethylhexanol, 1-hexen-3-one and *p*-xylene, which are associated with plastic- and solvent-like odor impressions. In one sample with a fatty and rancid odor, (*Z*)-4-heptenal could be identified as key compound. Next to the compounds mentioned, further VOC emissions from plaster, such as further aldehydes, ketones, (cyclo)-alkanes/alkenes, alcohols, amines, amides, esters, glycols, heterocyclic compounds, organic acids, phenolic compounds, benzene derivatives, phthalates, silanes, siloxanes and terpenes, have been identified. Most of these substances are potentially odor active, yet due to concentrations below their odor thresholds they are unlikely to contribute to the odor impression of the investigated materials. It is furthermore important to notice that the odor impact compounds could not be identified by means of classical VOC analysis, but only by means of gas chromatography-olfactometry [26.45].

A special type of plaster is gypsum. It has a salt structure based on calcium sulfate ($CaSO_4$) and emits a typical odor described as gypsum-like, slightly milky and chalk-like [26.13]. Several aldehydes, ketones, acetic acid, 2-acetyl-1-pyrroline as well as traces of sulfur-containing odorants (sulfides, disulfides, trisulfides, thiols and aromatic sulfur compounds) have been detected as odorous emissions in gypsum samples using gas chromatography-olfactometry [26.7, 46, 47]. Depending on the raw material and processing

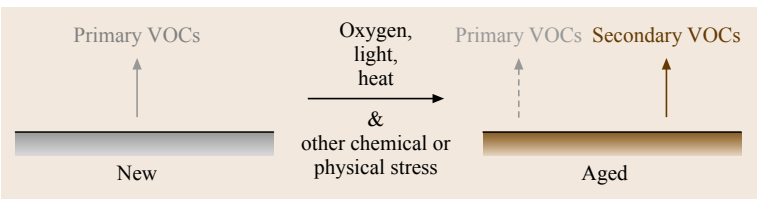

Fig. 26.11 Formation of secondary VOCs in materials

Table 26.1 Potential primary and secondary odorants emitted into indoor air by different sources

Odorant source	Primary VOCs potential odorants	Secondary VOCs condition	Potential odorants
Plaster/gypsum	Aliphatic aldehydes ((*E*)-2-butenal, (*Z*)-4-heptenal)	Oxidation	Increased benzaldehyde
	Aliphatic ketones (1-hexen-3-one)		
	Organic acids (acetic acid, pentanoic acid, hexanoic acid)		
	2-Ethyl-1-hexanol		
	p-Xylene		
	2-acetyl-1-pyrroline		
	Mono-/di-/trisulfides (dimethyltrisulfide)		
	Thiols (propanthiol)		
	Aromatic sulfur compounds		
Wood-based materials	Aliphatic aldehydes (pentanal, hexanal, heptanal, octanal, nonanal, (*Z*)-2-nonenal, (*E*)-2-nonenal, formaldehyde)	Oxidation	Increased aldehydes (several n-aldehydes, benzaldehyde, 2-nonenal, 4-nonenal, 6-nonenal)
	Terpenes (α-pinene, β-pinene, Δ3-carene, longifolene, β-phellandrene, camphene, myrcene, carvone, limonene, p-cymene, borneol, caryophyllene)	Microbiological degradation in combination with pentachloro phenol treatment	Chloro anisoles (2,3,4,6-tetrachloroanisole)
	Organic acids (acetic acid, butanoic acid)		
	Benzaldehyde		
	Acetophenone		
	1-Octen-3-one		
Linoleum	Aliphatic aldehydes (2-alkenals up to C_9)	Oxidation	Increased aldehydes
	Organic acids (up to C_6)		Increased organic acids (benzoic acid, propanoic acid)
	Ketones	Water	Increased organic acids (pentanoic acid)
	Toluene		Glycol ethers
	2-Pentylfuran		2-Decenal
Carpets	Esters (vinyl acetate)	Oxidation	Increased aldehydes (several n-aldehydes, 2-nonenal, acetaldehyde, methylbenzaldehyde)
	Alcohols (1,2-propandiole)		Ketones (2-butanone)
	Aliphatic aldehydes (up to C_{10})		Aliphatic acids
	Aromatic aldehydes (benzaldehyde, methylbenzaldehyde)	Heat	Benzothiazole
	Hydrocarbons (butylated hydroxytoluene, styrene, 4-phenylcyclohexene, 4-vinylcyclohexene)		
Polypropylene (PP)	Aliphatic ketones (C_6–C_9)	Oxidation	Increased ketones (C_6–C_9)
	Aliphatic aldehydes (C_6–C_9)		Increased aldehydes (C_6–C_9)
Polyethylene (PE)	Ketones (C_6–C_9)	Oxidation	Increased ketones (C_6–C_9)
	Aliphatic aldehydes (C_6–C_9, 8-nonenal)		Increased aldehydes (C_6–C_9)
	Sulfides	Thermal oxidation	Ketones (2,3-butanedione, 1-hexen-3-one, 1-hepten-3-one, 1-octen-3-one, 1-nonen-3-one)
	Thioles		Aldehydes (hexanal, octanal, nonanal, (*E*)-2-nonenal)
Thermoplastic polyolefin (TPO)	2-Acetyl-1-pyrroline		
	Ketones (2,3-butanedione, 1-hexen-3-one)		
	Aliphatic aldehydes (3-methylsulfanylpropanal, (*Z*)-2-nonenal, (*E*)-2-nonenal)		

Table 26.1 (continued)

Odorant source	Primary VOCs potential odorants	Secondary VOCs condition	Potential odorants
polystyrene (PS)	Hydrocarbons (styrene, α-methyl styrene)	Oxidation	Formaldehyde Benzaldehyde Benzoic acid
polyvinylchloride (PVC)	2-ethyl-1-hexanol	Water	Increased 2-ethyl-1-hexanol
	Phenol	Oxidation	2-Butanone
	1-Butanol		Increased aldehydes
polyurethane (PU)	Methylamines (trimethylamine)		
	Pyrazines (2-ethyl-3,5-dimethyl-pyrazine)		
polyphenylenoxide (PPO)	Phenols (guaiacols, cresols, xylenols)		
Rubber	Benzothiazole derivatives		
	Amines		
	Hydrocarbons (4-vinyl-cyclohexene, naphthalene, styrene)		
	Aliphatic aldehydes		
	Ketones		
	Esters (ethyl methylpropanoate, ethyl 2-methylbutanoate, ethyl pentanoate)		
	Phenols (*p*-/*m*-cresol)		
	Sulfides (hydrogen sulfide, carbon disulfide, dimethyl sulfide)		
	Benzaldehyde		
Electronics	Phenols (*p*-/*m*/*o*-cresol)		
Household and cleaning agents	Terpenes (citronellol, geraniol, linalool, α-terpineol, citronellal, geranial, neral, linalyl acetate, camphene, limonene, β-myrcene, α-phellandrene, α-pinene, β-pinene, α-terpinolene, α-terpinene)		
	Aliphatic aldehydes (octanal, nonanal, decanal)		
Human breath	Hydrocarbons (isoprene)		
	Aldehydes		
	Ketones (acetone)		
	Alcohols (ethanol, methanol)		
Human skin	Organic acids (3-methyl-2-hexanoic acid, 3-hydroxy-3-methylhexanoic acid)		
	Sulfanyl alcohols (3-sulfanylhexan-1-ol, 2-methyl-3-sulfanylbutan-1-ol, 3-sulfanylpentan-1-ol, 3-methyl-3-sulfanylhexan-1-ol)		
	16-androstenes (5α-androst-16-en-3-one)		
	Esters		
	Aldehydes (acetaldehyde, nonanal, decanal)		
	Hydrocarbons		
	Alcohols		
	Ketones (6-methyl-5-hepten-2-one, (*E*)-6,10-dimethyl-5,9-undecadien-2-one)		

methods, the concentration of sulfur-containing organic compounds can reach levels at which an unpleasant sulfurous off-odor is predominant [26.47]. After treatment with elevated ozone concentrations, a gypsum board sample showed increased secondary emissions of benzaldehyde, that could potentially lead to an off-odor [26.48].

Wood-Based Materials

Despite its wide use, the odor of wood itself has so far mainly been investigatedfor woods used for wine and spirits, where solvent extracts rather than emissions into air are used for analysis. Analysis of emissions from treated wood, wood products and furniture revealed mainly terpenes like α-pinene, β-pinene, Δ3-carene,

longifolene, β-phellandrene, camphene, myrcene, carvone, limonene, caryophyllene as well as saturated and unsaturated aldehydes, solvents and photo-initiator fragments such as benzaldehyde and acetophenone [26.13].

In parquet samples that were either oiled and waxed or varnished, the green-smelling short-chain aldehydes pentanal and hexanal, the wooden-smelling terpenes α- and β-pinene as well as 1-octen-3-one with a mushroom-like odor were identified as the most important odorants by means of GC-O and aroma dilution analysis. Important odorants that were specific for the used sealing were octanal, (*Z*)-2-nonenal and (*E*)-2-nonenal in oiled and waxed parquet and benzaldehyde and acetophenone in varnished parquet. Furthermore, not only differences in odor quality but also in intensity could be observed between the two samples. Since varnish prevents the release of odorants from the wooden parts to a higher extent than wax and oil, the overall odor of varnished parquet was less intense [26.49].

Wood-based panels, such as particle board, oriented strand board or medium density fiberboard are popular since they are less expensive, very versatile and still impart a nearly natural impression to furniture and construction materials. The panels are manufactured by binding small pieces of wood, often consisting of forest residues and sub-quality wood, with adhesives. Further additives such as wood preservatives, flame retardants, hardeners and hydrophobing agents might also be used. In these materials, formaldehyde and VOC emissions are of major concern [26.50]. Formaldehyde originates primarily from resins used to bind the wood particles, such as amino-formaldehyde, whereas other VOCs mainly originate from the wood itself [26.51]. Amongst other VOCs, further aldehydes (pentanal, hexanal, heptanal, octanal, nonanal) and terpenes (i. e., α- and β-pinene, camphene, 3-carene, p-cymene, limonene and borneol) originating directly from wood or from degradation of wood, have been detected and quantified in particleboard and medium density fiberboard products [26.52, 53]. Although these aldehydes and terpenes are well-known odorants, these studies did not assess the impact of these compounds on the overall odor of wood-based panels. Furthermore, organic acids like acetic acid and butanoic acid with a sweaty odor that originate from adhesives have been identified in oriented strand board samples [26.7].

Under the influence of ozone, it could be shown that terpene emissions decreased within 24 h while emissions of several aldehydes, such as *n*-aldehydes C_3 and C_5–C_{10}, benzaldehyde as well as 2-nonenal, 4-nonenal and 6-nonenal increased in different pine wood board samples [26.48].

In a case study, the use of unsuitably treated wood was identified as cause of a moldy off-odor in frame houses. The formerly used wood preservative pentachloro phenol can be degraded microbiologically to chloro anisoles, especially in damp areas with elevated microbiological activity. 2,3,4,6-tetrachloro anisole was identified as the main component in air samples taken from affected frame houses [26.54]. This substance has a very low odor threshold (0.01 ng/l air) and can therefore be the reason for the off-odor [26.55].

Linoleum

Linoleum is widely used as a flexible and durable floor covering. It is made of linoxyn (solidified linseed oil), natural resin, cork or wood particles, mineral fillers and pigments.

A gas chromatography-olfactometry investigation of linoleum samples revealed that mainly short-chain 2-alkenals (up to C_9) and fatty acids (up to C_6) with green, sour, citric and rancid odor attributes were responsible for the typical odor impression of linoleum [26.56]. As further odorous emissions from linoleum, saturated, branched and polyunsaturated aldehydes, ketones as well as toluene and 2-pentylfuran have been identified [26.13]. Oxidation of aldehydes to corresponding fatty acids has been observed in linoleum, which might cause a change in the odor impression of aged linoleum in comparison to new materials [26.44]. For example, increased emissions of benzoic acid and propanoic acid have been detected under the influence of ozone. Aldehyde emissions also increased whereas 1-penten-3-ol and 2-pentanone emissions were reduced [26.48]. Furthermore, the presence of water, either through wet cleaning or high water content in underlying concrete, has been shown to trigger secondary emissions of volatiles from linoleum, i. e., fatty acids, glycol ethers and 2-decenal. Especially pentanoic acid as well as 2-decenal with low odor thresholds (< 5 ng/l air [26.57–59]) can thereby generate an off-odor [26.60].

Carpets

For centuries, carpets have been valued as floor or wall coverings for their insulating and aesthetic properties. They consist of an upper layer of pile, made of natural or synthetic fiber, which is attached to an adhesive backing.

Odor emissions are mainly caused by the adhesive backing. For example, carpets with a polyvinylchloride (PVC) backing have been shown to emit primarily vinyl acetate and 1,2-propanediol whereas carpets with a polyurethane backing emit butylated hydroxytoluene [26.61]. Carpets with styrene-butadiene rubber latex backing have been shown to emit styrene, 4-phenyl cyclohexene and 4-vinyl cyclohexene [26.62].

Several n-aldehydes (up to C_{10}) as well as benzaldehyde and methylbenzaldehyde have been detected in carpets with rubber, textile, bitumen and PVC backings [26.48]. Under the influence of ozone, the total concentration of VOCs has been shown to increase in different carpets [26.62]. For example, emissions of saturated and unsaturated aldehydes (2-nonenal, acetaldehyde, methylbenzaldehyde) as well as branched and unbranched ketones (e.g., 2-butanone) and trace levels of aliphatic acids have been shown to increase in carpets that were incubated with ozone, depending on adhesives used as backing [26.48, 63]. Thereby, especially emissions of 2-nonenal can cause odor annoyances due to its persistence and very low odor threshold ($\leq$ 0.1 ng/l air for both (*E*)- and (*Z*)-isomer [26.64, 65]) [26.63]. Benzothiazole has been shown to be emitted in increasing amounts by a carpet with a styrene-butadiene rubber backing under the influence of heat [26.66]. While heat is of minor concern in building environments, temperatures up to 70 °C can be reached in parked cars, where textile floor coverings might then be a source of unwanted odorants.

Polymers

Plastics have become integral parts in many indoor environments. During the manufacturing of plastic materials from monomers using additives and accelerators, degradation or reaction products of raw materials can be generated, especially under the influence of heat, pressure, light or oxygen. Reaction products as well as the used raw materials themselves, especially in recycled products, may be odor active even in trace amounts.

Polypropylene PP. By means of GC-O, carbonyls with low odor thresholds, especially unsaturated C_6–C_9 ketones and aldehydes, were identified as major odor-active compounds emitted by polypropylene (PP). These are generated by oxidation processes from alkanes and alkenes and increase during aging of the material. Interestingly, commonly used stabilizers added during processing were unable to prevent the observed oxidation processes [26.67].

Polyethylene PE. Similar to polypropylene, unsaturated C_6–C_9 ketones and aldehydes were identified as the most important odor-active compounds emitted by polyethylene (PE), that are generated by oxidation independent of added antioxidants [26.67]. In another study, 8-nonenal has been identified as major contributor to the *plastic* off-odor in high density polyethylene (HDPE), although this study did not analyze air samples but used distillation to obtain volatiles [26.68]. Also, sulfurous smelling sulfides and thiols that originate from natural impurities in the mineral oil raw materials have been identified in PE samples [26.7]. Furthermore, the formation of secondary VOCs in PE after thermal oxidation has been studied. The treatment caused a strong waxy or burnt plastic off-odor. Using GC-O, several C_6–C_9 saturated or unsaturated aldehydes and ketones could be identified in the thermally oxidized samples. The most important were 2,3-butanedione, hexanal, 1-hepten-3-one, octanal, 1-octen-3-one, nonanal, 1-nonen-3-one and (*E*)-2-nonenal, whereof α-unsaturated aldehydes and ketones seem mainly responsible for the off-odor [26.69].

Further Polyolefines. In a thermoplastic polyolefin (TPO) sample, a strong roasty off-odor, rendering the product to be unacceptable for use, was identified as 2-acetyl-1-pyrroline, a nitrogen-containing molecule. Since none of the main ingredients of polyolefines contain nitrogen, the source of this off-odor has to be an additive. Due to the very low odor threshold of 2-acetyl-1-pyrroline (0.02 ng/l air [26.70]), even small amounts thereof can hence influence the sensory attributes of plastic materials [26.71]. Further important odor-active compounds identified in one TPO sample were 2,3-butanedione, 1-hexen-3-one, 3-methylsulfanylpropanal, (*Z*)-2-nonenal, (*E*)-2-nonenal and further unidentified compounds with roasty or geranium-like odors [26.72]. Other investigations of TPO materials showed that the odorant composition and overall odor perception is highly dependent on the formulation of raw materials, and on processing conditions, for example melt temperature and residence time during injection molding. The addition of a smell scavenger in TPO formulations has been shown to reduce amounts of acidic compounds, phenols and furanones, but not aldehydes [26.73]. Hence, the importance of elucidating the chemical structure of off-odors in order to find the right avoidance strategy becomes even more obvious.

Polystyrene (PS). Samples of polystyrene have been shown to emit the odor-active monomer styrene as well as α-methyl styrene that both cause acrid, solvent-like odor impressions [26.7]. After treatment with ozone for 24 h, styrene emissions decreased while emissions of formaldehyde, benzaldehyde and benzoic acid increased [26.48].

Polyvinylchloride PVC. A GC-O analysis of odorants emitted from polyvinylchloride (PVC) floorings revealed mainly 2-ethyl-1-hexanol (2E1H), phenol and 1-butanol as odor-active compounds. When in contact with wet construction materials, such as incompletely dried concrete with an alkaline pH, emissions of 2E1H increase sharply since this substance can be gener-

ated by hydrolysis of the ester di-2-ethylhexyl phthalate (DEHP), a plasticizer commonly used in PVC [26.74]. Contact with ozone led to secondary emissions of 2-butanone and increased emission of aldehydes in a PVC flooring sample [26.48].

Polyurethane PU. It could be shown that a polyurethane (PU)-specific odor is generated due to heat development during the manufacturing process. This odor arises from amine catalysts that are degraded to methylamines with low odor thresholds like trimethylamine, which can cause a fishy off-odor [26.75]. Furthermore, substituted pyrazines with extremely low odor thresholds (e.g., 2-ethyl-3,5-dimethyl-pyrazine; 7 pg/l air [26.76]) have been identified in PU samples causing earthy, nutty odor impressions [26.13, 71].

Polyphenylenoxide PPO. In Polyphenylenoxide (PPO), substituted phenols are used as raw materials and phenolic antioxidants are used as additives. Therefore, these phenols as well as degradation products thereof can be found to be emitted from the plastic material. For instance, odor-active guaiacols, cresols and xylenols have been identified by GC-O in emissions of PPO samples [26.71].

Rubber

The term rubber refers to polymers with elastic properties that can either be of natural or synthetic origin. To enhance the durability of these materials, they are often processed by vulcanization. During this process, the elastomers are cross-linked by addition of peroxides or sulfur together with nitrogen- and/or sulfur-containing accelerators like benzothiazole under heating. Therefore, benzothiazole derivatives as well as amines have been found to be emitted by rubber and seem responsible for the typical odor of rubber. Other compounds emitted by rubber are solvents, hydrocarbons like 4-vinyl-cyclohexene, aldehydes, ketones, esters like ethyl methylpropanoate, ethyl 2-methylbutanoate and ethyl pentanoate, phenolic compounds like *p*- and *m*-cresol, hydrogen sulfide, carbon disulfide, dimethyl sulfide as well as naphthalene, styrene and benzaldehyde [26.13].

Electronics

Electronics are active emission sources that develop heat during operation. Therefore, VOC emissions from electronic devices such as television sets, video recorders or computer monitors are especially high when switched on. A large number of VOCs from all kinds of substance classes, in total more than 350, have been shown to be emitted by electronics. Amongst them phenol-like compounds such as *m*-, *p*- and *o*-cresol, originating from printed circuit boards on phenolic resin basis, can be the source of odors [26.77, 78]. Additionally, it could be shown that the presence of operating personal computers can negatively affect perceived air quality and productivity in office environments. Since VOCs determined in this study could not sufficiently explain these adverse effects, the authors emphasize the inadequacy of analytical methods routinely used in indoor air quality investigations [26.79].

Household and Cleaning Agents

Cleaning agents, detergents and air fresheners are regularly used in indoor environments. These are usually scented to mask unwanted odors and to suggest cleanness and freshness. Odorants frequently used for this purpose are mainly terpenes like citronellol, geraniol, linalool, α-terpineol, citronellal, geranial, neral, linalyl acetate, camphene, limonene, β-myrcene, α-phellandrene, α-pinene, β-pinene, α-terpinolene and α-terpinene or aldehydes like octanal, nonanal and decanal [26.13, 80].

26.3.2 Human Emissions (Bio-effluents)

When entering a nonventilated, fully occupied room, the effect of human emissions on indoor air quality becomes obvious. In fact, humans continuously emit hundreds of VOCs through their breath and skin. Thereby, the composition of VOCs varies individually and is largely influenced by genetics, but also depends on age, health status, activity, personal hygiene and nutrition. Hence, human bio-effluents can also differ regionally and culturally. In an investigation of air samples collected in crowded rooms in Germany, indoor air contaminants that significantly correlate with human presence were identified. Those were ethanol, acetone and isoprene that originate from breath, nonanal and decanal that presumably originate from skin emissions as well as α-pinene, limonene and eucalyptol that are commonly used in personal care products. Although these substances are not willfully perceived by humans in the present concentrations since they do not exceed the odor thresholds, the combined presence of acetone, isoprene and limonene in elevated concentrations still correlates with the air quality being perceived as aversive [26.81].

Breath

Next to higher CO_2 levels in comparison to inhaled air, humans emit mainly isoprene, acetone, ethanol, methanol and other alcohols through their breath. Further compounds with molecular weights predominantly under 100 g/mol, including additional unsaturated as well as saturated hydrocarbons, aldehydes, ketones and

alcohols are emitted in minor quantities in human breath [26.82, 83]. While the major compounds are products of core metabolic processes and are similar in healthy humans, many minor compounds have exogenous sources such as food, medicine, skin care products or inhaled air and therefore underlie bigger qualitative and quantitative differences.

Skin

The axillary region is especially prone to the emission of human body odor (Chap. 49). The most important odor-active substances in human sweat are several carboxylic acids. Amongst them 3-methyl-2-hexenoic acid and 3-hydroxy-3-methylhexanoic acid are the most abundant ones, with very low odor thresholds in the range of 0.15–0.26 ng/l air [26.84–87]. Moreover, sulfanyl alcohols (3-sulfanylhexan-1-ol, 2-methyl-3-sulfanylbutan-1-ol, 3-sulfanylpentan-1-ol and 3-methyl-3-sulfanylhexan-1-ol) have been identified in trace amounts in human axilla secretions. Due to their extremely low odor thresholds in the range of 1–10 pg/l air, they are nonetheless likely to contribute to human body odor [26.88, 89]. Several volatile steroids of the 16-androstenes group with low odor thresholds (e.g., 5α-androst-16-en-3-one: 2.1 ng/l air [26.90]) have also been detected in human sweat [26.91–93].

Humans also emit various VOCs through non-axillary skin, amongst them several odorous compounds. As in breath, many of the emitted compounds are strongly dependent on individual factors, yet some are common to most humans. Studies that used headspace collection of volatiles emitted by skin surfaces found mainly carboxylic acids of various chain lengths, esters, aldehydes, alkanes, short chain alcohols and ketones. Thereby, 6-methyl-5-hepten-2-one, nonanal, decanal and (*E*)-6,10-dimethyl-5,9-undecadien-2-one (geranylacetone) have been frequently reported to be predominant in the volatile profile of human skin [26.94]. Furthermore, SPME (solid phase micro extraction)-measurements of skin emissions revealed acetone and acetaldehyde to be highly emitted next to the already mentioned 6-methyl-5-hepten-2-one [26.95].

26.4 Odor Avoidance and Reduction Measures

To minimize unwanted odorants in indoor air, basically two strategies can be followed. The most favorable is surely the avoidance of odorous emissions in the first place, by improving manufacturing processes or changing raw materials. The second strategy is the removal of odorous impact compounds from indoor air.

The ideal prerequisite for avoidance strategies is to know the nature of an unwanted off-odor in terms of its chemical structure, properties and concentration as well as to understand its generation pathway. Then, measures to eliminate the odor source from the material, for example by alterations in the production process, can be taken. Using this approach, an odor optimized PPO could be manufactured with improved values for odor intensity, hedonic odor tone and perceived air quality [26.71]. When recycled materials are used, the task of minimizing odorous emissions is especially challenging, since all kinds of odors might have been adsorbed to the raw materials during their previous use. The use of additives that bind specific odorants can help to minimize odors in the manufacturing of plastic materials. For example a combined zeolite called Abscents could effectively remove an off-odor in HDPE when added to raw materials during the manufacturing process [26.96].

A less cost-intensive and time-consuming strategy to control indoor odors is their removal from air. Therefore, mechanical filters that remove particles in combination with adsorbents that bind VOCs, such as activated carbon filters can be used. For example, mesophorous carbon materials such as activated CMK-3 and CMK-8 have been shown to remove indoor formaldehyde even at low concentrations [26.97, 98]. These filters need to be replaced from time to time when they have reached their capacity.

Other air purifiers use oxidation as a way to destruct VOCs and thereby allegedly remove pollutants from indoor air. For a long time, ozone-generating devices have been used, a strategy now severely criticized for potential health risks. For example, carboxylic acids, epoxides, organic peroxides, aldehydes and ketones have been shown to be formed by ozonolysis air purifiers [26.99]. Photocatalytic oxidation with active materials such as titanium dioxide (TiO_2) is also extensively used in order to destruct VOCs in indoor air [26.100]. Yet several studies have shown that this method does not always degrade the organic compounds completely to CO_2 and H_2O. Incomplete oxidation thereby generates aldehydes, ketones and organic acids, that might themselves be toxic, irritating or odorous, possibly even to greater extents than their precursors [26.101]. Plasma air cleaners as well as ionizer purifiers are suitable for particle removal, yet not for the removal of gas-phase pollutants. Plasma cleaners in combination with catalytic technologies have been shown to more efficiently remove VOCs [26.102].

26.5 Health Aspects and Sensory Irritations

Since this chapter is focused on odorants in indoor air, health concerns raised by other indoor VOCs such as benzene, carbon monoxide, formaldehyde, naphthalene, nitrogen dioxide, polycyclic aromatic hydrocarbons, trichloro ethylene or tetrachloro ethylene will not further be detailed, but are nonetheless of major concern. Information on this topic can be found elsewhere, for example from publications of the World Health Organization or other national authorities [26.103, 104]. Maximum levels of TVOCs, SVOCs (semivolatile organic compounds) and carcinogenic substances emitted from building products, as for example issued by the German Federal Environment Agency (Umweltbundesamt UBA), DIBt (Deutsches Institut für Bautechnik, German Institute for Building Technology) or the German Blue Angel Labeling System (Blauer Engel), as mentioned earlier, should generally be employed to minimize indoor air pollution [26.5].

When thinking about the specific health effects of odorants in indoor air, the main concern and most investigated effect is sensory irritation. Thereby, two important facts should be considered. First, it should be noted that an irritant is usually detected consciously in form of an odor – at least until the phenomenon of adaptation prevents awareness of the stimulus – since olfactory detection thresholds are generally one to four orders of magnitude lower than estimated thresholds for irritation effects of the upper airways [26.6, 105, 106]. A reason therefore is that sensory irritations in a person's ocular and nasal mucosae, also known as chemesthetic sensations, are transmitted through a different receptor system with different sensitivity than the olfactory system – the trigeminal nerve system [26.107] (Chap. 34). In several studies on sensory irritation of different vapors it could be shown that in homologous series the chemesthetic potency increases with chain length until a homolog is reached that fails to be detected (the cut-off homolog). For example, the cut-off homolog for a series of n-alcohols is 1-undecanol, for n-acetates decyl acetate, for *n*-butyrates hexyl butyrate, for 2-ketones 2-tridecanone, for *n*-alkylbenzenes heptyl benzene, for aldehydes dodecanal and for carboxylic acids heptanoic or octanoic acid, in ocular or nasal mucosa, respectively. Experiments suggest that these cut-off homologs exceed a critical molecular size that prevents them from interacting with the respective trigeminal receptors [26.108].

Second, it has to be noted that irritations may not originate from the odorant itself, but from reaction products being formed in indoor air, for example from reactions with ozone. Ozone present indoors may either be generated unwittingly from electronic devices such as photocopiers and laser printers or intentionally from air purifiers or it may simply infiltrate from outdoors [26.99, 109–111]. The reactions of ozone with some terpenes, such as d-limonene and α-pinene, that are common VOCs in indoor air originating for example from cleaning products and detergents, have been widely studied [26.111]. Due to C = C-bonds in their chemical structure, most terpenes easily react with ozone. In those reactions, several secondary pollutants such as various radicals, aldehydes (e.g., formaldehyde) and carboxylic acids as well as secondary organic aerosols (SOA), consisting of ultrafine ($< 0.1\,\mu m$) and lower fine ($0.1-2.5\,\mu m$) particle size ranges, are formed [26.112–117]. As opposed to their reactants, terpene/ozone oxidation products are associated with significantly higher sensory irritating effects [26.6, 118]. Thereby, the sensory irritating effect of formaldehyde is well documented in animal and human exposure studies [26.119–122]. Furthermore, it could be shown that terpene/ozone oxidation products of limonene, α-pinene and isoprene are causative of sensory irritations of the upper airways and airflow limitations in mice [26.105, 111, 123–127]. Although the ozone and terpene concentrations in these animal studies have been very high compared to realistic exposures of humans, derived human reference values for some common ozone initiated terpene reaction products indicated that 3-isopropenyl 6-oxo-heptanal, an oxidation product derived from limonene (Fig. 26.12), may contribute to sensory irritations in indoor environments [26.128, 129]. Further in vivo and in vitro studies of terpene oxidation products have observed health implications such as pulmonary inflammation, yet further data in order to substantiate these findings should be collected [26.111].

Overall, evidence for adverse health effects in rodents of terpene/ozone reaction products could be observed. Yet their impact on human health in an indoor setting depends on several factors such as concentrations of the reactants, relative humidity, reaction time (*fresh* or *aged* reaction products) and identity

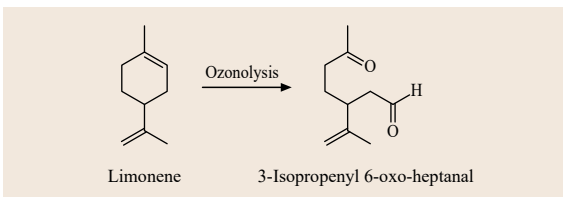

Fig. 26.12 Formation of the secondary pollutant 3-isoprenyl 6-oxo-heptanal from limonene through ozonolysis

of the terpene itself [26.111]. To fully understand the impact of ozone-initiated terpene chemistry on human health, multidisciplinary expertise from different scientific disciplines such as indoor and ambient air research, analytical and atmospheric chemistry as well as toxicology and medicine is required [26.111, 130].

As mentioned earlier, psychological stress as well as physiological symptoms such as respiratory problems, nausea and headache might also be caused by the simple association of unpleasant odors with negative health effects [26.8]. Notwithstanding, the presence of an unwanted odor in an indoor environment might also have a positive side-effect. For example the presence of earthy, moldy odors may be a hint for biological contaminations like mold with actual negative health implications, which would otherwise often remain undetected behind furniture or wallpapers.

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Odorant S Part D

Part D Odorant Sensing and Physiological Effects

Instrumental sensing has been highlighted in the previous section, together with the need for cross-linking such analytical data with human olfaction. Human sensing, however, is a non-linear process, being based on complex mechanisms that are intertwined with each other and regulated via complex feedback mechanisms. Right at the periphery, volatile molecules can be modified by physiological steps; these may influence the release, the interaction with receptors, and may even potentially generate new odorous molecules or degrade others due to biotransformation processes. The human body not only detects molecules, but also reacts to these, and these reactions may again modulate the primary and subsequent detection processes. Accordingly, instrumental sensors mainly measure and interpret what is present, while human sensing involves adaptive physiological mechanisms and processing steps related to the potential meaning of the molecules that are present in the immediate environment. Human olfaction is targeted at recognizing patterns and eliminating information that is of no relevance, or rather, information that has been learned and developed not to be relevant in the course of evolutionary processes and in a specific environment. Thereby, it is interesting to note that human olfaction is by far not as inferior to animal olfaction as is commonly believed.

On the other hand, olfactory detection systems, i. e., olfactory receptors, are not the only physiological targets that can be activated or modulated by odorants. Odorous substances are commonly small, extremely mobile, and lipophilic with some contribution of hydrophilic moieties that allow them to traverse the body, often without relevant barriers, and make them potential agonists for different physiological targets. Moreover, one needs to keep in mind that odorants do not simply travel the human body, but that there might be further strong biotransformation processes, irrespective of the point of entry of the compound, i. e., via the airways or via the gastrointestinal tract. Depending on their composition and concentration they may, for example, activate ion channels that play a role in trigeminal sensing, as well as in a series of other physiological processes or diseases; they also might act on pathways and cascades involved in central nervous and inflammatory processes, or allergies. In complex feedback loops and integration steps, such different ways of chemosensation might even interact with each other, through suppressive, enhancing or additive mechanisms.

Odorants may additionally serve other physiological functions in the human body, such as cell-to-cell chemo-communication and chemotaxis. On the other hand, if sensing of such chemo-stimuli is so complex, the likelihood of deviations between individuals, or even failures of parts of the physiological detection system due to innate or acquired defects, is high. Accordingly, disrupted chemosensory perception is a topic that is also addressed in the following selection of chapters.

27. Odorant Sensing

Heinz Breer, Jörg Fleischer, Jörg Strotmann

Chemosensory perception is one of the most important systems for appraisal of the environment. In contrast to physical sensory modalities, whose stimuli nature is constant (light, sound), the ever-changing odorous environment requires that the chemosensory system can cope with varying situations. This is accomplished by a large number of odorant receptors, most of them with a broad recognition spectrum due to a plasticity of the binding cavity. This apparent fuzziness is mandatory for the combinatorial mode of odorant recognition that allows reception of an almost infinite number of odorants with an enormous discriminatory power. Odorant receptors are expressed in olfactory sensory neurons (OSNs) following the one-neuron one-receptor rule; only one from more than a thousand receptor genes is expressed, in fact either from the maternal or the paternal allele. Thus, the receptor type determines the molecular receptive range of a particular chemosensory neuron. The choice and continuous expression of a particular receptor gene is supposed to result from a hierarchy of regulatory processes, involving cis-regulatory deoxyribonucleic acid (DNA) elements, epigenetics and a negative feedback mechanism mediated by the receptor protein itself. The chemoelectrical transduction process in OSNs is mediated by an intracellular reaction cascade leading to the generation of action potentials that are conveyed to the brain. An individual OSN extends its axon directly into the olfactory bulb where it targets onto a distinct spherical neuropil – the olfactory glomerulus – that connects sensory input with output neurons and local modulatory interneurons. The connectivity is remarkably precise such that only axons from neurons with the same receptor type innervate a specific glomerulus. The molecular determinants that control the complex process of axonal pathfinding, segregation, and targeting that lead to a receptor-specific wiring of the olfactory system have been only partially unraveled.

27.1 Structure of the Olfactory System

The structural design of the nose directs the inspired air toward the olfactory epithelium located in the roof of the posterior nasal cavity. In rodents, the paired nasal passages are highly convoluted with up to four cartilaginous structures, the turbinates, protruding into the nasal lumen. These intricate structures not only increase the surface area covered by the chemosensory epithelium, but also affect the airflow in the nasal cavity [27.1]. The olfactory epithelium is a pseudostratified columnar epithelium that comprises different cell types including basal cells, supporting cells, and olfactory sensory neurons (OSNs) (Fig. 27.1). The basal cells are in fact

stem cells capable of constant generation of new OSNs, allowing the olfactory epithelium to be reconstituted every 2–4 weeks. Because of its regenerative capacity, damage to the olfactory epithelium, by inhalation of toxic fumes or physical injury, is often temporary and only in extreme cases permanent, leading to anosmia. The supporting cells appear to function, analogous to neural glial cells, as metabolic and physical support for the OSNs; they are tall columnar cells with apical microvilli.

27.2 Olfactory Sensory Neurons

The OSNs are bipolar neurons; their unmyelinated axons congregate to form the olfactory nerve (cranial nerve I), which crosses the cribriform plate and projects to the target domain in the brain, the olfactory bulb (OB). All OSNs project a solitary dendrite to the epithelial surface of the nasal cavity that is covered with mucus secreted from Bowman's glands located in the lamina propria. The dendrite terminates in a spherical swelling called *knob* with a diameter of 1–2 μm that is decorated with a number of long, nonmotile cilia protruding into the mucus layer, thus considerably increasing the surface area [27.3]. This is of immediate importance as the cilia are considered as specialized compartments of the cells, capable to interact with odorous molecules and to convert the chemical signal into an electrical response. This concept was initially based on the observation that deciliated OSNs no longer respond to odorants [27.4], and moreover that a large inward current is only elicited when the odor stimulus is directed at the cilia, whereas the same stimulus fails to induce any current when directed at the soma [27.5]. Later on, it has been found that cilia are endowed with odorant receptor (OR) proteins [27.6, 7] – and also contain a number of signaling elements, including the G protein alpha subunit G_{olf}, the adenylyl cyclase type III (ACIII), the phosphodiesterase PDE1C2, and cyclic nucleotide-gated (CNG) channels [27.6, 8–14].

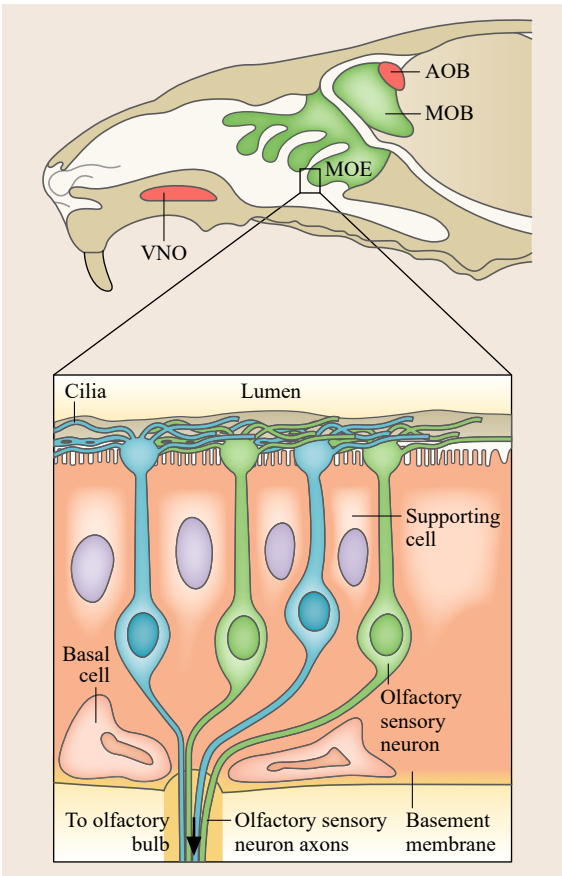

Fig. 27.1 Structure of the olfactory system: schematic sagittal representation of the head of a rodent. The main olfactory epithelium (MOE), the main olfactory bulb (MOB), the accessory olfactory system comprising the vomeronasal organ (VNO), and the accessory olfactory bulb (AOB) are highlighted (after [27.2])

27.2.1 Chemoelectrical Signal Transduction

In most cases, the process of odor sensing begins when the inhaled airstream containing scent molecules is flushed over the olfactory epithelium lining the nasal cavity. The odorous compounds then diffuse into the mucus, which is supposed to be facilitated by odorant binding proteins (OBPs). An interaction with appropriate chemosensory cilia triggers an intracellular reaction cascade converting the chemical stimulus into an electrical response.

The Canonical cAMP Cascade

The chemoelectrical transduction process is mediated via a transduction machinery that generates the second messenger cyclic adenosine monophosphate (cAMP), which in turn elicits a depolarization of the sensory cell [27.15] (Fig. 27.2).

First evidence for this so-called canonical cAMP pathway of olfactory signaling came from biochemical approaches demonstrating that odorants induce the synthesis of cAMP in olfactory cilia preparations [27.16, 17] and the accumulation of cAMP occurred within the

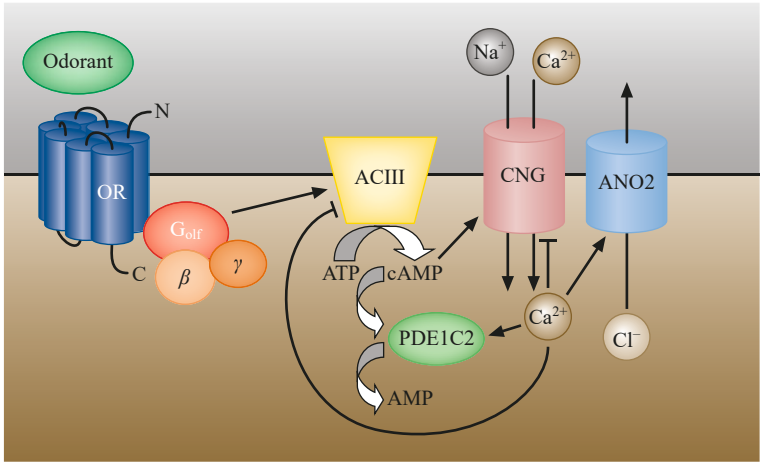

Fig. 27.2 Olfactory signaling cascade in OSNs

subsecond range [27.18]. This was in line with electrophysiological recordings demonstrating a latency of a few hundred milliseconds between odorant application and the electrical response [27.19]. The notion that the primary transduction process is based on a G-protein-coupled reaction cascade was substantiated by the findings that inhibition of G_{olf} by a specific antibody [27.20, 21] or deletion of the G_{olf} gene [27.9] led to a substantial reduction of odorant-evoked responses. G_{olf}, which is closely related to the G-protein G_s, is supposed to activate ACIII in the olfactory cilia [27.10], because a disruption of the ACIII gene in transgenic mice ablates odorant-evoked electrical responses [27.22]. As essential elements for converting elevated concentrations of cAMP into electrical currents of the sensory cells, CNG channels were identified in olfactory cilia [27.5, 23, 24]. The CNG channel of OSNs is a tetramer of three distinct subunits: two CNGA2, one CNGA4, and one CNGB1b [27.25–30]. Within the tetrameric complex, the two CNGA2 appear to be the principal channel subunits relevant for the activation by cAMP; the subunits CNGA4 and CNGB1b play a modulatory role contributing to the cAMP sensitivity, ion selectivity and permeability, as well as negative feedback control [27.27, 31, 32]. The olfactory CNG channel is permeable not only for K^+ and Na^+ ions but also for Ca^{2+} ions [27.4, 25, 33, 34]. The conductance of the channel was found to be relatively low ($\approx$ 0.5 pS), leading to a contribution of less than 0.05 pA of each individual channel [27.35]. Yet, conductance of Ca^{2+} by the CNG channel leads to an influx of Ca^{2+} ions from the mucus into the cilia, which evokes a rapid increase in the ciliary Ca^{2+} concentration [27.36, 37]. This is of particular importance since the ciliary membrane is endowed with Ca^{2+}-gated Cl^- channels that can be directly opened by an increase of cytoplasmic Ca^{2+} concentration [27.38]. Although diffusion of Ca^{2+} within the cilia appears to be limited [27.39] and Ca^{2+} ions are buffered by Ca^{2+}-binding proteins [27.40], the odorant-evoked Ca^{2+} influx via CNG channels clearly activates Ca^{2+}-dependent Cl^- channels in the ciliary membrane [27.41–43]. Under physiological conditions, opening of Ca^{2+}-activated Cl^- channels evokes a depolarizing inward current, an efflux of Cl^- ions. Such an efflux of Cl^- ions is based on an elevated intracellular Cl^- concentration in OSNs that is (at least) in a similar range as the Cl^- concentration of the mucus surrounding the cilia [27.44, 45]. This unusually high concentration of intracellular Cl^- of OSNs is believed to be mediated by the $Na^+K^+2Cl^-$ co-transporter Nkcc1 [27.46–48]. Two candidate proteins have been identified that may serve as Ca^{2+}-gated Cl^- channels in olfactory cilia; bestrophin-2 and transmembrane protein 16B (TMEM16B) (anoctamin-2 (AN02)) [27.49–51]. However, based on recent studies, a role of bestrophin-2 has been excluded [27.52]. Thus, currently, ANO2 seems to be the most promising candidate; in particular as a recent study has demonstrated that Ca^{2+}-activated Cl^- currents are absent in ANO2-deficient OSNs [27.53]. With a channel conductance of 0.5 pS [27.35] and a half-maximal activation at 5–20 µM Ca^{2+} [27.38, 54], ciliary Ca^{2+}-activated Cl^- channels are supposed to mediate up to 90% of the total odorant-induced current [27.46, 55]. In fact, more than 80% of the response was found to be blocked by the Cl^- channel inhibitor niflumic acid [27.56]. Therefore, it has been proposed that the Cl^- current provides a substantial amplification of the primary CNG-mediated cationic current [27.42] enabling a higher signal-to-noise ratio than the primary signal [27.35]. Yet, recent findings challenge the concept that Ca^{2+}-evoked Cl^- currents are essential for olfactory signaling: disruption of the ANO2 Cl^- channel reduced fluid-phase electro-olfactogram

responses by only ≈ 40%. Moreover, elimination of ANO2 did not affect air-phase electro-olfactograms and did not reduce performance in olfactory behavioural tasks [27.53]. Consequently, the authors of this study concluded that there may be no need for a boost by Cl^- channels.

Termination of Signaling Processes

For repeated stimulation and for encoding temporal information, it is essential that the odorant-induced cellular response in OSNs is terminated rapidly. The termination of the olfactory reaction cascade is primarily mediated by Ca^{2+}-dependent processes. At elevated Ca^{2+} concentrations, binding of Ca^{2+}/calmodulin inactivates CNG channels via channel closure. This is apparently due to the fact that binding of Ca^{2+}/calmodulin reduces the affinity of the channel for cAMP by up to 20-fold [27.57, 58]. Moreover, another Ca^{2+}/calmodulin-controlled protein – the Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase II) – seems to play a role in signal termination. At increased Ca^{2+} concentrations, phosphorylation by CaM kinase II inactivates ACIII in olfactory cilia preparations [27.59, 60]; thus terminating ciliary cAMP generation. Also the calmodulin-dependent phosphodiesterase subtype PDE1C2 present in the cilia of OSNs may contribute to signal termination and/or adaptation to repeated stimulation [27.12, 61]. Since the ciliary concentration of Ca^{2+} is of central importance for olfactory signaling, a rapid removal of Ca^{2+} from the cilia is essential. This process seems to be mediated by the $Na^+/K^+/Ca^{2+}$-exchange protein 4 (NCKX4) (SLC24a4) [27.62]. In addition to Ca^{2+}-regulated processes, also phosphorylation of ORs by protein kinase A (PKA) and the G-protein-coupled receptor kinase 3 (GRK3) has been proposed to contribute to adaptation and/or signal termination in OSNs via receptor desensitization [27.63, 64]. Yet, further studies are required to unravel in more detail the relevance of receptor phosphorylation by different kinases for termination and adaptation of olfactory signaling.

27.3 Recognition of Odorants

The perception of smell is based on the highly sensitive recognition and precise discrimination of myriads of foreign molecules from the environment. This task is analogous to the recognition of diverse antigens by the immune system. The olfactory system, as well as the immune system, accomplishes this formidable challenge by means of many different receptors. In the immune system, the receptors are generated by a combinatorial arrangement of constant and variable immunoglobulin chains, whereas for the olfactory system more than a thousand genes are set aside to encode a repertoire of receptors that collectively can recognize and discriminate what seems to be an unlimited number of odorous compounds.

Table 27.1 Odorant receptors (OR) gene repertoire of selected vertebrate species

Species	Number of OR genes	Number of intact OR genes
Human	851	384
Chimpanzee	899	353
Dog	971	713
Mouse	1375	1194
Rat	1576	1284
Opossum	1516	899
Chicken	554	78
Pufferfish[a]	94	40
Zebrafish[a]	133	98

[a] After [27.65] and [27.66]

The notion that receptors for odorants may comprise a large family of seven-transmembrane-domain G-protein-coupled receptors (GPCRs) expressed in the olfactory epithelium has led to the discovery of the first few OR genes in rat [27.67]. This seminal finding opened the avenues for the identification of a whole family of OR genes that, on the basis of genomic library screenings, was predicted to comprise several hundred, may be more than a thousand members in mammals [27.68].

27.3.1 Diversity of Genes Encoding Odorant Receptors

More recently, detailed assessments of complete genome sequences revealed that the OR gene family accounts for about 1500 genes in mice. Thus, the multigene family that encodes the receptors for odorants represents the largest gene family in the mammalian genomes; collectively they represent up to 3% of all genes. The total length of the olfactory subgenome is estimated to be about 30 Mb and all OR genes occupy about 2% of the genomic DNA. As the genome sequences of additional species became available, a comparison of different olfactory subgenomes was possible. It turned out that the size of the OR repertoires (Table 27.1) varies considerably among different species; the reason for this remains elusive, as the number of OR genes often does not correlate with the apparent importance of olfaction. While humans and other primates,

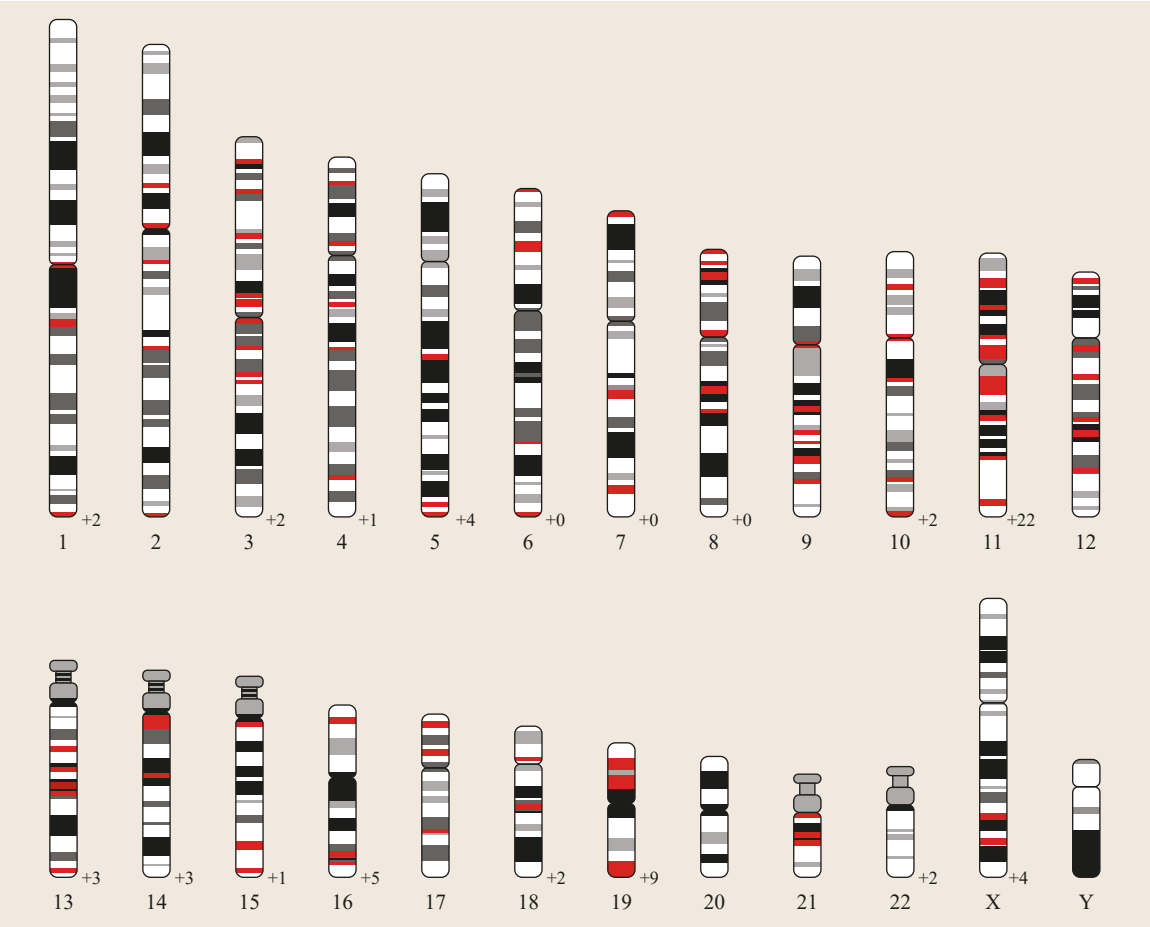

Fig. 27.3 Distribution of genes encoding ORs in the human genome. Genes for ORs are organized in clusters (labeled in *red*) with a bias for the pericentromeric and subtelomeric regions (after [27.69])

like chimpanzee, have about 800 OR genes [27.70, 71], the dog, being well-known for its excellent smelling capability has about 970 ORs [27.72]. The chicken's OR repertoire consists of 554 genes; only 78 among them are intact [27.73]. Lower vertebrates, such as fish have only a few dozens of OR genes, the Pufferfish has 40 ORs while the Zebrafish has 98 [27.65].

Genomic Organization

Each OR is expressed from a separate gene; the coding region is an intronless single exon of about 1 kb, as typical for many GPCRs [27.74]. There is a polyadenylation signal less than 1 kb downstream from the stop codon and a variable number of upstream exons [27.75]. The noncoding exons in the 5′ untranslated region may undergo alternate splicing leading to different isoforms of mRNAs [27.76]. Within the genome, the OR genes are distributed in multiple clusters, which are dispersed among various loci that contain anywhere from 1 to 100 genes [27.66]. In human, these clusters are found in more than 40 locations on almost all chromosomes, except chromosome 20 and the Y chromosome (Fig. 27.3). The largest clusters are localized on chromosomes 2 and 7, which harbor 344 and 267 OR genes, respectively. Within a cluster, spacing between adjacent OR-coding regions varies between 5 and 70 kb, and non-OR genes are usually excluded from OR gene clusters. While some chromosomes [27.1, 7, 10, 12, 15, 20] comprise many genes for ORs, chromosome 22 contains only a single OR-encoding gene.

Odorant Receptor Pseudogenes

A significant portion of the OR gene family has been pseudogenized. This is particularly striking in humans, where around 55% of the OR genes has degenerated to pseudogenes, leaving only about 350 apparently functional OR genes. Other primates, such as the chimpanzee, have a similar percentage of pseudo-

genes [27.77]. The proportion is much lower in rodents, where pseudogenes account for only about 15% of the OR genes [27.78]. These results imply that the mouse possesses over three times as many intact genes as human. In spite of the reduced number of OR subtypes, the human olfactory system retained the ability to recognize a broad spectrum of chemicals; however, it is probably less discriminating between closely related odorous compounds. The high proportion of pseudogenes [27.79] and the unusually high rate of single nucleotide polymorphisms (SNP) in human OR genes [27.80] indicate a variable repertoire of functional ORs in the human population. Interestingly, the decline of the OR gene family in some primates has been found to coincide with the acquisition of trichromatic vision, suggesting that better visual capability makes olfaction partially dispensable [27.81].

Odorant Receptor Gene Classes

Exploring candidate ORs in different species has led to the discovery of two unambiguous subdivisions of ORs, designated class I ORs that were first found in fish [27.82] and in the water-accessible nasal cavities of amphibia [27.83], and clearly distinguished from class II ORs typical for terrestrial vertebrates [27.84]. Genome database mining has subsequently shown that both classes do also exist in mammals. Based on sequence homology, 90% represent class II ORs and 10% are class I ORs. Interestingly, the more than 100 class I OR genes are assembled in a large cluster on human chromosome 11, as well as in the syntenic locus on mouse chromosome 7. Class I ORs in mammals were originally thought to be evolutionary relics; however, the relatively large number of intact class I OR genes in the mammalian genomes suggests an important role in mammalian olfaction [27.85]. This notion is supported by the finding that the percentage of pseudogenes among class I receptor genes is significantly lower compared to class II genes [27.86]. Class I genes are expressed exclusively in the most dorsal zone of the olfactory epithelium [27.87, 88] which has led to the speculation that they may be tuned to moderately hydrophobic odorants. Although the functional importance of class I ORs in mammals is still unclear, it is interesting to note that a recent study has demonstrated that dorsal-zone-depleted mutant mice lack the innate responses to aversive odorants [27.89].

The observation that class I ORs in mammals share the greatest sequence homology with fish ORs has raised the possibility that the class II ORs may be a more recent adaptation to terrestrial life. Consistent with this notion, class II ORs in mammals occurred at about the time during evolution as the common ancestor with amphibians. Moreover, it was found that in *Xenopus laevis*, class I ORs are exclusively expressed in the water-filled nasal diverticulum, whereas class II ORs are expressed in the air-filled diverticulum [27.83]. These observations suggest that when our ancestors began to occupy niches on land, much of the current diversity seen in the mammalian class II repertoires arose, and these more recently evolved ORs were adapted for the detection of airborne (or volatile) odorants.

Relationship to Invertebrate Odorant Receptors

ORs of invertebrates were identified first in the genomes of worms and flies. It turned out that both of them do not share any sequence homology to each other or to the vertebrate ORs; the fruit fly has about 60 and the mosquitos about 80 ORs [27.90, 91]. The repertoire of chemosensory receptors in *Caenorhabditis elegans* is quite large, more than around 800 functional OR genes that appear to have arisen independently from vertebrates and insects [27.92]. These findings emphasize that the absolute size of OR repertoires does not correlate with the complexity of the olfactory driven behavior. Moreover, the lack of homology between ORs from nematodes, insects and vertebrates implies that the evolutionary requirement for distinct olfactory abilities is met by recruiting novel gene families, rather than exploiting pre-existing gene families in the ancestral genomes.

27.3.2 Molecular Structure of Odorant Receptor Proteins

Sequence Motifs

The OR genes encode receptor proteins of 300–350 amino acids that are devoid of N-terminal signal peptides. They comprise all structural hallmarks typical for GPCRs, including seven hydrophobic transmembrane (TM) domains, a potential disulfide bond between conserved cysteines in the extracellular loops 1 and 2, a conserved glycosylation site in the N-terminal region, and potential phosphorylation sites in intracellular regions [27.94]. In addition, there are a few consensus motifs that allow to classify a vertebrate sequence as candidate OR, in particular MAYDRY at the end of TM3 that is considered as a sequence signature for the mammalian OR family [27.95, 96]. Moreover, motif FYVPAIFLSLTHRFGKHVPPLV on TM6 appears to be characteristic for class I ORs and the motifs MSPRVCVLLVAGSW (intracellular loop 2-TM4) and IFYGTAIFMY (TM6) are hallmarks for class II ORs [27.71] (Fig. 27.4). The TM domains 3, 4, and 5 are particularly variable and are considered as hypervariable regions and part of the ligand-binding pocket. The high variability of distinct positions within this do-

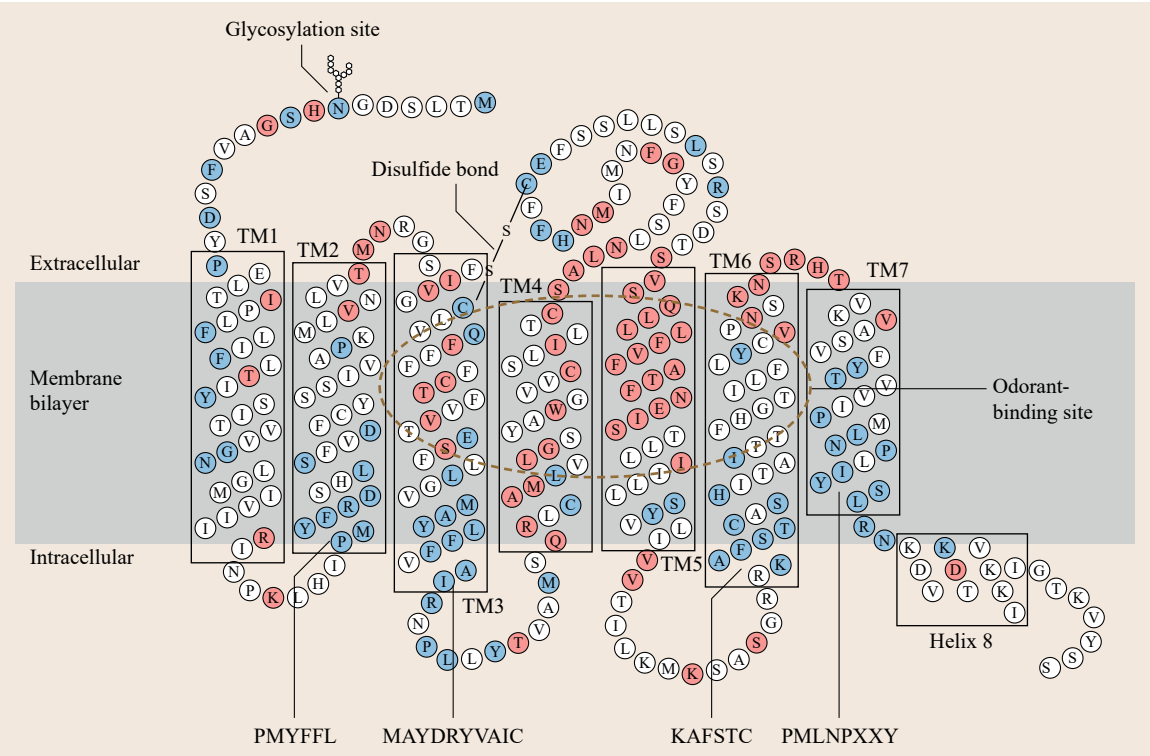

Fig. 27.4 Schematic representation of OR topology. The amino acid sequence of an exemplary OR, some conserved sequence motifs as well as the sites for glycosylation and disulfide bonds are depicted (after [27.93])

main may be the basis for the recognition of highly diverse ligands by a particular OR type [27.96].

Ligand–Binding Mechanisms

Exploring the intermolecular interactions between the odorous molecules and the receptor protein is essential not only for understanding the basis for the molecular recognition and discrimination, but also for deciphering the molecular basis for the receptor activation. This is a particularly intriguing task, because unlike most other GPCRs, ORs are activated by exogenous and mostly hydrophobic molecules. Moreover, individual receptor types are activated by many different odorants and the same odorant molecule can activate many different ORs [27.97]. Thus, the molecular features determining the specificity of an individual OR type for a distinct set of odorants are key parameters for the coding, and ultimately perception of odors. However, due to the lack of high-resolution structures of the OR proteins, it is still unknown how the protein structure determines its function, that is, which amino acid residues of the receptor protein interact with the odorous molecules, and how that determines the different ligand profiles of individual OR types. The highly variable residues in three of the seven TM domains (TM3-5) have led to the notion that these domains contribute to the binding pocket for a variety of odorants [27.98, 99]; however experimental proof for this concept is just emerging. Although a reliable structural basis for the ligand recognition of ORs is not available, the recent advances in computational power and bioinformatics methodology to perform molecular modelling approaches have opened new avenues for elucidating the molecular basis of odorant/receptor interaction. On the basis of known 3-D structures of other GPCRs, attempts have been made to get a first glimpse into the molecular interactions between receptor protein and odorous molecules [27.100, 101]. ORs belong to class A of the large GPCR superfamily and high-resolution structures of a few receptors in this class are currently available that have allowed to perform homology modelling studies using the high-resolution protein structure of rhodopsin or the $\beta 2$-adrenergic receptor [27.102] as a template to predict amino acid residues of the OR-binding pocket [27.101]. A combination of computational methods for the prediction of functionally important amino acids, together with site-directed mutagenesis and functional assessments, has allowed identifying 11 amino acid residues that seem to be part of the ligand-binding pocket and contribute to the inter-

action of a receptor protein with its cognate ligands. Based on the molecular models, 10 of these residues are located in the transmembrane helices TM3–TM6 and one in the extracellular loop between TM2 and TM3 [27.99, 101]. Moreover, docking studies have allowed some estimates for apparent affinities of an odorant toward the receptor. Subsequent studies have approached the question how the broadly tuned ORs can respond to chemical compounds with different structures. The results indicate that the recognition mode differs from those encountered in more specific systems, showing an opportunistic mode of binding. While receptors usually recognize their ligand through a network of oriented interactions, such as hydrogen bonds, the binding feature of broadly tuned ORs is dominated by hydrophobic contacts that favour multiple binding modes through opportunistic interactions [27.103]. Interestingly, a lack of an H-bonded interaction has also been observed in OBPs, where the odorous molecule is stabilized through a network of hydrophobic contacts within a solvent-occluded cavity [27.104, 105]. The domination of binding by nonpolar contacts provides an adaptability of the ORs such that they can bind several types of odorants with equivalent affinities, and nonetheless discriminate between very closely related odorants [27.103]. Molecular dynamics methodologies previously employed to study the opsins [27.106] and β2-adrenergic receptor [27.107] have recently been used to simulate the interaction of odorants with appropriate receptors [27.108]. This advanced technology has recently been extended allowing a so-called all-atom simulation at a nanosecond scale to simulate the molecular dynamics in a more realistic environment [27.108, 109]. These approaches may eventually lead to quantitative thermodynamic and kinetic constants for interactions between ORs and their cognate odorous ligands and their downstream signaling proteins.

27.4 Expression of Odorant Receptor Genes

The genes that encode ORs are expressed in OSNs that reside in the olfactory epithelium located on the so-called turbinates. A given OR gene is expressed in only a small fraction of the several million OSNs that are present in the epithelium; a population of neurons expressing the same OR gene typically comprises several thousand cells.

27.4.1 Spatial Expression Patterns

In the olfactory epithelium of mammals, each receptor-specific neuron population is restricted to an area that is called the expression zone for the corresponding OR gene, leading to a complex spatial pattern within the epithelium [27.110–112] (Fig. 27.5a). Expression zones are arranged as parallel stripes that run along the anterior–posterior axis of the nose. When viewed in both nasal cavities, the stripes are arrayed like a semicircular ring that is located at a distinct position along the dorsomedial to ventro-lateral axis of the nose. The zones are populated by OSNs with different OR types, and within a zone OSNs with the same OR are broadly and randomly distributed. The different zones are arranged like consecutive bands from dorsal to ventral, with adjoining zones overlapping to quite a degree [27.113, 114]. This spatial organization of OR gene expression results in a tessellate organization of the olfactory epithelium with OSN populations having distinct receptor types (Fig. 27.5b). There are, however, a few exceptions from this general principle: OSNs that express a member from the OR37 subfamily are restricted to a small patch in the center of the nasal turbinates [27.115, 116] (Fig. 27.5a). Here, these OSNs are concentrated in a small area at a much higher density than other receptor populations. In different mammalian species, the OSNs expressing OR37-type receptors are located at a similar central position, although the anatomical structures of the turbinates vary considerably [27.117]. The conservation of such a unique spatial organization across species supports the view that the locations of OSNs within the nasal epithelium are important for the proper function of the olfactory system.

Also the dorsomedial region of the nasal cavity is considered as unique regarding the expression of OR genes. Within this area, there is no zonal segregation; therefore the whole region is commonly referred to as the dorsal zone and distinguished from the ventral part. Moreover, the majority of sensory neurons within the dorsal zone express class I ORs that are evolutionary related to the ORs of fish.

The functional relevance of the spatial segregation of OSNs with distinct ORs is elusive. It has been speculated that such an arrangement may be important to position neurons with certain ORs at defined locations along the respiratory airflow. This may be important to match the proposed *chromatographic separation* of the volatile scent compounds according to their physicochemical properties, such as volatility and water solubility, along the air passage [27.118]; however, there is some controversy in the literature regarding this aspect [27.119]. Experimental evidence for a functional

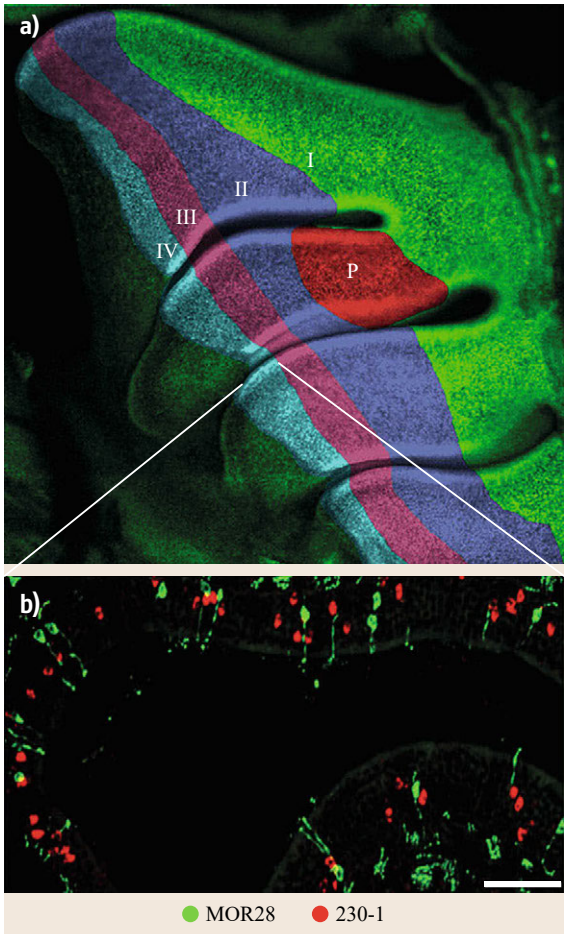

Fig. 27.5a,b Spatial expression patterns of ORs. **(a)** Neurons expressing the same receptor type are segregated in distinct zones, which are arranged from dorsal to ventral and extend along the anterior–posterior axis of the nose. In each zone, a distinct subset of receptor genes is expressed. *P* denotes the patch region, an exception from the otherwise elongated parallel organization of zones. **(b)** Within a given zone, neurons expressing a distinct receptor type are randomly distributed, as shown for MOR28 and MOR230-1 (after [27.120])

implication at this point only exists for the dorsal zone, where the ablation of all OSNs eliminates the innate avoidance behavior of mice to predator odors [27.89].

27.4.2 The One Neuron–One Receptor Rule

Although inherently difficult to prove, most of the available data currently favors the concept that each OSN expresses only a single OR gene from the huge repertoire [27.97, 121] (Fig. 27.6a); moreover, it is one allele of that gene, either the paternal or the maternal one that a cell selects [27.122]. Monoallelic expression is considered to prevent OSNs from having two ORs that are very similar in sequence, but still may differ in their odorant response profile. The mechanisms that enable an individual OSN to express only one OR gene are still at the beginning to be deciphered.

Control Mechanisms for Odorant Receptor Gene Expression

The fact that OSNs expressing the same OR gene are located within a defined zone of the olfactory epithelium, and this is the same across individuals, has led to the concept that this topography is genetically determined. This view implies that there are positional cues within the epithelium that operate in selecting the subset of OR genes suitable for expression within a given zone. Still, there is no evidence for such cue(s). However, in this context it is interesting to note that in some mouse lines an OR transgene is not expressed in its *receptor specific* zone, but in ectopic positions within the epithelium [27.124–127]. The reason for such aberrant spatial expression is unclear; it is hypothesized that the transgene integrated in the vicinity of an OR gene cluster and that the genomic locus exerted a significant influence on the transgene, thereby resulting in a corresponding spatial expression pattern [27.128]. The experiments indicate that the molecular machinery underlying the selection of a distinct OR gene for expression is not restricted to the zone in which the gene is typically expressed. Moreover, recent results suggest that OR genes may initially be chosen for expression in broader regions of the epithelium and only subsequently restricted to one defined region of the epithelium [27.129].

DNA-Regulatory Elements. One of the intriguing possibilities that DNA rearrangement may be responsible for the singular expression of an OR gene per OSN has been ruled out by cloning mice from nuclei of OSNs that already expressed a particular gene [27.130, 131]. In the cloned mice the expression pattern of ORs was normal, a result which strongly argues against the DNA rearrangement hypothesis.

Compared to most other genes, those encoding ORs are very compact units; they are comprised of only a single coding exon and a few short noncoding upstream exons (see above). The transcriptional start site (TSS) has been mapped to a few kilobases upstream, thus in very close proximity to the coding sequence [27.132, 133]. Bioinformatic analyses have revealed that the preceding promotor is frequently an adenin-thymin (AT)-rich region and that some promotors contain a canonical TATA-box which in most cases contains the core DNA sequence 5′-TATAAA-3′. The most prominent features of promotor regions for OR

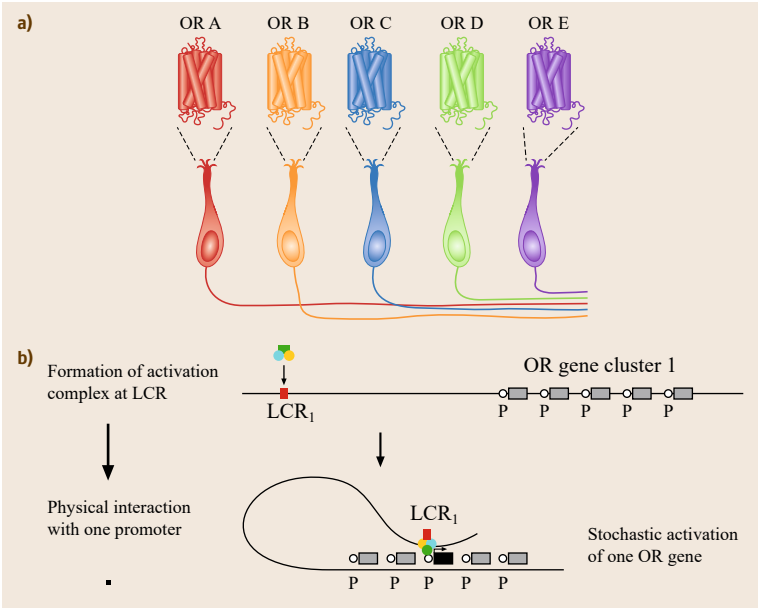

Fig. 27.6a,b Control of OR expression. **(a)** Each OSN expresses just one OR type, and only one allele of the more than 1000 receptor genes is expressed. **(b)** The process of a selective OR gene expression is assumed to be initiated by an interaction of the locus-control region (LCR) with the promotor region of a distinct OR gene within a gene cluster (after [27.123])

genes are the binding sites for homeodomain (HD) transcription factors and so-called O/E-(olf1/early B-cell factor) like sites. O/E-like sites are believed to be involved in determining the specific expression of OR genes in OSNs, because they are also found in promotors of other genes expressed in OSNs [27.134, 135]. Whether these elements are actually involved in the regulation of the spatial patterns is not yet clear; the unique organization of such elements in the putative promotors of genes with distinct expression patterns has been viewed in favor of this concept [27.136].

Experimental data concerning the mechanisms of OR gene regulation are still limited. Transgene technologies in mice have allowed to analyze a few fragments of OR gene loci for their capacity to reproduce the features of OR gene expression. Transgenes of around 10 kb are the smallest genomic fragments (minigenes) that closely reproduce the expression features of endogenous OR genes [27.125–127]. Very short DNA elements of about 150 base pairs upstream of the TSS turned out to be sufficient for eliciting the transgene expression. These elements contain the putative HD and an O/E-binding sites, and by site-directed mutagenesis they were shown to be important for in vivo expression of OR genes [27.125].

The finding that very short OR transgenes do not recapitulate all expression features of an intact gene implies that additional, more distantly located sequence elements are involved in controlling the expression of an OR gene. Indeed, such elements have been identified far more distant from the coding sequence than the typical promotor. One element (the *H-element*) is positioned in neighborhood to the MOR28 gene cluster on mouse chromosome 14 [27.137] and a second one (the *P-element*) within the cluster containing the P2 gene [27.138]. The deletion of these elements affects the expression of OR genes from the nearby cluster [27.139, 140]; therefore, they are considered as locus-control-regions (LCR), regulatory elements known for example, from the globin-gene cluster [27.141]. The finding that both, the *H-* and the *P-element*, share a conserved HD-binding site that is similar to the one in promotors of OR genes [27.132] led to the concept that the putative LCRs may physically interact with promotors of OR genes by looping onto these positions (Fig. 27.6b). When a multimerized HD-binding site from the *P-element* (19 bp) is added upstream of a minigene containing the MOR23-coding sequence, the transgene is expressed in a significantly higher number of neurons; elements with a mutant HD-binding site do not have this effect [27.142]. Based on these results, it was hypothesized that HD-binding sites in the *H-* and *P-elements* and in the OR promoters affect the probability of an OR gene to be chosen. In line with this idea, the frequency how often a gene from the MOR28 cluster is expressed correlates with its distance to the *H-region*. So far, only these two putative LCR elements have been identified in the vicinity of OR genes; thus it remains unclear whether they represent a general regulatory principle for controlling their expression.

Epigenetics. The results of very recent studies imply that also epigenetic mechanisms are involved in regulating the expression of OR genes. In olfactory progenitor

cells, all OR genes are associated with heterochromatin containing the repressive histone methylation mark H3K9me3 (tri-methylation of lysin at position 9 of histone 3) [27.143], suggesting that all OR genes are repressed prior to the onset of expression, and that the chosen OR gene is de-repressed by demethylation of H3K9 by the histone demethylase Lsd1 [27.144]. The downregulation of Lsd1 in mature OSNs apparently contributes to maintain the repressive histone methylation marks in chromatin associated with all other OR genes [27.144].

Odorant Receptor-Dependent Mechanisms. The results of several studies indicate that the receptor itself may play a role in maintaining the singularity of expression. A cell that expresses an OR pseudogene will switch to another OR gene [27.128, 137, 145]. Such a gene switch ensures that each mature OSN expresses a functional receptor protein. This model implies that a functional OR, once it is produced, contributes to silence the expression of all other OR genes, thus preventing the expression of additional ORs and ensuring the stable expression of a single receptor type in each neuron. This effect extends even to OR transgenes that are randomly inserted into other locations of the genome [27.146], implying that the sequence(s) involved in this control are within or very close to the transcribed regions of OR genes [27.147].

27.5 Odorant Receptor-Specific Wiring of Olfactory Sensory Neurons

Each OSN extends a single unbranched axon into the OB, the first relay station of olfactory information processing; thus, olfactory information is conveyed directly into the brain. Within the OB the axon terminal forms synapses with second order neurons in distinct anatomical structures, called glomeruli. A defined glomerulus harbors only the axons from OSNs that express the same OR [27.148], and a population of OSNs with the same OR typically projects to two distinct glomeruli, one is located in the medial and the other one in the lateral hemisphere of the OB [27.149]. An exception from this general dual-glomerular-projection rule are OSNs that express a member from the OR37 subfamily; these cells target only a single glomerulus that is located in the ventral domain of the bulb [27.116]. The location of a receptor-specific glomerulus within the OB is very similar among individuals; however, it is not entirely fixed; variations within an area of approximately 20–30 glomeruli have been observed. Also the location of glomeruli relative to each other can vary; these local permutations can result in different arrangements of glomeruli even in the two bulbs from one individual [27.116]. How this influences the processing of incoming information is not known.

Within the glomerulus, information conveyed by the OSNs is processed by a network of interneurons, before the projection neurons (mitral and tufted cells) relay it onto higher brain centers. The glomerulus-related circuitry is highly organized and the different cell types form a columnar structure with each glomerulus [27.150]. The neuronal networks beneath the two related glomeruli are interconnected through a set of intrabulbar projections mediated by tufted cells [27.151].

27.5.1 Organization of the Olfactory Sensory Map

The axonal projection pattern from the epithelium to the OB forms the anatomical basis of the olfactory sensory map. The formation of this complex projection pattern of OSNs along the axes of the OB has been intensively explored during recent years.

Glomerular Positioning Along the Bulbar Axes

OSNs located in the dorsal part of the olfactory epithelium send their axon to the dorsal domain of the OB, whereas neurons situated in the ventral region of the epithelium project to ventral aspects of the bulb; thus the receptor-related zonal organization of the olfactory epithelium is maintained in the dorsal-ventral topography of the OB; *zone-to-domain topography* [27.114, 153] (Fig. 27.7a). OSNs expressing a given receptor type are scattered within spatially defined but overlapping zones of the epithelium along the dorsal-ventral axis, and send their axon to a glomerulus at a corresponding position within the dorsal-ventral domains in the bulb. Each glomerulus is formed exclusively from neurons expressing the same receptor type. The topographic projection pattern along the dorsal-ventral axis is in part sculpted by complementary gradients of chemorepellent proteins and their receptors (Slits/Robo, Semaphorin/Neuropilin), indicated by the shaded bars. The notion that cues specific for the epithelial region in which an OSN is located may contribute to the process of axonal targeting was confirmed by the discovery of guidance factors that establish characteristic dorsal-ventral patterns [27.154–156]. It has been demonstrated that sets of repulsive ligand/receptor molecules, most notably Slit1/Robo2 and

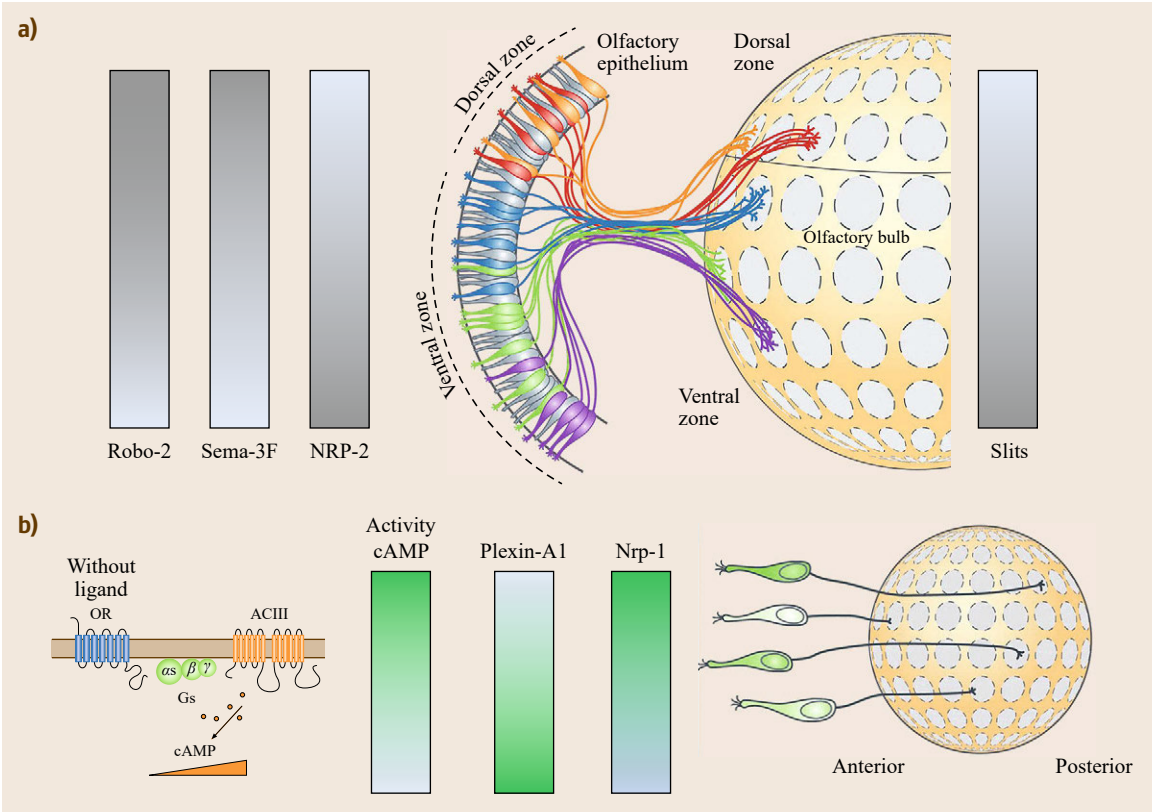

Fig. 27.7 Receptor-specific wiring in the olfactory system (after [27.152])

Neuropilin 2/Semaphorin3F, influence the projection of axons along the dorsal-ventral axis. For example, axons positive for Robo2 navigate to the dorsal domain of the bulb due to the repulsive effect of the Robo-ligands Slit1 and Slit3 present in the ventral domain of the bulb.

The second dimension of the axon guidance is the projection to either the medial side or to the lateral side of the bulb, where the glomeruli of the receptor-specific population are established (see above). Although this aspect of axon targeting is less well understood, it has been shown that the medial glomerulus is innervated by OSNs located in medial region in the olfactory epithelium, and the lateral glomerulus by OSNs located in the corresponding lateral region [27.157]. More recent studies suggest that the insulin-like growth factor (IGF1) plays a role in this process; in mouse mutants with a disrupted IGF1 signaling, the axons that are supposed to project onto the lateral region of the bulb are found in ectopic positions in the ventro-medial region of the bulb [27.158].

The receptor-specific wiring pattern of olfactory axons has led to the speculation that the receptor itself may be a critical determinant in the process of pathway and target finding of the outgrowing nerve fibers. This notion is supported by experiments in which the coding sequence of one receptor type is replaced with that of another receptor type [27.159], demonstrating that with a new receptor the OSNs send their axons to a novel glomerulus, and thereby indicating that the receptor indeed plays an instructive role in positioning of the glomerulus. The precise molecular mechanism how the receptor contributes to this process remained elusive, however. It was proposed that the ORs may recognize and respond to axon guidance cues, thus not only serving as OR in the ciliary membrane, but also as receptor for guidance cues in the axon membrane. Consistent with this hypothesis, the receptor protein is in fact present in the axon terminals of OSNs [27.7, 160]. Moreover, even mRNA for ORs is present in the axon [27.161, 162], suggesting that a local translation of the receptor protein may be possible [27.163].

In search for the intracellular mechanisms underlying the targeting of olfactory axons, the level of cAMP appears to be a critical determinant for the glomerular position along the anterior–posterior (a–p) axis of the OB [27.164, 165] (Fig. 27.7b). The topographic projection pattern along the anterior–posterior axis of the bulb is not determined by the position of a neuron within

the epithelium, but rather by the spontaneous activity of an OR type. This agonist-independent activity leads to a characteristic, receptor-related concentration of intracellular cAMP in an OSN (high or low, indicated by the shaded bar in Fig. 27.7b). The level of cAMP in turn controls the expression of specific axon guidance cues, like Neuropilin1 (Nrp1) and thereby determines the sensitivity of an axon for guidance cues along the projection pathway into the OB. Olfactory neurons expressing a receptor with low spontaneous activity form glomeruli in the anterior region of the bulb, those with high-activity receptors form glomeruli in the posterior region of the bulb. An attenuated cAMP-signaling in OSNs results in an anterior shift of glomerular position, whereas an enhanced cAMP signaling results in a posterior shift [27.166]. Thus, the capacity of an OSN population to generate cAMP determines the position of its target glomerulus along the a–p axis. This phenomenon appears to be due to the fact that the level of cAMP determines the level of axon guidance cues, such as Neuropilin 1, a receptor for the repulsive Semaphorin 3A. OSNs with high levels of Neuropilin 1 project to posterior positions in the bulb, whereas neurons with low levels project to anterior positions. The question how the cAMP level within a particular OSN population is controlled turned out to be very challenging to answer. Only very recently, in elegant approaches it was discovered that the OR itself determines the cAMP level, and most interestingly not via a ligand-induced activation, but rather by its spontaneous, thus agonist-independent activity [27.167]. Indeed, the intrinsic activity of a particular OR protein correlates very well with the glomerular position of the corresponding OSNs. In this way, the position of a glomerulus along the anterior–posterior axis is not based on the location of the corresponding OSNs within the epithelium, but determined by the intrinsic activity of the OR protein.

Development and Regeneration

During development of the olfactory system, the axons of OSNs expressing a given OR project into the prospective OB; initially the projection is not as precise as in adult stages [27.168, 169]. In early stages, some adjacent glomeruli are targeted, resulting in heterogeneous glomeruli, that is, neuropil structures composed of axons from different OSN populations. These heterogeneous glomeruli are supernumerary and eliminated as the glomerular map matures after birth. If odor-evoked activity is blocked at birth, for example, by naris occlusion, the heterogeneous glomeruli persist [27.124]; conversely, if odorants are present at higher doses during the early postnatal phase, the glomeruli mature more rapidly [27.170, 171]. These observations suggest that odor induced activity is involved in the refinement of the innervation.

OSNs have only a limited life span of about 90 days and throughout life they are continuously replaced from stem cells, the globose basal cells, that reside at the base of the olfactory epithelium [27.172–174]. The newly generated cells migrate up into higher layers and differentiate to mature OSNs that express an appropriate OR type. Under normal conditions the generation of new OSNs occurs asynchronously, with only 1−2% of the cells being replaced at a given point in time. Because the vast majority of cells in the olfactory epithelium are mature OSNs that are connected to their target glomerulus, it is supposed that the axon of a newly generated neuron can grow along established tracks and most likely associates with preexisting fibers. This could either be based on a homotopic attraction between axons from OSNs with the same OR, mediated by the OR itself or appropriate cell adhesion molecules. Alternatively, residual fragments of fibers from replaced neurons may label the tracks, for example, by inducing molecular cues in the surrounding extracellular matrix.

In addition to this normal scenario, the olfactory system has the remarkable capacity to re-establish the system even after an extensive damage to the olfactory epithelium; this could either be due to an injury, a virus infection or an exposure to toxins which can cause a loss of virtually all OSNs. During a reasonable period of time, a complete population of OSNs is reproduced and the system is re-established to a level that is nearly indistinguishable from the original in terms of neuron number and spatial organization [27.113, 175]. In addition, the axonal projection patterns are newly formed and the basic olfactory functions, such as odor detection and discrimination of odorants are recovered. Only after severe events, the targeting of olfactory axons to the glomeruli seems imperfect, reminiscent of the projection during development. However, during the regeneration, these errors are apparently not repaired and receptor-specific neuron populations do not project to only two glomeruli, but multiple glomeruli are formed [27.176]. The impact of this mistargeting on odor representation is largely elusive; however, it has been reported that in humans smell deficiencies may remain after severe traumata of the olfactory epithelium.

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28. Nasal Periceptor Processes

Boris Schilling

There are myriads of odorous molecules that we perceive and it is remarkable that most of us seem to have very similar odor impressions that originate from a specific stimulus and the sense of smell appears to be robust during much of a lifetime. When perceiving scents, olfactory receptor (OR) proteins are at work to translate chemical information into neuronal signals that are decoded in the olfactory cortex to provide us with an odor image. Proposed in the middle of the last century but only substantiated with intriguing laboratory data during the last decade, there are enzymes expressed at high levels in the olfactory mucosa, and they metabolize xenobiotics including odorants and produce many new chemical species. Examples demonstrate that such perireceptor events can alter the receptor-dependent activation pattern in the olfactory neuroepithelium, which has an impact on the quality and the intensity of odor stimuli. Results that do not seem to fit a model or a hypothesis may make sense if perireceptor events are brought into the equation.

Our senses provide us with an internal representation of the outside world with a straight impact on our behavior and decision-making processes. While it is generally appreciated that sight and sound are crucial for our quality of life, the important role of the sense of smell is often forgotten and goes far beyond enjoying a subtle splash of a luxury perfume. The perception of fragrances is inevitably linked with joy, well-being, mood, emotions, memories, and both physiological and psychological reactions are responsible for the power of the sense of smell. There is a strong personal flavor to the perception of odors and learning, association, context as well as a genetic predisposition all contribute to a unique individuality for olfaction that is not observed for other senses, such as vision and audition. Evidence for a striking variability for the perception of β-ionone, a floral and woody odorant with a strong freesia character, was based on a large sensory study conducted during a flower show in New York City in 1935 [28.1]. The term *specific anosmia* describes the fact that many people are *odor blind* for specific molecules. The first thorough investigation was published in 1967 by *Amoore* who described anosmia for the sweat odorant isovaleric acid [28.2]. He expanded the studies to other odorants and also discovered that specific anosmia is genetically inherited [28.3, 4]. Today, it is generally known that even for perfumery ingredients, specific anosmia exists for instance for β-ionone, salicylates, musks, and amber odorants. Androstenone is an interesting body odor, that is perceived as unpleasant/urinous/sweaty or pleasant/sweet/floral or odorless, and it has been shown that a genetic variation in one human odorant receptor (OR) is responsible for the difference in odor perception [28.5]. Besides single-nucleotide polymorphisms, receptor gene copy number variation is a possible factor for phenotypic difference in odor perception [28.6]. There is some decrease in the performance of the sense of smell during aging which is generally not dramatic unless the cause is a neurodegenerative disease, such as Alzheimer and Parkinson where an impaired sense of smell is one of the earliest symptoms [28.7]. The decline in the ability to detect and discriminate odors in aged humans is not well understood, and latest investigations suggest

that it is multifactorial, and includes reduced neurogenesis, altered synaptic organization as well as modified odor representation in primary olfactory cortices and beyond [28.8].

Many excellent studies on the sense of smell have been based on sensory behavioral experiments, psychophysical measurements, and electrophysiological and anatomical investigations. A review in National Geographic Magazine in 1986 provides a superb overview on various aspects of olfaction that were known at the time and emphasizes that the mechanisms by which odorous molecules activate neurons that convey respective information to the brain were still to be elucidated [28.9]. An explosion of research in olfaction followed the discovery of the odorant receptor proteins in 1991 by *Buck* and *Axel* [28.10]. Olfactory receptor genes are the largest gene family in the human genome with close to 1000 genes that are scattered across most chromosomes. In humans, the majority of the receptor genes have been mutated to pseudogenes leaving us with close to 400 functional genes [28.5, 11–15]. Interestingly, segregating pseudogenes have been identified, indicating that different people may have a slightly different number of pseudogenes on top of the occurrence of various alleles for each of the functional olfactory receptor genes [28.16]. Investigating and understanding the olfactory gene expression in olfactory sensory neurons, signal transduction, receptor agonist patterns, axonal projections to glomeruli in the olfactory bulb and signal processing in the olfactory cortical areas are fascinating topics that are reviewed and explained in other chapters in this book. There is a chemotopic map preserved on the level of olfactory bulb, the first relay station in the brain [28.17, 18]. Information processing beyond the olfactory bulb toward the olfactory cortical areas is multifaceted and surprisingly, the activation patterns in the piriform cortex, the next processing center for olfactory information get much more complex. The same cortical neuron responds to diverse odorants and it appears that odorants are represented by unique ensembles of active neurons in the piriform cortex. When moving from single odorants to odor mixture, strong suppression and some minor synergistic effects are observed, demonstrating that the representation of complex odors in the piriform cortex is not the integration of individual activation patterns [28.19]. Previously, Buck et al. proposed that cortical neurons might be acting as *coincidence detectors* when receiving inputs that originate from more than one receptor. More recently, it has been elegantly demonstrated that neuronal information from the olfactory bulb is conveyed to multiple cortical centers where odor representation is differently organized. While activated neurons in the piriform cortex show no discernible spatial order, the representation in the cortical amygdala exhibits spatially stereotyped projections from olfactory bulb glomeruli which overlap and allow for the local integration of neuronal signals [28.20]. The authors propose a model, where the representation of activated ensembles of neurons in the piriform cortex is vital for experience and learned olfactory responses, while the odor representations in the cortical amygdala are linked to innate behavioral responses.

The activation of olfactory receptors by odorants at the periphery of the olfactory system is the essential step toward smell perception. Yet there is evidence that perireceptor events also contribute to the shape of the olfactory percept, and this understanding may be important to compare in vitro results derived from receptor screenings and in vivo psychophysical studies, such as the determination of odor detection thresholds, as well as the assignment of odor descriptors to fragrance molecules. It has been speculated for years that enzymes in the respiratory tract and in particular in the olfactory epithelium could have an impact on the perception of odorants. The initial hypothesis that enzymatic activities (or their inhibition) are involved in the nature of the sensation of smell was proposed by the chemist *G.B. Kistiakowsky* from Harvard University more than 60 years ago [28.21]:

> *On the Theory of Odors: I cannot resist the temptation to add on more hypothesis on the nature of the sensation of smell to speculation of others to this subject. Several characteristic traits of this sense can be accounted for without infringing on the basic physical principles if it is attributed to the inhibition of certain enzymes contained in the olfactory organs* [. . .].

He proposes that sequences of metabolism, on the one hand, and inhibition of enzymes on the other contribute to high impact odors, form the basis of the large collection of molecules having a smell and determines that the complexity of odors also results from the inhibition of various enzymes to different extents. Interestingly, he also proposes that such enzyme inhibition can change the quality of smell, a concept that will be revisited later in this chapter. Around the same time, enzymes were localized in and around the gustatory and olfactory organs of the rabbit, including the olfactory mucosa, and it was suggested that they may be associated in some way with the mechanisms of smell and taste [28.22].

The first evidence of the in-nose metabolism of a volatile compound was provided at the 6th International Symposium of Olfaction and Taste [28.23]. The scientists observed that upon channeling tritium-labeled octane through a frog's nose, some of the labeled mate-

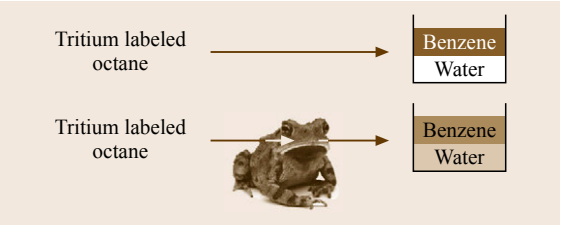


Fig. 28.1 Indication of in-nose oxidative metabolism (after [28.23])

rial became water soluble, and they speculated that the chemical got somehow transformed at the olfactory receptor site (Fig. 28.1). From today's perspective, it can be concluded that the nonwater-soluble alkane was oxidized by cytochrome P450 monooxygenases (CYPs) to produce the water soluble alcohol.

Octane is soluble in the organic solvent (benzene); however, following exposure to the olfactory tissue of a frog, some of the tritium-labeled material is soluble in the aqueous phase, indicating in-nose oxidative metabolism [28.23].

Only a few years later, it was demonstrated that high concentrations of CYPs and other enzymatic activities are located in the nasal cavity of animal species [28.25]. One paper was published showing that the fragrance material heliotropin (piperonal) inhibits rat nasal CYP activity and the author is putting forward the idea that part of the effectiveness of heliotropin as a perfumery ingredient may result from prolonging the half-life and residency time of other odorants in the nasal cavity by inhibiting their enzymatic oxidation and degradation [28.26]. An excellent review by *Alan Dahl* describes animal studies conducted in the 1980s to investigate whether nasal metabolism influences the biological fate and toxicity of inhaled materials as well as to address a potential impact on olfactory physiology [28.24]. Selected substrates and metabolites reported in this review and references cited therein are shown in Fig. 28.2 together with the original classification of olfactory mucosal enzymes.

It is further postulated that olfactory xenobiotic metabolizing enzymes might have an effect on the characteristic odors of compounds, and the author makes suggestions how to direct research efforts to provide data on the role of nasal metabolism in olfaction [28.24]. He lists five specific effects where xenobiotic-metabolizing enzymes may influence odor perception:

1. Conversion of a nonodorant into one or more odorants.
2. Conversion of odorants to nonodorants.
3. Transformation of odorants to other odorants (change in quality).
4. Transformation of lipophilic compounds into more water-soluble ones (change in physicochemical properties and elimination).
5. Inhibition of the metabolizing enzymes (may alter all the previous effects). These possibilities will be discussed in more detail in this chapter.

Another group of proteins which is prone to play a role in perireceptor events are the so-called odorant-binding proteins (OBPs) which are small soluble carrier proteins with binding activity toward volatile compounds [28.27]. These proteins belong to the family of lipocalins which are known to transport small ligands in other body fluids but their role in mammalian ol-

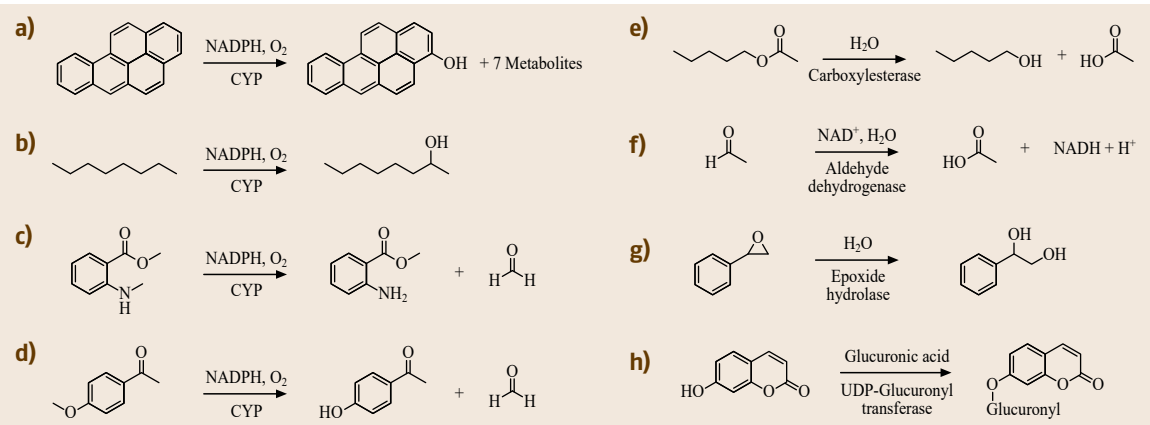


Fig. 28.2a–h Metabolic reactions catalyzed by olfactory mucosal enzymes (after [28.24]): **(a)** hydroxylation of benzo[a]pyrene, **(b)** hydroxylation of octane, **(c)** *N*-demethylation of dimethylanthranilate, **(d)** *O*-demethylation of *p*-methoxyacetophenone, **(e)** hydrolysis of amyl acetate, **(f)** oxidation of acetaldehyde, **(g)** hydrolysis of styrene oxide, **(h)** glucuronidation of 7-hydroxycoumarin (umbelliferone)

faction is still unclear. Proposed functions include the transport of the hydrophobic molecules from the nasal air space across the viscous, hydrophilic mucus layer to the ciliae of olfactory sensory neurons where receptor proteins are located, as well as a role as scavengers for excess odorants and removal of the stimuli. Since the studies on heterologously expressed olfactory receptor genes clearly indicated that they function in the absence of OBPs, their role in vertebrate olfaction is still unanswered. More evidence for the function of lipocalins in chemoreception was found for insects [28.28]. The first identified insect OBP was the pheromone binding protein (PBP) of the silk moth *Antheraea polyphemus* [28.29] shortly before the first mammalian OBP was described. Later, compelling evidence was provided that the *Drosophila* insect pheromone 11-*cis* vaccenyl acetate is requiring a specific OBP for the activation of pheromone-sensitive neurons [28.30] and it has been demonstrated that the detection of the *Drosophila* pheromone is mediated directly by the OBP upon a pheromone-induced conformational change [28.31]. Binding of the pheromone converts an inactive ligand into an activator of pheromone-sensitive neurons, which is in contrast to the general assumption that olfactory neurons are activated by volatile odorants as a result from the direct activation of the membrane-embedded receptor by the volatile stimulus.

It has been nicely demonstrated that OBP/PBP-enabled perireceptor events play an essential role in insect olfaction where a large repertoire of OBP genes are expressed; however, their role in mammalian olfaction is still not clear. Therefore, the upcoming sections are dedicated to review the current understanding on biotransformation reactions that take place in the olfactory mucosa with emphasis on the role of cytochrome P450 enzymes (CYPs) in nasal metabolism, and the potential impact of xenobiotic-metabolizing biochemical reactions for olfaction research.

28.1 Xenobiotic-Metabolizing Enzymes in the Olfactory Epithelium

Metabolism of xenobiotic molecules is primarily assigned to a role of hepatic phase-1 and phase-2 biotransformation enzymes. In phase-1 metabolism, molecules are made reactive and during phase-2 metabolism sugar or peptide moieties are added to make water soluble catabolites and allow excretion via urine. However, there has been strong evidence that xenobiotic metabolism is taking place outside the liver, and various reviews have described that biotransformation enzymes are found in the respiratory tract and in particularly high concentrations in the olfactory mucosa [28.32, 33]. In order to identify the enzyme families that are involved in xenobiotic metabolism, gene expression patterns were compared between human fetal and adult olfactory mucosa and liver specimens, using a combination of gene array analysis and ribonucleic acid polymerase chain reaction (RNA-PCR) [28.34]. A series of biotransformation enzymes which were identified in the nasal tissue are shown in Table 28.1. The family of CYPs is of specific interest, since they are involved in the phase-1 metabolism of very diverse chemical species. About one dozen CYP genes are expressed in the human olfactory mucosa and amongst them, CYP2A13 has been identified to be specifically expressed in the human respiratory tract, predominantly in the olfactory mucosa [28.35], therefore being a primary candidate to explore and test odorants as substrates or enzyme inhibitors.

Liver metabolic enzymes are known to be upregulated as a response to exposure to xenobiotic compounds, including drugs. However, the regulation of xenobiotic-metabolizing enzymes in the olfactory mucosa has been little explored. There are studies indicating an enhanced expression of specific genes following

Table 28.1 Metabolic enzymes that are expressed in the human nasal mucosa

Enzyme	References
Aldehyde dehydrogenase (ALDH6)	[28.34, 36]
Aldehyde dehydrogenase (ALDH7)	[28.34, 36]
Carboxyl esterase (CE)	[28.37, 38]
Cytochrome P450 monooxygenase (CYP1B1)	[28.34]
Cytochrome P450 monooxygenase (CYP2A6)	[28.32, 33]
Cytochrome P450 monooxygenase (CYP2A13)	[28.32, 33, 35]
Cytochrome P450 monooxygenase (CYP2B6)	[28.33]
Cytochrome P450 monooxygenase (CYP2C)	[28.33]
Cytochrome P450 monooxygenase (CYP2E1)	[28.34]
Cytochrome P450 monooxygenase (CYP2F1)	[28.34]
Cytochrome P450 monooxygenase (CYP2J2)	[28.33]
Cytochrome P450 monooxygenase (CYP2S1)	[28.39]
Cytochrome P450 monooxygenase (CYP3A)	[28.33]
Cytochrome P450 monooxygenase (CYP4B1)	[28.34]
Epoxide hydrolase (EH)	[28.36, 40]
Flavin-containing monoxygenase (FMO1)	[28.34]
Glutathion-*S*-transferase (GSTP1)	[28.34, 36, 40, 41]
Nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase (POR)	[28.32, 36]
Glucuronyl transferase (UGT2A1)	[28.34, 36, 42]

treatment with chemicals [28.43] and a recent publication reports that inducers known to regulate hepatic gene expression also worked in the rat olfactory mucosa and phase-1, phase-2, and transporter genes were up-regulated [28.44].

28.2 Cytochrome P450 Enzymes

CYPs are peripheral membrane proteins anchored to the membrane bilayer of smooth endoplasmic reticulum by their amino-terminal domain. An iron–heme cofactor is present in the catalytic center where the oxygen-dependent monooxygenation of suitable substrates takes place. For a full catalytic cycle, two electrons are required which are supplied via an electron-transfer system from an NADPH-cytochrome P450 reductase (POR) which is also membrane-anchored and in a close proximity to the CYP. NADPH is the ultimate electron donor, and electrons are channeled via two flavin cofactors (flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN)) in POR to the catalytic center of the CYP. The overall reaction is shown in Fig. 28.3 and further described, for instance, in the following reviews [28.45, 46].

A comprehensive overview on classical and particularly uncommon CYP-catalyzed reactions has been published by *Guengerich* [28.47]. This family of enzymes shows low substrate specificity and frequently produces multiple products. The availability of several crystal structures of human CYPs allows rationalizing the fate of substrates and the binding site of inhibitors.

Pharmacological metabolism research and the role of CYPs in chemical toxicology have been the area of strong interest. Various excellent reviews describe recent developments in metabolism studies and safety testing, adverse effects of drugs through biotransformation, and bioactivation of chemicals. Besides identifying metabolites of active pharmaceutical ingredients (APIs) and determining the pharmacogenetics and clearance of drugs, various groups also investigated the role of CYP polymorphisms in the onset and progression of cancer and the role of genetic variability in human CYP genes [28.48–50]. It is remarkable that nasal cytotoxicity and carcinogenic activities are originating from systemically distributed organic chemicals, confirming the metabolic power of the nasal mucosa [28.51]. Several publications conclude that the respiratory tract CYP2A enzymes and particularly CYPA13 play a role in the metabolic activation of nasal toxicants [28.52] and are involved in the bioactivation of tobacco-specific nitrosamines [28.35, 53–57]. Genetic polymorphism of *Cyp2a13* can be linked to lung cancer susceptibility [28.58–64] and CYP enzymes have been mentioned as potential targets for chemoprevention of lung cancer by the use of selective inhibitors (see the following paragraphs). Active site mutations of CYP2A13 influence the orientation and results in altered kinetics for metabolite formation which can be rationalized by docking studies using the CYP2A13 crystal structure [28.65].

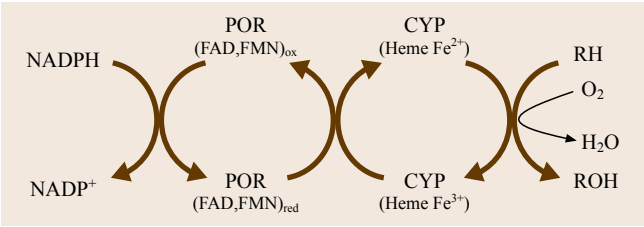


Fig. 28.3 Substrate oxidation by CYP with the concomitant reduction of oxygen to water. Electron transfer from NADPH is taking place via two flavin cofactors (FAD, FMN) which are present in POR. The iron-heme cofactor is the site of chemical oxidation in CYP

28.3 Exploring the Substrate and Inhibitor Range of Olfactory P450 Enzymes

CYP members of family 2 are strongly expressed in nasal tissue, and also known to bind small molecular weight compounds as substrates and inhibitors, including volatile odorants. Studies were conducted with various commercially available CYP sources; however, the respiratory tract-specific CYP2A13 was selected as the primary candidate for further investigations and produced from *Spodoptera frugiperda* cell line 9 (Sf9) insect cells together with the reductase partner POR [28.66, 67]. A library of odorant molecules was used to identify substrates of CYP2A13. For many molecules, a molecular weight increase [M+16] was

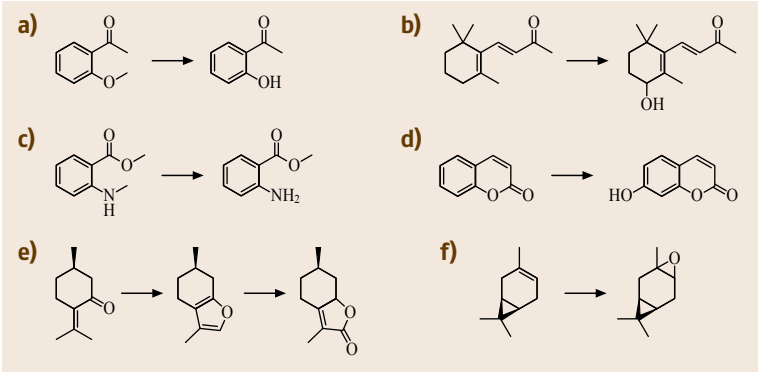


Fig. 28.4 CYP2A13-catalyzed oxidation of odorants: **(a)** demethylation of 2-methoxyacetophenone, **(b)** allylic hydroxylation of β-ionone, **(c)** demethylation of dimethylanthranilate, **(d)** hydroxylation of coumarin, **(e)** oxidation and cyclization of (*R*)-(+)-pulegone to menthofuran and further oxidation to mintlactone, **(f)** epoxidation of delta-3-carene

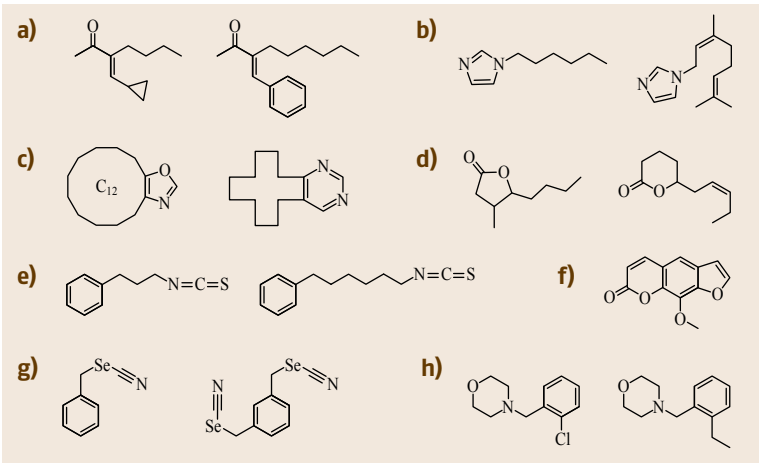


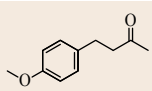
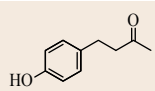
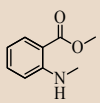
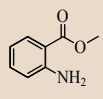
Fig. 28.5 Inhibitors of CYP2A13 exhibiting IC50 values in the low μM range: **(a)** ketone types, **(b)** *N*-heterocycles, **(c)** macrocyclic heterocycles, **(d)** lactones, **(e)** isothiocyanates, **(f)** 8-methoxypsoralen, **(g)** organoselenium types, **(h)** benzylmorpholine types

found indicating a monooxygenation reaction (either hydroxylation or epoxidation). Furthermore, demethylation of methoxy- and *N*-methyl groups was observed. A selection of odorants that are metabolized by CYP2A13 is shown in Fig. 28.4.

Two examples of CYP substrates with available odor intensity and quality data are shown in Table 28.2. In the case of methoxyphenylbutanone (Ketanone), the metabolite is the powerful raspberry ketone, whereas in the case of dimethylanthranilate, the metabolite has a slightly lower threshold and distinct but small differences in the odor description. Depending on the extent of nasal metabolism, one is never smelling the substrate alone, but always a combination of the substrate and the metabolite, which may differ between individuals.

Libraries of diverse chemical classes of small molecular weight compounds were screened for inhibitors of CYP2A13, CYP2A6, and CYP2B6 enzymes [28.68–75]. Chemically diverse inhibitors as

Table 28.2 Odor thresholds and qualities of selected substrate–metabolite pairs

Substrate		Metabolite	
	OTH: 0.05 ng/l Odor description: raspberry, floral, green		OTH: 0.005 ng/l Odor description: raspberry, fruity, sweet
	OTH: 0.59 ng/l Odor description: floral, neroli, sweet, warm		OTH: 0.12 ng/l Odor description: floral, orange blossom, neroli, mandarin

OTH: Odor detection threshold in ng/l air, determined using an olfactometer

well as various substrates were identified, further confirming that the respiratory tract-expressed enzymes CYP2A13 and 2A6 are able to catalyze detoxification as well as metabolic activation reactions of environmental molecules and are also subject to inhibition by xenobiotic compounds. Selected examples of CYP2A13 inhibitors having half minimal inhibitory concentration (IC50) values in the low micromolar range are shown in Fig. 28.5. Different small molecular weight inhibitors have recently been evaluated for their selectivity toward human CYP2A enzymes [28.76–78]. Odors are generally blends of a series of volatile molecules which activate ORs. However, all of them can also be substrates or inhibitors of metabolic enzymes. Odorants which are also CYP inhibitors will reduce the metabolism of other odorants, which can impact the intensity or the quality of odors.

The earliest demonstration of nasal bioactivation took place using insecticides and herbicides. The herbicide 2,6-dichlorobenzonitrile (DCBN) was known to cause tissue-specific toxicity at very low doses in the olfactory mucosa of rodents. Amongst all tested heterologously expressed CYP variants, the 2A subfamily showed strong activity toward DCBN [28.79]. It has recently been shown that the bioactivation of DCBN is also catalyzed by human nasal mucosa microsomes [28.80]. The study was run in parallel using wild type or *Cyp2a5*-null mice (with CYP2A5 being the mouse ortholog of human nasal CYPs 2A13/2A6) demonstrating strong olfactory tissue-specific and CYP-dependent bioactivation of systemically applied DCBN. Metabolites were identified in nasal-wash fluid and these results are particularly interesting, since they demonstrate that products that originate from metabolism in the olfactory sustentacular cells are secreted into the nasal mucus, where metabolites could act as ligands of olfactory receptor proteins, either as agonists or antagonists.

28.4 Evidence for the Role of Biotransformation Enzymes in Olfaction from Animal Studies

It is generally assumed that odorant identity is represented in the chemotopic map by the glomerular activation pattern (see also Chap. 27). *Touhara* et al. reported that there are differences between OR-derived glomerular activation in the olfactory bulb (OB) and response patterns derived from in vitro assays [28.81]. For instance, only modest or no responses were observed for a mouse olfactory receptor protein (mOR-EG) in olfactory glomeruli following exposure of the animal's olfactory system to vanillin, although this odorant was shown to be a potent agonist of mOR-EG in isolated olfactory sensory neurons, as well as in the human embryonic kidney cell line 293 (HEK293) expressing mOR-EG. Most interestingly, it was reported that the nasal olfactory mucus influences the responsiveness to some but not all odorants indicating that some metabolic enzymes appear to be present in the mucus that is surrounding the ciliae where the olfactory receptor proteins are embedded. Later, the same group demonstrated that the enzymatic conversion of odorants in the nasal mucus affects both the olfactory glomerular activation patterns and odor perception in mice [28.82]. It was presented that mucus-secreted enzymes oxidized aldehydes to the corresponding acids and hydrolyzed esters; and that selected inhibitors reduced the metabolism of the odorants. The effect of metabolism taking place at the periphery was shown to influence the pattern of glomerular responses in the olfactory bulb as monitored by calcium imaging. The final study aimed to demonstrate that the enzymatic conversion of odorants in the mucus effects perception. Mice trained to recognize the ester acetyl isoeugenol showed a clear deficit to recognize the target odorant when treated with a carboxylesterase inhibitor, while they behaved no different to the control group when exploring odorants that were not metabolized [28.82]. This study elucidated for the first time that modulated peripheral metabolism in the olfactory epithelium is manifested in the first relay station in the brain, and is influencing the perception and behavior of the animal.

A study in rats further investigated the role of xenobiotic-metabolizing enzymes in the olfactory mucosa including activities of enzymes that are not secreted into the mucus [28.83]. The two CYP substrates coumarin and quinoline, as well as the carboxylesterase substrate isoamyl acetate were investigated. CYPs produced hydroxylated metabolites, while esterase activity resulted in isoamyl alcohol and acetic acid. Electro-olfactogram (EOG) recordings on the olfactory epithelium allowed to determine the activation of olfactory sensory neurons by either substrates or metabolites. When identified metabolites were tested separately in control experiments, the EOG responses were generally lower and weaker amplitudes were recorded, indicating that metabolites are less efficient agonists. In order to determine the functional role of olfactory metabolic enzymes, EOG studies were run in the presence of CYP- or carboxyl esterase-specific inhibitors which in-

hibit the enzymes as demonstrated in in vitro assays. Interestingly, in all cases the recorded EOG signal increased when using specific inhibitors, while in controls where the substrate and the inhibitor were not targeting the same enzymatic activity, no effects were observed [28.83]. This study revealed that peripheral olfactory responses are modulated by enzymes that are located in sustentacular cells.

28.5 Human Sensory Studies

While studies with human beings must be less invasive than the above described studies, several investigations have demonstrated that the respiratory tract-specific metabolism of volatiles is fast and can influence odor perception. The human olfactory mucosa has a very high metabolic activity, and in particular, CYP2A13 acts to oxidize a broad range of substrates and is itself subject to inhibition by small molecular weight compounds.

Two approaches allowed to monitor in vivo formation of metabolites [28.84]. In one case, a mass spectrometer was used to analyze exhaled air in real-time. Saturated headspace of the odorant 2-methoxyacetophenone was inhaled, and the breath exhaled into a glass funnel that was connected to a quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) ion source. Exhaled breath was monitored over several minutes, and the metabolite 2-hydroxyacetophone was already detectable in the first exhalation cycle [28.67]. A second approach enabled better quantification of metabolites, and exhaled breath was captured on a resin, followed by thermal desorption and analysis by gas chromatography-mass spectrometry (GC-MS), where metabolite formation was monitored, for example, for the CYP substrate 2-methoxyacetophenone, or the carboxylesterase substrate styrallyl acetate [28.84] as shown in Fig. 28.6.

Intensity rating is more challenging for panelists than detecting a change in olfactive character. While the former was successfully done for fragrance accords [28.69] the latter demonstration was important to provide evidence that mucosal biotransformation of odorants can impact the olfactory percept. During the substrate screening for CYP2A13 (see above) a metabolite was identified by GC-sniff analysis that had a strong, characteristic raspberry odor, while the substrate is commonly described as woody, fruity with raspberry aspects. The hydroxylated metabolite was isolated, its structure elucidated, and a reference material synthesized to confirm the characteristic raspberry smell of this molecule (Fig. 28.7). In order to determine if indeed that substrate is *woody*, *fruity*, *raspberry*, or if the raspberry note originates from the formation of the metabolite, a volatile odorless inhibitor was selected and used in a sensory experiment. A miniaturized olfactometer was used where the substrate was present in one channel, and the inhibitor in a second one, and a panelist could smell the odorous substrate, the odorless inhibitor, or a combination of the two by switching the pressure control valves. The majority of panelists reported that the raspberry note was reduced or completely eliminated when smelling the inhibitor together with the odor stimulus [28.67, 69]. During the study, some panelists reported that they could only identify a woody smell, while a few individuals described that odor as fruity/raspberry but without any woody facets; a possible explanation is that these panelists are hypo- or hyper-metabolizers of the substrate. This one sensory demonstration further supports the role of biotransformation enzymes as a perireceptor event that contributes to odor perception.

When designing and synthesizing novel odorants, fragrance chemists are building olfactophore models in analogy to pharmacophores, and the question is to what extent metabolism in the olfactory neuroepithelium needs to be considered in such studies to strengthen the model. The above example demonstrates that one needs to know the hydroxylated ketone metabolite to correlate the structure with other odorants that are described as having a raspberry odor. An interesting case is the search for novel green and fruity odorants,

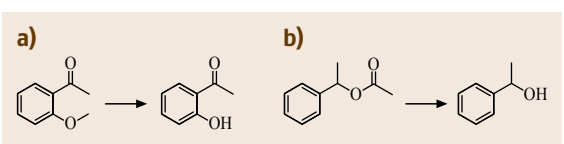


Fig. 28.6a,b Two different odorants were inhaled and exhaled breath was analyzed by real-time mass spectrometer analysis (a), or by trapping on a resin followed by thermal desorption and analysis by GC-MS (a,b)

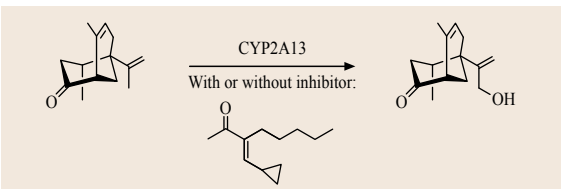


Fig. 28.7 The ketone substrate is olfactively described as woody and raspberry, while the hydroxylated metabolite has a pure raspberry note

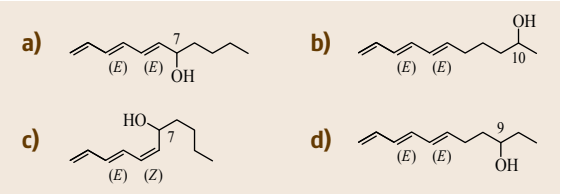


Fig. 28.8a–d Green smelling metabolites of undecatriene, which are produced by CYP2E1. Olfactory descriptions: **(a)** green, floral, metallic, **(b)** undecatriene-like, fatty, green, metallic, **(c)** green, pineapple, **(d)** fatty, oily, green, undecatriene-like, pineapple, fruity, metallic

starting from the signature hydrocarbon odorant 1,3,5-undecatriene which is found in galbanum oil. Series of molecules were synthesized over the years and an olfactophore model was developed that worked well for most molecules, but not for undecatriene that lacks a hydrogen-bonding function, which is present in other prototypic fruity, galbanum-type odorants, and it was speculated that enzymatic oxidation could take place prior to receptor interaction [28.85]. Several CYPs were tested with undecatriene as substrates, and in particular CYP2E1 which is expressed in the human olfactory mucosa produces multiple metabolites, that are described as having a fruity, green, and galbanum smell [28.67, 86] (Fig. 28.8). While the identified metabolites fit the galbanum olfactophore model much better, there is no experimental evidence to date that 1,3,5-undecatriene does not have those odor characteristics.

28.6 Discussion

At first glance, one may assume that deciphering the *olfactive code* is mastered when determining the molecular receptive range of the repertoire of roughly 380 different olfactory receptor proteins that convert the chemical information of odorants into neuronal signals and chemotopic maps in the olfactory bulb. However, recent results clearly demonstrate that the sense of smell is more complex than anticipated and is going to stay a fascinating area of research for many years to come. Latest studies demonstrate that the transformation and coding of neuronal activation patterns in the olfactory cortex is multifaceted and it has been proposed that different cortical areas are involved in learned versus innate behavioral responses providing another scientific approach to investigate emotional components of odor perception [28.20]. Olfactory receptor research has gained much interest in the last two decades, because of their discovery in 1991 and generally because of all the advancements in G-protein coupled receptor (GPCR) research, such as functional, heterologous expression and the growing number of crystal structures that are available for modeling and rationalizing agonist and antagonist interactions in the ligand binding domain of receptor proteins.

The fact that xenobiotic-metabolizing enzymes occur in high concentrations in the olfactory mucosa made scientists speculate on their role in olfaction since the middle of the last century. Recent animal studies demonstrated that inhibiting specific enzymatic activities in the olfactory mucosa changes the olfactory receptor response, the activation pattern in the olfactory bulb and even animal behavior [28.82]. Together with other data reported in this chapter, one can conclude that indeed, the in-nose biotransformation of odorants can modify the quantity (intensity) and the quality of odor stimuli. Since we are normally exposed to complex scents, such biochemistry has little impact on our perception of odors; however, when correlating olfactory receptor response patterns with the hedonics of an odorant this perireceptor event may well play a role and should not be neglected, and ideally one could include a metabolic interface that is mimicking the biotransformation events. When using receptor screening data to predict novel odorants for the fragrance industry, a solid understanding of the structure–activity and the structure–odor relationship is critical, and future activities in cheminformatics and computer modeling will help further valorize the growing number of datasets.

The destiny of xenobiotics that reach the olfactory epithelium can be manifold: receptor agonists and antagonists, enzyme substrates and inhibitors, OBP-ligands, precursors of bioactive compounds and allosteric modulators of receptors, enzymes, and other targets in the signal transduction cascade, such as the ion channels that are exposed to the mucus. The CYP2 family, and in particular the CYP2A subfamily of enzymes shows strong activity toward volatile organic molecules, and does not only oxidize odorants, but is also involved in the activation of nasal toxicants and carcinogens, and those enzymes have been proposed as pharmaceutical targets. There is evidence that olfactory biotransformation enzyme concentrations are regulated on the gene transcription level by chemicals acting as inducers of gene expression. This indicates a chance for plasticity and fast adaptation to the environment which impacts the metabolic capacity of this tissue. It is interesting to mention that the olfactory neuroepithelium is constantly regenerating and, in a way, it is remarkable that our sense of smell does not change much as a function of time.

As it invariably happens in scientific research, when answering one question, two more questions arise. Our current understanding on the code of smell advanced significantly over the last two decades and there is still much to learn and to be discovered. Hypotheses, including the ones expressed in this chapter, will be proven incomplete and there is still much incentive to further investigate chemoreception and in particular olfaction.

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29. Metabolism of Odorants in Humans

Michael Rychlik

This chapter outlines the metabolism of important food odorants and its impact on their bioactivity. The first section describes general metabolic pathways including functionalization (phase 1), conjugation (phase 2) and export (phase 3). These pathways are intended to excrete the compounds, which can be regarded as xenobiotics. In the second section, the metabolism of important classes of odorants, that is, alcohols and aldehydes, esters, thiols, terpenes, and phenylpropanoids is presented in detail. Among the terpenes, the focus lies on the monocyclic monoterpene hydrocarbon carvone, the monocyclic monoterpene ketone pulegone, the bicyclic monoterpene oxide 1,8-cineole, the bicyclic monoterpene ketone thujone, and on the allylalkoxybenzenes estragole and methyleugenol and coumarin among the phenylpropanoids. Recent studies are presented and each pathway is depicted in a separate reaction scheme. The contribution of each path either to detoxification or to toxification is discussed. The last section finally deals with conclusions and an outlook for further research.

Physiology of odorants is generally regarded as interaction of the odorant molecules with odor receptors in the olfactory tissue. While interaction of these ligands with the receptors is being intensely investigated [29.1], metabolism of odorants with respect to odor activity has only scarcely been addressed. Just recently, *Thiebaud* et al. [29.2] reported on odorant metabolism in the olfactory tissue and hypothesized that the reactions observed serve to clear the receptors from its ligands to avoid permanent receptor stimulation. Further aspects in relation to such peri-receptor events are discussed in detail in the chapter of *Schilling* in this book (Chap. 28). Besides their interaction with olfactory tissue, odorants are ingested with foods along with common nutrients and are subject to common metabolic reactions. Subsequently, as potential further physiological functions in the organism are still not clearly understood, odorants can be regarded as xenobiotics and follow the general principle of xenobiotic metabolism, which intends to enable or facilitate excretion and will be explained in the next chapter. There are only few exceptions from this rule, for example, short-chained fatty acids as abundant odorants in cheese, which contain nutritive value and are transferred into lipid metabolism.

As mentioned above, the physiology of odorants apart from olfaction is still unclear, but recent reports on odorant receptor expression in many tissues point to their function in skeletal muscle regeneration, cell–cell adhesion, and sperm migration [29.3]. Apart from their bioactivity in odor sensation, odor-active compounds exhibit pharmacological properties as components of essential oils. The latter show antimicrobial or antiviral or antioxidative activities and are essential elements of phytotherapy. More specifically, in aroma therapy essential oils are applied as remedies for many different disorders [29.4]. This knowledge has not only evolved in the last decade but has been in use for centuries in different cultures thus being an important component of ethnomedicine.

Pharmacological activity of odorants has been studied almost exclusively for the unmodified molecules. However, metabolism aims at excreting these compounds by transforming them into structurally different molecules, the activity of which is mostly unclear yet.

29.1 General Principles of Metabolism and Absorption

Similarly to other xenobiotics, odorants may enter the organism by different ways with absorption from the gastrointestinal tract being the most prevalent one. As odorants are at least partly lipophilic and have a rather low molecular weight, they are absorbed from foods and subsequently distributed in the organism mainly by passive diffusion. Almost exclusively, odorants will be identified as xenobiotics and the organism intends to excrete them as fast as possible. To accomplish straightforward excretion and also to prevent accumulation of these lipophilic compounds in tissues, metabolic processes are directed toward increasing hydrophilicity to facilitate biliary and renal excretion. During this process, three different phases can be differentiated, that is, functionalization as phase 1, conjugation as phase 2, and export as phase 3.

29.1.1 Phase 1: Functionalization

The first step toward increasing hydrophilicity is introducing a polar functional group or clearing an inherent one. The former alternative is achieved either by introducing oxygen into the xenobiotic or by oxidation or by reduction. Examples for odorants being subject to introduction of hydrophilic functional groups are the hydroxylation of estragole to hydroxyestragole [29.5] or the reduction of *Strecker* aldehydes such as methional to methionol [29.6]. Hydroxylations are mainly catalyzed by cytochrome P450 monooxygenases (CYPs), which are present in a huge variety of isoforms with different specificities and catalytic efficiency. These enzymes are expressed in all tissues, but the overwhelming part of turnover takes place in the liver.

An example for clearing a functionality of odorants is the hydrolysis of esters, which is catalyzed by carboxylesterases. These enzymes are present in all types of cells and recently their activity has been detected in olfactory cells, when isoamyl acetate was found to be hydrolyzed into acetic acid and isoamyl alcohol [29.2].

Regarding influx and metabolism of odorants, functionalization already starts in saliva, where esters have been found to be hydrolyzed, thiols to be degraded, and aldehydes reacted to the respective alcohols [29.6, 7].

In some cases, functionalization decreases the odor activity of odorants. When regarding common metabolic ways of odorants, hydroxylated terpenes show significantly higher odor thresholds than their nonhydroxylated precursors, and the same is valid when comparing alcohols as the metabolic products of the respective aldehydes [29.8]. However, depending on the individual enzyme expression and the odorant, changes in odor quality were reported and metabolites may show similar or even lower odor thresholds. This aspect will be detailed in this book's chapter *Nasal Periceptor Precesses* (Chap. 28).

Functionalization leads to increased hydrophilicity, which already may be sufficient for substantial excretion. However, this is not always the case. Therefore, conjugation reactions with highly hydrophilic molecules follow phase 1 to further increase hydrophilicity and to facilitate excretion.

29.1.2 Phase 2: Conjugation

Following functionalization, many metabolites are still not sufficiently hydrophilic for excretion or show increased toxicity due to instability. Examples are epoxides, for example, coumarin epoxide (see below) with subsequent nucleophilic addition to deoxyribonucleic acid (DNA) bases, which may induce genotoxicity. Therefore, functionalization is followed by coupling of the functional group with hydrophilic molecules such as glucuronic acid, glutathione, glycine, or sulfate. These reactions are mainly catalyzed by transferases using conjugating agents such as uridine diphosphate (UDP)-α-glucuronic acid. An example is the odorant furaneole, an odor impact compound of strawberries and pineapple, which is transferred into its glucuronide that is detectable in urine [29.9]. Conjugation with glutathione is followed by further metabolism to N-acetylcystein conjugates, the so-called mercapturic acids.

Conjugates may be directly excreted by passing the kidney into the urine. The other alternative is the active transport in the liver into bile by transport proteins, which is called phase 3.

29.1.3 Phase 3: Export

Conjugates are often highly hydrophilic and are not able to pass cell membranes to be efficiently excreted. Therefore, transport mechanisms for these conjugates exist, which require energy in the form of adenosine triphosphate (ATP) as molecular pumps. During phase

3, no further metabolism takes place. These proteins are classified as ATP-binding cassette (ABC)-transporters and are highly expressed in liver and kidneys. Apart from transporters for xenobiotics, also multidrug resistence (MDR) transporters belong to this class of proteins [29.10].

29.2 Metabolism of Important Classes of Odorants

29.2.1 Alcohols and Aldehydes

Odor-active alcohols and aldehydes in foods often originate from the *Strecker* reaction of amino acids. The respective aldehydes, the so-called *Strecker* aldehydes, show lower odor thresholds than the respective alcohols with the potato-like smelling methional showing the most intense odor. Further *Strecker* aldehydes along with their precursors and odor thresholds are presented in Table 29.1. Due to their partly lipophilic character, alcohols and aldehydes can be expected to be absorbed by passive diffusion after ingestion. Thereafter, both classes of compounds are oxidized to the corresponding carboxylic acids by highly expressed nicotinamide adenine dinucleotide (NAD^+)/NADH-dependent enzymes [29.11]. Of these, alcohol dehydrogenases (ADH) are cytosolic enzymes catalyzing the oxidation of alcohols to their corresponding aldehydes. Thereafter, the latter are oxidized by aldehyde dehydrogenases (ALDH) to the acids. Linear as well as branched-chain aliphatic alcohols and aldehydes are good substrates for ADH and ALDH. The generated branched-chain and linear carboxylic acids then undergo general metabolism of fatty acids.

29.2.2 Esters

Esters are character-impact odorants particularly in fruits such as apples [29.12], and, due to their lipophilicity, are easily absorbed from foods by passive diffusion. The most effective way to transform esters into hydrophilic compounds is hydrolysis of the ester bond, which is catalyzed by carboxylesterases.

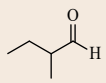
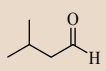
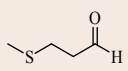
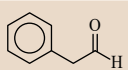
The generated alcohols and acids undergo further metabolism. Alcohols are oxidized to acids as detailed above and transferred into fatty acid metabolism.

Hydrolysis of esters already starts in saliva, where odor-active ethyl esters of butanoic acid, hexanoic acid and octanoic acid were degraded to an extent of max. 49% within 10 min [29.7]. Considering a structure activity relationship, ethyl octanoate was more efficiently degraded than ethyl hexanoate and ethyl butanoate.

29.2.3 Thiols

Thiol metabolism (Fig. 29.1) includes various reactions such as S-oxidation, oxidative desulfuration and dealkylation, methylation, and disulfide formation with glutathione (GSH). Chemical reactivity of thiols is

Table 29.1 Structures, odor qualities, odor thresholds and precursors of important *Strecker* aldehydes

Aldehyde	Structure	Odor quality	Odor threshold in water[a] (µg/L)	Precursor
Acetaldehyde		Pungent	6–25	L-Alanine
2-Methylpropanal		Malt-like	1–10	L-Valine
2-Methylbutanal		Malt-like	1–3.7	L-Isoleucine
3-Methylbutanal		Malt-like	0.4–3.1	L-Leucine
Methional		Boiled potato-like	0.2–1.8	L-Methionine
Phenylacetaldehyde		Honey-like	4	L-Phenylalanine

[a] Orthonasal thresholds [29.8]

mainly based on their easy dissociation under physiological pH to form a thiolate anion, which is highly nucleophilic. On this basis and given their reactivity with weak oxidants, thiols easily form mixed and non-mixed disulfides by reacting with endogenous thiols such as GSH or cysteine or with proteins bearing thiol groups.

In the course of S-oxidation, thiols are transferred enzymatically into reactive sulfenic (R–S–OH) acids, the further oxidation of which can lead to sulfinic (R–SO_2H) acids. Alternatively, sulfenic acids can react with excess thiols (e.g., GSH) to give the corresponding disulfides. The latter can either be reduced back to the thiols (enzymatically by thiol-transferase or chemically by exchange with GSH or endogenous thiols), or can be oxidized to thiosulfinic acid, hydrolysis of which gives rise to sulfinic acids and further oxidation to sulfonic (R–SO_3H) acids. Thiol-S-methylation as a special form of conjugation for simple aliphatic and aromatic thiols is catalyzed by thiol-S-methyl-transferases and subsequent S-oxygenation may lead to water-soluble methylsulfoxides and/or sulfones [29.13]. Parts of these pathways have been reported for methylmercaptan in microsomes [29.14] and rats [29.15] and for diethyl disulfide in rodents [29.16]. The other reactions can be deduced from pharmacokinetic studies of thiol-containing drugs.

29.2.4 Terpenes

Terpenes encompass a wealth of compounds essential in animal and plant metabolism. Their structures vary from monoterpenes containing 10 carbon atoms to tetraterpenes bearing 40 carbons. While the latter are active as natural colorants, antioxidants, and precursors

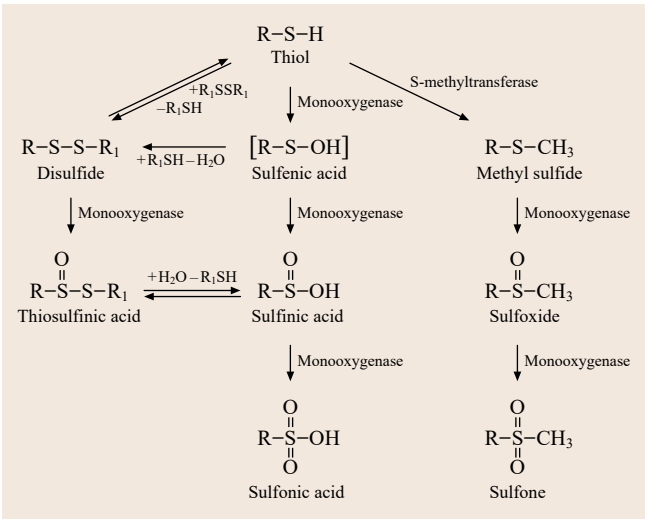


Fig. 29.1 Metabolism of thiols in humans

of vitamins, mainly monoterpenes and sesquiterpenes are regarded as odorants due to their sufficient volatility. Terpenes may bear several functional groups at their hydrocarbon backbone, the monoterpene alcohols, ketones, phenols, esters, and oxides. Moreover, monoterpenes can be acyclic or consist of one ring (monocyclic monoterpenes), two rings (bicyclic monoterpenes), or three rings (tricyclic monoterpenes). In plants, terpenes play a major role as attractants or repellents for insects, protectants from fire, hormones, antimicrobials and, therefore, are also attractive for human use in spices, aromatherapy, and as antioxidants. Generally, terpenes as lipophilic compounds are assumed to be absorbed abundantly in the gastrointestinal tract. Metabolism is often initiated by oxidation to more polar metabolites by CYP-450 enzymes.

These phase 1 reactions involve hydroxylation or epoxidation of the exocyclic or endocyclic double bond. Hydroxylated metabolites may be excreted in conjugated form or undergo further oxidation, giving rise to more polar metabolites that are also excreted, mainly in urine. Epoxides may be further metabolized either by hydrolysis to yield diols or by conjugation with glutathione to be finally excreted as mercapturic acids. Aromatic hydrocarbons may undergo epoxidation and dihydroxylation of the ring to form phenolic metabolites, which subsequently may be conjugated with sulfate or glucuronic acid and excreted in the urine. Saturated alkanes may be metabolized via omega oxidation to give the respective carboxylic acids or via so-called omega-1, -2, -3, or -4 oxidations giving rise to secondary alcohols and ketones. The generated carboxylic acids are, as mentioned above, expected to participate in the endogenous fatty acid metabolism.

Most of metabolic data for terpenes have been obtained from rats, rabbits, possums, various antipodean species or cell models by administration of terpene-rich diets or high doses of single terpenes. However, it has to be criticized that the data from these studies may not be applied to humans due to possible interspecies differences in metabolism. Moreover, these data may be restricted to pharmacological applications as the doses applied to animals or cellular models were much higher (up to 1000 mg/kg of body weight) compared to doses occurring in a normal diet. However, dose matters as for terpenes various positions of hydroxylations or epoxidations are possible and in higher dosages the capacities of single enzymes may be exceeded. This can favor pathways, which are different to those occurring in low dosage trials. Evidence for these relationships has been found for carvone [29.17] and estragole [29.5] among others. In order to refer as close as possible to the situation present in daily diet, the metabolism of ingestion correlated amounts (MICA) approach has been devel-

oped [29.17]. Quantitation often has been accomplished by using stable isotopologs as internal standards, which offers many advantages to compensate for matrix interferences and low recoveries [29.18]. As essentially no conjugates of terpenes are commercially available, the sum of conjugated and nonconjugated metabolites was determined after deconjugation using glucuronidases and/or sulfatases [29.5].

A Monocyclic Monoterpene Hydrocarbon: Carvone

Carvone is a monoterpene hydrocarbon occurring in many essential oils with the (*S*)-(+) enantiomer being the main constituent of caraway oil and dill seed oil. The (*S*)-(+) enantiomer, however, is the major impact compound of spearmint oil and due to its refreshing odor it is widely used as a flavor compound, for example, for toothpaste and other cosmetics.

In an MICA experiment (Fig. 29.2), the major in vivo metabolites of *S*-(+)- and *R*-(−)-carvone were newly identified as dihydrocarvonic acid, carvonic acid, and uroterpenolone [29.17]. In two different approaches, the author administered (1) pure carvone enantiomers [29.17] and (2) $^{2}[H]_{2}$-carvone and $^{13}[C]_{1}$-carvone [29.19] and analyzed the metabolites via gas chromatography-mass spectrometry (GC-MS) to clarify the metabolic pathways. Carvonic acid was generated by oxidation at the methyl carbon of the side chain of carvone, whereas dihydrocarvonic acid arose from oxidation at the methylene position, supposedly by an intermediate carvone epoxide. Labeling experiments indicated a *nonaromatic* National Institute of Health (NIH) shift in the pathway yielding dihydrocarvonic acid. Moreover, the former study proved dehydrogenation of dihydrocarvonic acid and hydrogenation of carvonic acid as minor metabolic ways. Oxidation at the methylene carbon of the isopropenyl group of carvone gave uroterpenolone probably formed by hydrolysis of the supposed carvone epoxide. No differences were found between the metabolism of the enantiomeric *S*-(+)- and *R*-(−)-carvone.

A Monocyclic Monoterpene Ketone: Pulegone

(*R*)-(+)-Pulegone is a major compound of the essential oils of peppermint and pennyroyal besides its occurrence in many other herbs such as oregano and tea. Its characteristic minty odor is the background of the frequent use of (*R*)-(+)-pulegone as component in mint-flavored beverages, sweets, and chewing gums. However, due to its hepatotoxicity in higher dosages, the European Union has set the following maximum levels for pulegone in foodstuffs: 25 mg/kg in foodstuffs in general; 100 mg/kg in beverages; 250 mg/kg in peppermint- or mint-flavored beverages; and up to 350 mg/kg in mint confectionery [29.20]. Toxicokinetic studies dating back to 1987 identified menthofuran as a metabolite of pulegone at high exposure to pennyroyal oil [29.21], and it is assumed that hepatotoxicity of pulegone is due, at least in part, to this metabolite [29.22].

While many studies on pulegone metabolism were performed using rats, only one human study has been reported [29.23], the results of which will be presented in the following.

In MICA experiments, both enantiomers of pulegone were applied to humans. The major in vivo metabolites (Fig. 29.3) of (*S*)-(−)-pulegone in humans were identified as *E*-2-(2-hydroxy-1-methylethylidene)-5-methylcyclohexanone (10-hydroxypulegone, P1), 2-(2-hydroxy-1-methylethyl)-5-methylcyclohexanone (8-hydroxymenthone, P2), 3-hydroxy-3-methyl-6-(1-methylethyl)cyclohexanone (1-hydroxymenthone, P3), and 3-methyl-6-(1-methylethyl)cyclohexanol (menthol, P4) on the basis of GC-MS analysis in combination with syntheses and nuclear magnetic resonance (NMR) experiments. In contrast to the studies performed before, menthofuran was not found to be a major metabolite of pulegone. On the contrary, the author assumed this compound to be an artifact formed during workup from at least one metabolite (P1). The author deduced the differences in toxicity between (*S*)-(−)- and (*R*)-(+)-pulegone from the strongly diminished ability for enzymatic reduction of the double bond in (*R*)-(+)-pulegone. In consequence, further potential oxidation of 10-hydroxypulegone (P1) to the reactive 10-pulegonealdehyde was hypothesized to ac-

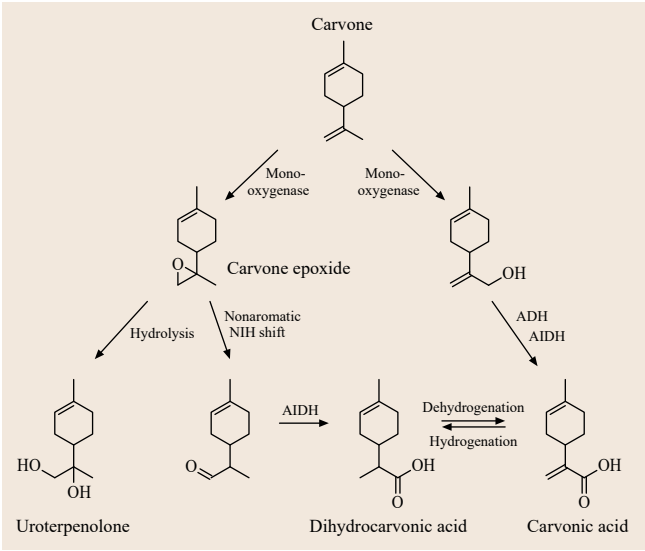


Fig. 29.2 Proposed metabolic pathways of carvone in humans (after [29.17, 19])

count for the observed hepatotoxic and pneumotoxic activity of (*R*)-(+)-pulegone in humans.

A Bicyclic Monoterpene Oxide: Cineole

A major component of essential oils from *Eucalyptus polybractea* is the monoterpene 1,8-cineole (Col), also known as eucalyptol. Besides this, 1,8-cineole is present in numerous spices, such as rosemary, sage, basil, and laurel. It has a characteristic fresh and camphoraceous odor quality and, therefore, is used for flavoring of different foods and cosmetics. Apart from its flavoring applications, 1,8-cineole is used in pharmaceutical preparations to treat cough, muscular pain, neurosis, rheumatism, asthma, and urinary stone [29.4, 24, 25]. Originally, biotransformation studies of 1,8-cineole have been performed in brushtail possum and rabbits and identified 2α-hydroxy-Col, 2β-hydroxy-Col, 3α-hydroxy-Col, 3β-hydroxy-Col, 7-hydroxy-Col, 9-hydroxy-Col (Fig. 29.4) and the respective diols, cineolic acids, and hydroxyl cineolic acids as phase I metabolites in urine and blood plasma [29.26–28]. Regarding toxicity of 1,8-cineole, the oral acute LD_{50} in rats is reported to be 2480 mg/kg bw [29.29]. Subacute toxicity was shown in rats for dose levels of 600 mg/kg bw and higher. There is no evidence for chronic or genotoxic effects of 1,8-cineole [29.30]. For the hydroxylated metabolites, no toxicological data is available.

In an approach modeling the realistic dosage from a normal diet, the metabolism of 1,8-cineol after ingestion of sage tea (dose 1.02 mg 1,8-cineole; 19 µg/kg bw) was studied [29.31]. After application of the tea,

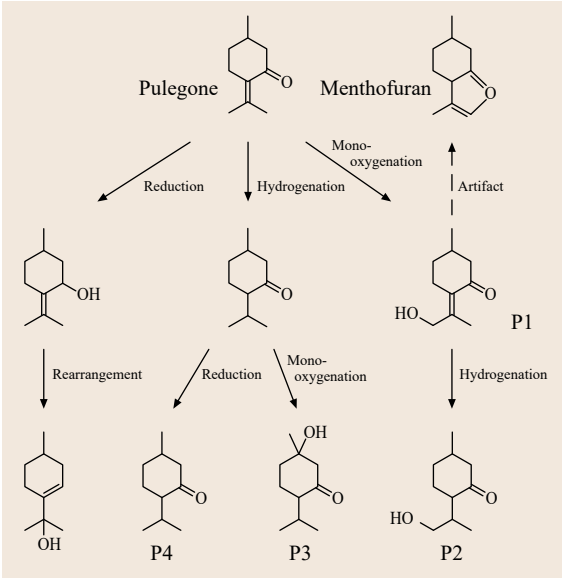


Fig. 29.3 Hypothetical metabolic pathway of pulegone (after [29.23])

the metabolites 2-hydroxy-Col, 3-hydroxy-1,8-Col, 9-hydroxy-Col, and 7-hydroxy-1,8-Col were identified in plasma and urine of one volunteer (Fig. 29.4, full arrows). For quantitation of these metabolites and the parent compound, stable isotope dilution assays were developed after synthesis of [$^2H_3$]-Col, [9/10-$^2H_3$]-2-hydroxy-Col, and [^{13}C,$^2H_2$]-9-hydroxy-Col as internal standards. Quantitation of 1,8-cineole was accomplished by solid phase microextraction (SPME) GC-MS and of the hydroxyl-1,8-cineoles by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after deconjugation in blood and urine of the volunteer.

In urine, 2-hydroxy-Col showed highest contents followed by its 9-isomer. Summing up the urinary excretion over 10 h, 2-hydroxy-Col, the 9-isomer, the 3-isomer, and the 7-isomer accounted for 20.9%, 17.2%, 10.6% and 3.8% of the cineole dose, respectively. These results were not only obtained after deconjugation treatment of urine but also by direct analysis of the hydroxycineole glucuronates after synthesis of the reference compounds according to Königs–Knorr and analysis via LC-MS/MS (Fig. 29.5). Interestingly, the double peak of 9-hydroxy-Col glucuronate indicated separation of anomers. This is one of the rare examples of a direct detection of terpene conjugates.

In a recent pharmacological investigation [29.32], the metabolite profiles of 1,8-cineole in human milk were detected after lactating mothers ingested a non-prescription pharmaceutical (Soledum) containing this substance. Apart from the metabolites found before in urine (α-2-hydroxy-Col, β-2-hydroxy-Col, α-3-hydroxy-Col, 7-hydroxy-1,8-Col, 9-hydroxy-Col), three of the metabolites hitherto only found in microorganisms and insects (2-oxo-Col, 3-oxo-Col, 2,3-dehydro-Col) and the two additional derivatives 2,3-α-epoxy-Col and 4-hydroxy-Col never have been identified before as metabolites of 1,8-cineole (Fig. 29.4, open arrows). A very recent investigation was dedicated to elucidate the enantiomeric ratio of the metabolites in urine [29.33]. Interestingly, all metabolites showed an excess of one enantiomer with 3-oxo-Col revealing almost exclusive formation (99.5% enantiomeric ratio) of the (+)-enantiomer.

A Bicyclic Monoterpene Ketone: Thujone

α-Thujone is a bicyclic monoterpene, which is a common constituent in herbal medicines, essential oils, foods, flavorings, and beverages [29.4] along with its epimer at the C-4 methyl group, β-thujone. The isomer ratio is variable, with high contents of α-thujone in the essential oils of cedarleaf oil and β-thujone in that of wormwood oil [29.4]. α-Thujone is known as the active component of the emerald-green liqueur absinthe,

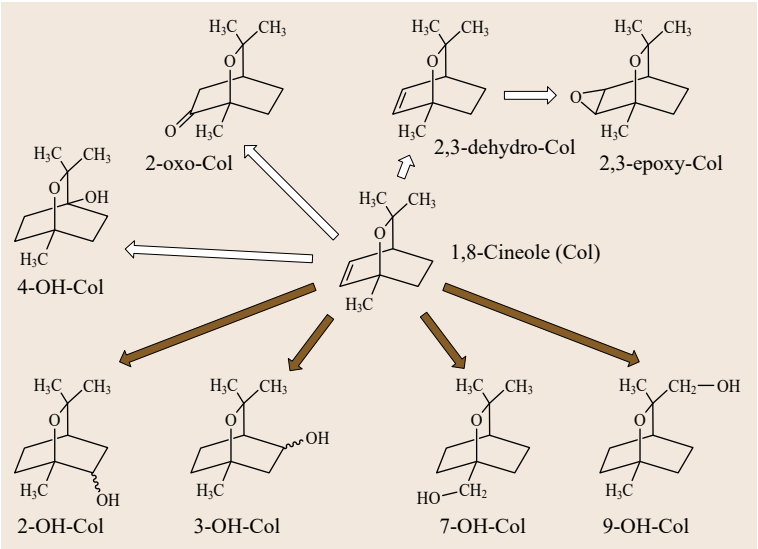


Fig. 29.4 Metabolism of 1,8-cineol after administration of a sage infusion (*full arrows*); administration of a pharmaceutical, the metabolites indicated by open arrows additionally were detected in human milk

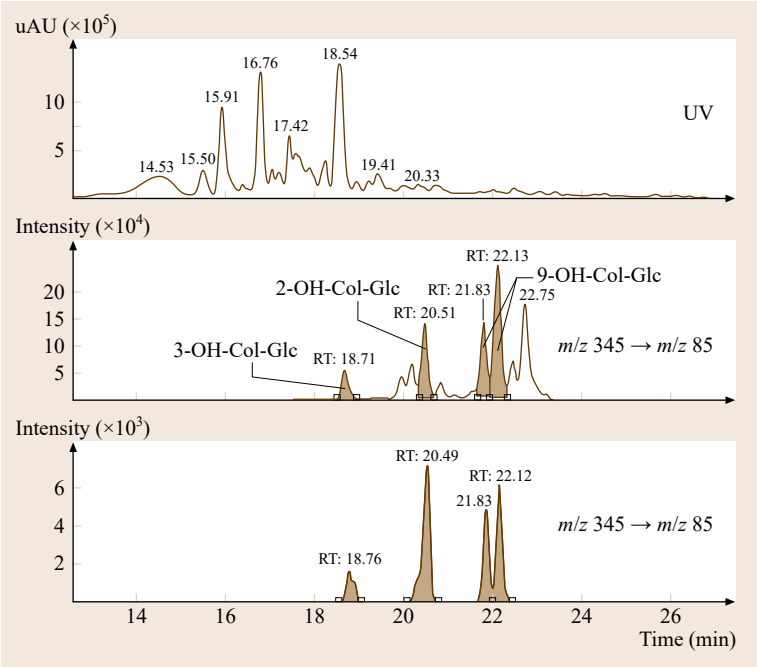


Fig. 29.5 LC-UV (*upper trace*) and LC-MS/MS (*middle trace*) chromatograms of hydroxycineole glucuronates (Glc) in human urine after ingestion of sage infusion. The *lower trace* shows an LC-MS/MS chromatogram of a mixture of the reference compounds

which was very popular in the 19th and early 20th centuries as a preferred drink of artists and writers including Vincent van Gogh, Henri de Toulouse-Lautrec, and Charles Baudelaire [29.34]. Abuse of absinthe often induced fits and hallucinations and sometimes contributed to psychoses and suicides, thus leading to a ban in many countries early in the 20th century. The actual regulation in the European Union prohibits to add thujone as odorant to foodstuffs or flavorings and it is only allowed in foods originating from flavorings or components from natural raw materials. In general, the sum of alpha- and beta-thujone may not exceed 0.5 mg/kg in foodstuffs and beverages with following exceptions: Maximum thujone levels of 5 mg/kg in alcoholic beverages with not more than 25% volume of alcohol, of 10 mg/kg in beverages with more than 25% volume of alcohol, of 25 mg/kg in foodstuffs containing preparations of sage and of 35 mg/kg in alcohol labeled as bitters (40% volume of alcohol and more) are allowed [29.20].

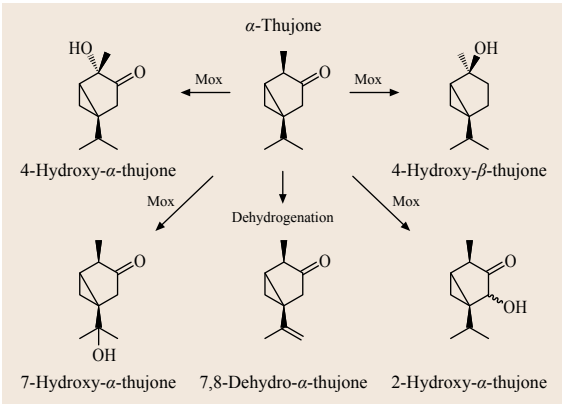


Fig. 29.6 Metabolism of α-thujone in mammals, Mox: monooxygenation

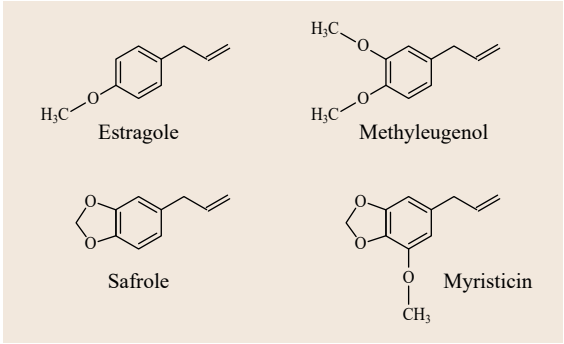


Fig. 29.7 Structures of important allylalkoxybenzenes occurring in foods

The toxicity of absinthe was attributed to wormwood oil as ingredient, which itself is a herbal medicine for treating dyspeptic disorders, liver and gallbladder complaints and to control gastrointestinal worms with records back to the ancient world [29.35]. The principal bioactive ingredient of wormwood oil and neurotoxic principle in absinthe is generally considered to be α-thujone. The acute toxicity of α-thujone has been attributed to blocking the γ-aminobutyric acid (GABA)-gated chloride channel [29.36]. However, some recent studies indicate that the toxic symptoms after consumption of absinthe were mainly due to its alcohol content. Studies of the fate of α-thujone and β-thujone in vitro and in rodents led to the identification of the hydroxythujones and dehydrothujones shown in Fig. 29.6 [29.36, 37] with different abundancies depending on the species under study. In mice urine after administration of α-thujone, mainly 2-hydroxythujone could be detected along with minor amounts of 4-hydroxythujone, 7-hydroxythujone, and 4,10-dihydrothujone. In contrast to this, the respective experiment in rats resulted in 4-hydroxythujone and 4,10-dehydrothujone as the two major metabolites along with 7-hydroxythujone at minor amounts. 2-Hydroxythujone was not detectable. In vitro studies using human microsomes indicated 7-hydroxythujone along with 4-hydroxythujone and 7,8-dehydrothujone as possible human metabolites of α-thujone. Recent syntheses of 2[H]6-isotopologues of α-thujone and of its hydroxylated major metabolites [29.38] will enable quantitation of α-thujone in future MICA studies on herbs containing thujone.

29.2.5 Phenylpropanoids

Phenylpropanoids consist of a structure that is derived from a propyl side chain attached to a benzene backbone showing several variations in its oxidation state, attachment to other phenolic systems and attachments of hydroxyl- and methoxy groups to the aromatic moiety. These compounds are important odorants of several plants and originate from metabolism of amino acids such as phenylalanine or tyrosine. Here, two examples for classes of phenylpropanoids are presented: (1) the allylalkoxybenzenes estragole and methyleugenol (Fig. 29.7) and (2) coumarin.

Allylalkoxybenzenes: Estragole and Methyleugenol

Estragole is a component of several herbs such as tarragon, basil, fennel, and anise [29.39–41]. Of these, the fruits of fennel and anise are commonly used to treat catarrh of the respiratory tract and gastrointestinal disorders. Therefore, fennel extractions are the classical infusion for nursing babies to prevent flatulence and spasms.

However, estragole as a ring-substituted allylbenzene along with the structurally similar safrole and methyleugenole have been reported as potent carcinogens in rodents [29.42]. The reason for their hepatotoxic properties is the specific 1′-hydroxylation of the side chain of allylbenzenes. In consequence, conjugation of 1′-hydroxyestragole with sulfuric acid is assumed to result in a carcinogenic potential as the sulfate might decompose readily to an electrophilic cation reacting easily with the DNA [29.43]. Reports on 1′-hydroxyestragole showing a higher carcinogenic activity than its precursor [29.42] are in line with this hypothesis. However, due to its lability, the identity of the sulfate has not yet been unequivocally proved.

The formation of 1′-hydroxyestragolesulfate has been suggested due to scavenging reactions indicating its formation [29.44] and due to the decrease in incidence of hepatocellular carcinoma by application of the sulfotransferase inhibitor pentachlorphenol to rats [29.45]. Further evidence for adverse effects comes

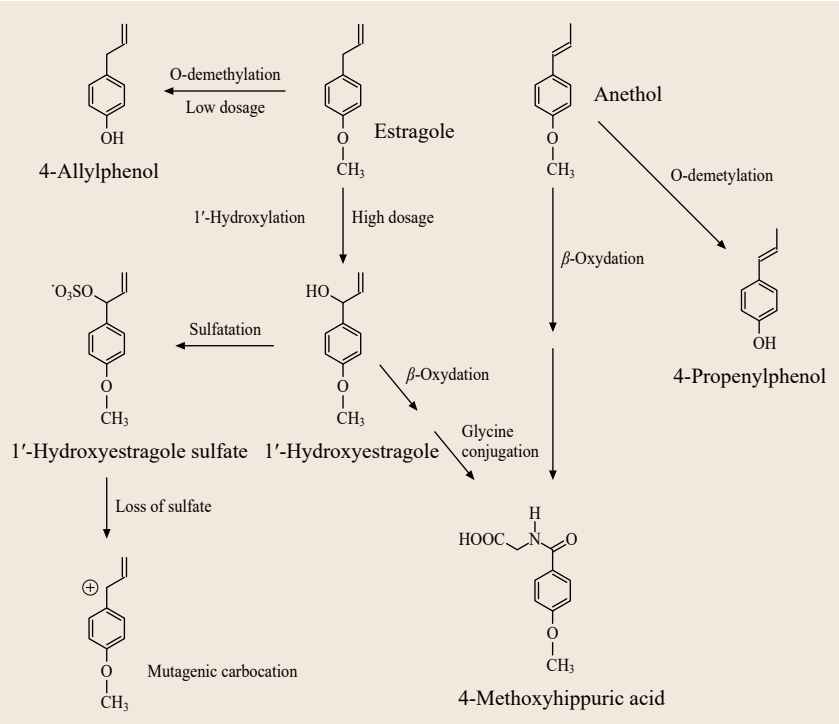


Fig. 29.8 Metabolism of the fennel odorants estragole and anethole showing the proposed generation of a mutagenic agent resulting from decomposition of 1′-hydroxyestragole sulfate

from the detection of adducts of 1′-hydroxyestragole with DNA [29.46] and DNA modifications following the carbocation formation mediated by sulfotransferase 1A1 [29.47]. Apart from the open unequivocal identification of the 1′-hydroxyestragolesulfate, the interaction of estragole metabolism with that of other highly abundant terpenes such as *trans*-anethole in doses usually administered with herbal infusions was not clarified. Therefore, in a human study, the metabolism of estragole was investigated after consumption of fennel tea (Fig. 29.8) by analyses of its metabolites in blood plasma and urine [29.5]. Stable isotope dilution assays based on LC-MS/MS detection revealed that 1′-hydroxylation of estragole happened very fast as the concentration of conjugated 1′-hydroxyestragole in urine peaked after 1.5 h, whereas it was no longer detectable after 10 h. Besides the formation of less than 0.41% conjugated 1′-hydroxyestragole of the estragole dose administered, the further metabolite *p*-allylphenol was generated from estragole in a higher percentage (17%). Both metabolites were also detected in blood plasma in less than 0.75 to 2.5 h after consumption of fennel tea. From these results, it can be concluded that an excess of the major fennel odorant *trans*-anethole principally does not interfere with estragole metabolism. Moreover, in these low dosages no evidence for the 1′-hydroxyestragolesulfate were found as the applied deconjugation for urine analyses showed no differences in the treatments with pure glucuronidase alone and that with a mixture of glucuronidase and sulfatase.

Methyleugenol is an allylalkoxybenzene occurring naturally in a variety of spices, herbs, and essential oils, such as clove oil, nutmeg, pimento, basil, anise and mace. Moreover, it is also present in blackberries, bananas, black pepper, bilberries, and walnuts [29.4].

Similarly to estragole, genotoxicity of methyleugenol is attributed to 1′-hydroxylation, sulfatation and the labile sulfate being the putative genotoxic carcinogen. In analogy to estragole, the respective sulfate of hydroxymethyleugenol has not been detected directly, but the number of evidence is also increasing to support this hypothesis. Most recently, also for methyleugenole DNA adducts and the influence of sulfotransferases on their formation have been found [29.48]. A risk assessment of genotoxic compounds such as methyleugenole is difficult as for these no zero threshold in a dose–effect relationship can be deduced. In these cases, the joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) expert committee on food additives (JECFA) and the European Food Safety Authority (EFSA) agreed upon the *margin of exposure* (MoE) approach [29.49]. In this approach, the MoE is defined as the ratio of a benchmark dose as estimation for the no-observed-adverse-effect-level (NOAEL)

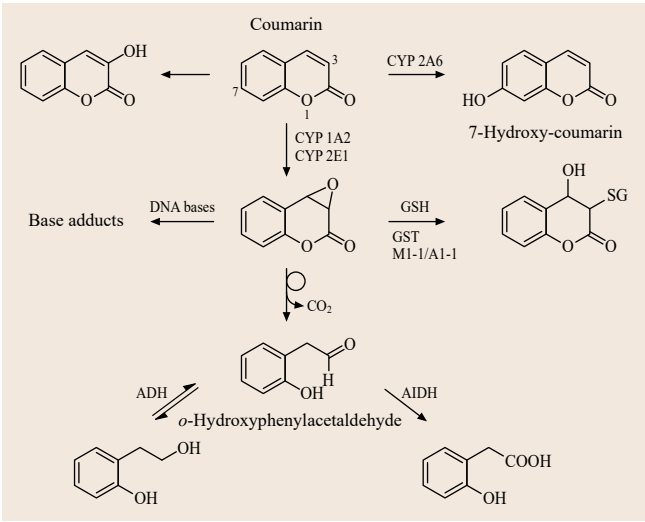


Fig. 29.9 Proposed metabolism of coumarin to hepatotoxic *o*-phenylacetaldehyde

and the estimated exposure. A relevant exposition of the consumer in Europe was found to originate from spices, particularly basil and foods flavored with spices and essential oils. From the modeling of the benchmark dose lower confidence limit at 10% (BMDL10) and the exposure data the calculated MoEs ranged from 100 to 800 depending on the assumptions used in the exposure estimation [29.50]. As a considerable risk for the consumer is assumed for MoEs below 10 000, the EFSA considers methyleugenol as one of the food components showing a distinct carcinogenic risk potential for the consumer along with acrylamide, aflatoxin B1 and furan [29.49].

Coumarin

The phenylpropanoid coumarin is a constituent of several spices, the respective essential oils, and other flavoring foods such as *Cinnamomum aromaticum* (cassia bark), *Asperula odorata* (sweet woodruff), *Dipterix odorata* (tonka bean), and species of clover [29.51]. Besides occurring naturally in these foods, coumarin has been widely used as a flavoring compound due to its sweet and aromatic odor. However, since the early 1950s, the odorant has been found to exert hepatotoxicity and was suspected to be mutagenic and carcinogenic [29.52]. Toxicity of coumarin cannot be attributed to the parent compound but to metabolites and for the metabolic pathway significant inter-individual variations have been found (Fig. 29.9). Although coumarin in humans is mainly metabolized to 7-hydroxycoumarin [29.53], a subpopulation lacks these detoxification enzymes, which have been characterized as CYP 2A6. This subpopulation has been estimated to account for 10% of Germans. This susceptible subpopulation metabolizes the odorant to its 3,4-epoxy derivative, which was suspected to form DNA adducts and may react to hepatotoxic *o*-hydroxyphenylacetaldehyde [29.54]. While the former reaction was found not to be the cause for carcinogenic effects in rodents, the latter product was confirmed to evoke hepatotoxicity by coumarin [29.55].

For these reasons, a maximum level of 2 mg/kg for foods generally and 10 mg/kg in alcoholic beverages has been set in the European Union [29.20]. Moreover, coumarin is not allowed to be used as flavoring additive to foods.

The issue of coumarin's hepatotoxicity arises particularly in winter as cassia bark increasingly substituted true cinnamon in baked goods, particularly in seasonal products such as gingerbread or cinnamon star cookies. Moreover, indications to use cassia bark powder as a supplement and remedy against type 2 diabetes mellitus [29.56] increased consumption of this spice in the Western countries. As a tolerable daily intake of 0.1 mg/kg body weight has been established by the Scientific Panel on Food Additives, Flavorings, Processing Aids, and Materials in Contact with Food (AFC) [29.57], a longer lasting consumption of products high in cassia can be expected to provoke hepatotoxic effects. Therefore, supplements containing cassia have been classified as drugs.

Traditional and seasonal bakery such as gingerbread or cinnamon stars are now restricted to 50 mg coumarin/kg, breakfast cereals to 20 mg/kg, other fine bakery to 15 mg/kg and desserts such as sweet rice flavored with cinnamon to 5 mg/kg [29.58]. Since 2012, the German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung, BfR) recommends for children not to exceed a daily consumption of 6 small cinnamon stars and advices adults to carefully use cassia as spice [29.59].

29.3 Conclusions and Outlook for Further Research

According to the current knowledge, metabolism of odorants serves, on the one hand, to reduce or modify the odorant's affinity to the odor receptor and to clear the latter from the ligand to avoid receptor blockage. On the other hand, metabolism intends to help excreting the odorants, which almost exclusively are not

endogenously formed. The consequences of these reactions are only poorly understood. For some examples, lower bioactivity of the metabolites compared to that of the odorant has been shown, but in some cases the metabolites reveal increasing toxicity (allylalkoxybenzenes, coumarin). Moreover, the molecular mechanisms of these activities are often still unclear and, therefore, the effect of metabolism is still under discussion.

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30. Olfactory Subsystems

Marc Spehr

A plethora of structurally diverse environmental chemosignals convey critical information for survival, health, and reproduction. To meet the bewildering structural complexity of the chemical *odor space*, distinct cellular mechanisms and, ultimately, sensory subsystems have evolved to detect and discriminate these varied chemostimuli. Mammalian olfactory subsystems can be categorized by the stimuli they detect, the signaling proteins they express, and the central circuits that process these information. This chapter is centered on noncanonical olfactory subsystems and their peripheral sensory structures – the vomeronasal organ, the septal organ of Masera, and the Grüneberg ganglion.

Animals, including humans, detect and respond to a plethora of structurally diverse environmental chemical cues. These chemosignals convey critical information for survival, health, and reproduction:

- Food type and quality
- The existence (and concentration) of toxins
- The presence of prey, predators, competitors, or potential mates
- Social cues that elicit stereotyped, genetically preprogrammed behaviors or hormonal responses.

To meet the bewildering structural complexity of the chemical *odor space*, distinct molecular and cellular mechanisms have evolved to detect and discriminate these varied stimuli [30.1, 2].

In recent years, it has become increasingly clear that the mammalian olfactory system is organized into multiple subsystems that can be categorized by the chemostimuli to which they respond, the receptors and downstream signaling proteins they express, and the brain circuits that process these information [30.3–6]. The diversity of olfactory subsystems manifests both in the occurrence of anatomically distinct chemosensory structures within the mammalian nose and in the coexistence of unique neuronal subpopulations within individual tissue(s).

In the following, this chapter will focus on the noncanonical chemosensory subsystems that, in part, have only recently (re)gained scientific attention. The majority of experimental research on olfactory subsystems has been carried out in mice. As their genetic amenability allows integrated investigations that span the molecular, cellular, and systems level [30.7], the findings presented below are largely restricted to this model organism.

30.1 The Subsystem Organization of the Sense of Smell

At least four different chemosensory structures are found in the rodent nose (Fig. 30.1):

1. The main olfactory epithelium (MOE)
2. The septal organ (SO) of Masera

3. The vomeronasal organ (VNO)
4. The Grüneberg ganglion (GG).

The newfound appreciation of an organizational concept of olfactory subsystems – or noses within noses [30.8] – comes with the recognition of profound differences in the molecular and cellular mechanisms that, at least theoretically, confer a substantial degree of stimulus selectivity for each subsystem and, thus, allow dedication of each structure to a particular role in chemosensation [30.9]. The high level of specialization perhaps becomes most apparent when considering the rapidly expanding repertoire of chemosensory receptor gene/protein families employed by the different systems. While the majority of putative chemoreceptor genes encode for members of the *classical* odorant receptor [30.10] gene family – which, in most mammals, accounts for as much as 2% of the whole genome – noncanonical chemosensory genes devoted to encoding the proteins of smell also make up a significant proportion of many genomes [30.11, 12]. This clear indication of the importance of the sense of smell and its underlying olfactory (sub)systems contrasts our, at present, still largely fragmentary conception of sensory signaling in many noncanonical olfactory cells and tissues.

Not too long ago, it was widely accepted that the mammalian olfactory system had only two anatomical and functional divisions: a main and an accessory (vomeronasal) olfactory system [30.9, 13]. In the 1970s, anatomical description of segregated parallel projections of the main and accessory systems to different *telencephalic* and *diencephalic* nuclei then founded the dual olfactory hypothesis [30.14]. Accordingly, the main system was believed to predominantly detect general environmental odor cues, whereas the accessory system and its peripheral sensory structure – the VNO – were considered to serve a crucial function in the detection and communication of social chemosignals that elicit stereotyped social and sexual behaviors and/or hormonal responses among conspecific animals [30.14, 15]. These two divisions of the olfactory system are by no means homogeneous [30.1]. The MOE contains various sensory neuron subpopulations that respond to distinct classes of chemostimuli, express different receptors – both *Gprotein-coupled receptors* (GPCRs) as well as non-GPCRs – and utilize a variety of transduction pathways. Similarly, the VNO contains at least three different groups of neurons that also vary in stimulus selectivity and transduction mechanisms.

As it has now become apparent that the organization of the sense of smell does not adhere to a strict anatomical and functional dichotomy, but is rather much more complex, several exciting questions now rank high on the agenda of (chemo)sensory neurobiologists:

1. Which receptor structures and signaling strategies are implemented by each different sensory tissue/cell population?
2. What coding logic underpins the anatomical segregation of the different subsystems in the nasal cavity?
3. How is subsystem-specific parallel information routed to and integrated by higher order circuits?
4. What is the exact role that each subsystem plays in regulating chemosensory-dependent behaviors?

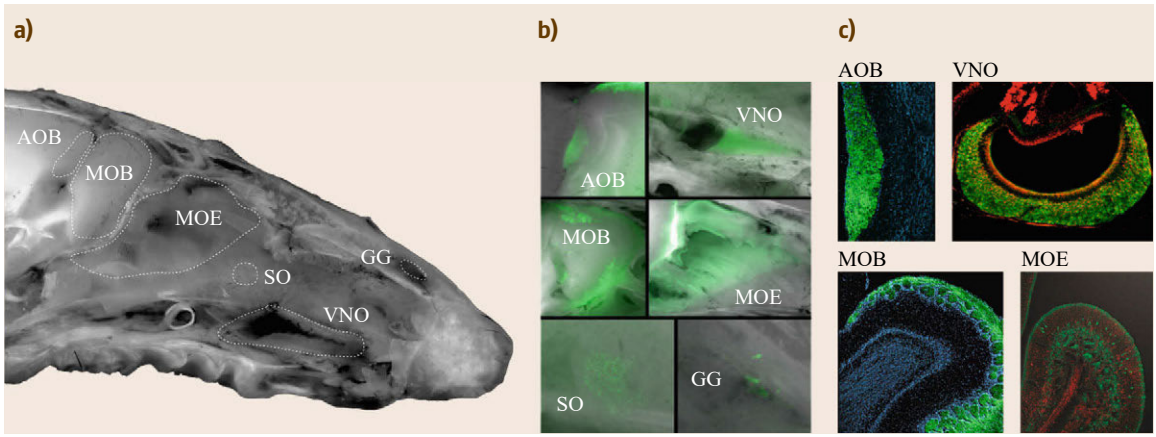

Fig. 30.1a–c Subsystem organization of the sense of smell. **(a)** Para-sagittal hemisection of the rostral head of a mouse that expresses the green fluorescent protein under transcriptional control of the olfactory marker protein promotor. **(b)** Merged *en-face* images under brightfield and epi-fluorescence illumination illustrate the different olfactory subsystems as well as their primary central projection areas. **(c)** Cryosections reveal GFP fluorescence of olfactory tissues: Grüneberg ganglion (GG), VNO, septal organ (SO), main olfactory epithelium (MOE), main olfactory bulb (MOB), accessory olfactory bulb (AOB)

30.2 The Vomeronasal System

In 1813, the Danish anatomist Ludvig L. Jacobson described a novel mammalian organ *located in the foremost part of the nasal cavity, in close contact with the septum, on palatal elongations of the intermaxillary bone* [30.16]. He concluded from comparative investigations in various species that *the organ exists in all mammals* and most likely functions as a *sensory organ which may be of assistance to the sense of smell* [30.16]. Originally designated as the *organ of Jacobson*, this enigmatic structure was (re)named in 1895 *organon vomeronasale* (*Jacobsoni*) or *VNO*.

The VNO is a bilaterally symmetrical paired tubular structure at the base of the anterior nasal septum, located just above the palate [30.17, 18] (Fig. 30.1). Enclosed in a bony capsule, the blind-ended organ opens anteriorly into either the oral or the nasal cavity via the nasopalatine or vomeronasal duct, depending on the species examined. Viscous lateral gland secretions fill the VNO lumen [30.1] which separates a medial crescent-shaped pseudo-stratified sensory neuroepithelium from lateral cavernous tissue that harbors a large blood vessel that is extensively innervated by nerve fibers from the superior cervical ganglion [30.19, 20]. The sensory epithelium is mainly composed of basal cells, sustentacular cells, and mature *vomeronasal sensory neurons* (VSNs). The small bipolar VSNs each extend an unbranched apical dendrite that ends in a microvillous swelling (*knob*) at the epithelial surface. At the basal soma, VSNs extend single unbranched axons that gather into nerve bundles that project below the septal epithelium, dorsally pass the cribriform plate, and project along the olfactory bulb's medial sides to the glomerular layer of the *accessory olfactory bulb* (AOB) [30.18]. The mouse VNO consists of a few hundred thousand VSNs in total [30.21]. Each neuron gains structural and metabolic support from sustentacular cells in the most superficial epithelial layer. Even in aged animals, VSNs are continuously replaced from a vomeronasal pool of adult pluripotent cells [30.22] that are located along the basal epithelial membrane as well as in the marginal zones [30.17].

How is VNO stimulus uptake achieved? In situations of stress and/or novelty-induced arousal, sympathetic activity triggers adrenergic release and, consequently, a peristaltic vascular pumping mechanism causes massive fluid entry into the VNO. This way, following direct contact with urine deposits, vaginal secretions, facial gland secretions or saliva, relatively nonvolatile stimuli, such as peptides or proteins gain access to the VNO lumen [30.23, 24].

Initially observed between embryonic days 12 and 13 in the rat [30.25], the vomeronasal neuroepithelium is embryologically derived from an *olfactory placode* evagination. As assessed by expression of the olfactory marker protein (OMP), mouse VSNs are first identified by embryonic day 14 [30.26]. The lateral vascular pattern is completed later in prenatal *ontogeny*, around embryonic day 18 [30.27]. At this stage, the vomeronasal nerve appears fully developed. While all structural vomeronasal components thus seem present at birth [30.28], VNO function in neonates and juveniles is still debated [30.29–31].

30.2.1 Molecular and Cellular Mechanisms of Vomeronasal Signaling

Vomeronasal Chemoreceptors

In most mammalian species, the VNO displays both a structural and functional dichotomy [30.31–33]. At least two topographically segregated neuronal subpopulations express distinct repertoires of receptors and other putative signaling molecules [30.34–36]. In the sensory epithelium's apical layer, VSNs express the G protein α-subunit $G_{\alpha i2}$ together with phosphodiesterase 4A (PDE4A) and a member of a multigene family that encodes class-A (rhodopsin-like) GPCRs: the V1Rs [30.37–39]. Vomeronasal receptor types 1 (V1R) appear exclusively expressed in the VNO [30.38] in a punctate, nonoverlapping pattern. The murine *V1r* gene family contains more than 150 potentially functional members and a similar number of pseudogenes [30.39, 40]. These genes are highly divergent and polymorphic. They share intron-free open reading frames that are found on most chromosomes in a clustered organization [30.40, 41]. Each of the 12 relatively isolated *V1r* families contains between 1 and 30 members [30.39, 42]. In a given VSN, *V1r* gene choice is tightly controlled. *Monoallelic expression* results in a distinct V1R chemodetector *morph* for each neuron [30.43] ensuring that individual VSNs obtain a unique functional identity. *Gene cluster* deletion of all (but one) *V1r*a and *V1r*b family members has shown that VSN chemoresponsivity critically depends on at least some V1Rs [30.44]. To date, however, the majority of *V1r* genes products remain *orphan receptors*. Remarkably, Catherine Dulac and coworkers recently determined sensitivity of ≈ 50 individual V1Rs to a variety of ethologically relevant cues [30.45]. Their data reveal that individual *V1r* subfamilies could have evolved toward the recognition of specific groups of animals or behaviorally relevant chemical structures.

The molecular mechanisms underlying monoallelic gene choice – a hallmark of *V1r* expression [30.40, 43, 46] – remain elusive. The interrupted cluster organiza-

tion of many *V1r* genes may not just reflect multiple gene-duplication events, but may also allow choice regulation to function at the cluster level [30.40]. As shown for mutually exclusive OR gene choice [30.47, 48], transcription of a nonfunctional *V1r* allele triggers coexpression of a second, functional *V1r* gene. This, in turn, then drives a negative feedback mechanism that maintains monoallelic expression. Remarkably, this negative feedback is also maintained when an exogenous OR gene is expressed from a *V1r* locus [30.41]. This surprising finding suggests a common mechanism of monoallelic chemoreceptor transcription in both olfactory sensory neurons (OSN) and VSNs.

A molecularly distinct population of neurons forms the basal VNO layer. Basal VSNs express $G_{\alpha o}$ and members V2R family of GPCRs [30.49–51]. Approximately 120 intact *V2r* genes are found in the mouse genome, whereas an additional ≈ 160 appear pseudogenized [30.52]. Members of the V2R family are typical class-C GPCRs that share a large hydrophobic N-terminal extracellular domain. This extracellular *venus flytrap* module has been attributed to function as the primary site of ligand binding [30.12]. While there is no apparent sequence homology with *V1r* genes, *V2r* gene family members are distantly related to metabotropic glutamate receptors, Ca^{2+}-sensing receptors, metabotropic glutamate receptors, and *T1r* taste receptors [30.12, 32]. Like the *V1r*s, *V2r* genes are mostly organized in clusters on many chromosomes. Based on sequence homology, four distinct *V2r* families are distinguished: family-A, -B, and -D, as well as family-C (a.k.a. *V2r2*) [30.52–55]. With more than 100 members, the vast majority of *V2r*s are family-A genes. By contrast, only four genes constitute family-D. With the exception of family-C receptors, mutually exclusive monoallelic transcription of *V2r* genes underlies a punctate expression pattern. By contrast, family-C receptors do not *obey* the chemosensory *one neuron – one receptor* rule [30.1, 5]. The seven highly homologous (> 80%) family-C proteins are found in most, if not all, $G_{\alpha o}$-positive VSNs [30.53]. Somewhat reminiscent of the atypical insect olfactory coreceptor Orco [30.56], this rather unusual coexpression of family-C *V2r* genes in basal VSNs could indicate chaperoning and/or dimerization function. Whether this holds true, however, remains to be determined [30.2].

H2-Mv (or *M10*) genes are members of a family of nine nonclassical class Ib *major histocompatibility complex* (MHC) genes that, as concurrently reported by two groups in 2003 [30.36, 57], are expressed by V2R-positive basal VSNs. Initially, H2-Mv proteins were believed to associate with V2Rs and, together with β2-microglobulin, serve as essential chaperones for V2R trafficking and surface expression [30.32, 36]. However, a considerable fraction of basal VSNs lacks *H2-Mv* gene expression [30.34] and while the *H2-Mv* family is exclusively found in rodents, other mammals such as the opossum express putatively functional *V2r* genes without intact *H2-Mv* genes [30.52, 58]. Moreover, a recent study suggests that H2-Mv proteins, while not absolutely essential for the generation of physiological responses, are required for ultrasensitive chemodetection by a subset of VSNs [30.35].

As for *V1r*s, gene deletion studies have provided direct evidence that at least some V2Rs mediate VSN chemosignals. Knockout of the *Vmn2r26* (*V2r1b*) or *Vmn2r116* (*V2rp5*) genes results in drastically diminished VSN sensitivity to the male-specific exocrine gland-secreting peptide 1 (ESP1 [30.59]) or MHC class I peptide ligands [30.60], respectively. Both stimuli, however, trigger highly sensitive VSN responses in wildtype mice.

With the notable exception of a recent study that reported promising advances in recombinant V2R plasma membrane targeting [30.61], V1R/V2R *deorphanization* attempts in *heterologous expression* systems have largely failed. We still lack a mechanistic understanding of V1R/V2R cell-surface expression.

In 2009, two groups identified a third family of five putative VNO chemoreceptors [30.62, 63]. These candidate chemosensory GPCR-encoding genes are all members of the formyl peptide receptor (FPR)-like gene family (*Fpr-rs1*, *rs3*, *rs4*, *rs6*, and *rs7*). Their predicted seven-transmembrane topology, their selective, punctate and *monogenic* vomeronasal expression pattern, and their localization in microvillous dendritic VSN endings [30.62, 63] strongly suggest a functional role of FPR-like receptors in vomeronasal chemosignaling. Interestingly, while *Fpr-rs1* is coexpressed with $G_{\alpha o}$ in basal sensory neurons, the remaining vomeronasal *Fpr-rs* genes all coexpress with $G_{\alpha i2}$ in the apical layer of the VNO neuroepithelium [30.8].

The two prototypical FPRs, FPR1 and FPR-rs2, are known for their function in immune cells, such as neutrophils and monocytes [30.64]. Here, both receptors serve crucial functions in host defense against pathogens [30.65]. A characteristic of immune system FPRs is their ligand promiscuity; a wide range of compounds are detected and function as leucocyte chemoattractants that signal direction toward sites of infection or tissue damage. Mitochondrially encoded peptides, the formylated bacterial peptide *N*-formylmethionine-leucine-phenylalanine (fMLF), and various other antimicrobial/inflammatory modulators activate immune system FPRs [30.66], suggesting functionally, rather than structurally defined ligand spectra. Interestingly, neither FPR1 nor FPR-rs2 was found transcribed in VSNs [30.62, 63].

Neither V1/2Rs nor ORs share significant sequence homology with immune system *Fpr*s or vomeronasal *Fpr-rs* genes. Liberles and coworkers suggested that vomeronasal *Fpr*s evolved from several recent gene duplications and positive Darwinian selection in the rodent lineage [30.62] since the single *Fpr-rs* gene cluster is directly adjacent to a stretch of more than 30 *V1/2r* genes. Together with recent functional data obtained from recombinant FPR expression [30.67], these theoretical considerations argue for neofunctionalization of vomeronasal *Fpr* genes. By contrast, VSNs are activated in situ by fMLF as well as the mitochondria-derived formylated peptides [30.68] and heterologously expressed FPR-rs proteins retain agonist spectra that share some similarities to immune system FPRs [30.63]. As true for most vomeronasal chemoreceptors, the exact biological role of vomeronasal FPRs remains to be determined.

Signaling Cascade(s) and General VSN Physiology

Gaining detailed conceptual insight into the transduction mechanisms downstream V1R, V2R, or FPR-rs receptor activation is central to understanding how chemosignals control social and sexual behavior. So far, translation of the chemical binding energy between any VNO receptor and its cognate ligand into a meaningful electrical signal that can be read out by the brain is only partly understood and many critical aspects of VSN physiology are yet to be revealed. Upon exposure to natural sources of semiochemicals such as urine, vaginal secretions, or saliva, VSNs depolarize, display an increased action potential firing rate and, consequently, a transient increase in cytosolic Ca^{2+} [30.62, 69–80]. A physiological hallmark of VSNs is their extraordinarily high *input resistance* of typically several gigaohms [30.81–84]. Consequently, receptor current amplitudes of only a few picoamperes generate robust trains of *action potentials*. This passive membrane property contributes considerably to the exquisitely high electrical sensitivity of VSNs, rendering vomeronasal neurons among the most sensitive sensory structures.

In addition to *conventional* Hodgkin-Huxley type voltage-dependent conductances, such as *TTX*-sensitive Na^+ and delayed rectifier K^+ currents [30.85], several other ion channels shape the electrophysiological input-output function of VSNs. Action potential discharge is, in part, driven by *L*- and *T*-type Ca_v^{2+} currents that generate low-threshold Ca^{2+} spikes [30.82, 84]. Moreover, functional coupling of these Ca_v^{2+} currents to large-conductance Ca^{2+}-sensitive K^+ (BK) channels was proposed to maintain persistent spiking [30.84]. By contrast, a similar coupling mechanism was suggested to underlie sensory adaptation via arachidonic acid-dependent BK channel recruitment during stimulation [30.86]. Another K^+ channel, the ether-á-go-go-related (ERG) K^+ channel, is expressed in a layer-specific and activity-dependent manner, serving a homeostatic function by controlling the output characteristics of basal VSNs [30.9, 81]. ERG channel expression is poised to adjust VSNs to a target output range in a use-dependent manner, thus, extending the dynamic range of the neurons' stimulus – response function. In addition, hyperpolarization-dependent I_h currents are yet another voltage-gated conductance that shapes VSN excitability [30.87].

Ever since the initial reports on layer-specific $G_{\alpha i2}$ and $G_{\alpha o}$ coexpression in dendritic tips of V1R- and V2R-positive VSNs, respectively [30.37, 88, 89], speculation thrived on the notion of a functional role of either G protein α-subunit in apical and basal signaling pathways, respectively. While being an attractive model, conclusive evidence in favor of this concept was only recently reported. *Chamero* and coworkers demonstrated an essential requirement of $G_{\alpha o}$ in VSN responses to MHC I antigens, MUPs, mitochondrially encoded FPR-rs1 ligands, as well as ESP1 [30.68]. However, neuronal responses to fMLF, a stimulus shown to activate at least some of the four $G_{\alpha i2}$-coupled FPRs [30.63], were unaltered in $G_{\alpha o}$-deficient mice [30.68]. Similar studies supporting a role of $G_{\alpha i2}$ in V1R-mediated signaling are currently lacking. As expected for unconditional knockout of abundantly and promiscuous signaling proteins such as $G_{\alpha i2}$ and $G_{\alpha o}$, constitutive gene deletion models are likely to bear a range of phenotypic defects. Accordingly, global $G_{\alpha i2}$ and $G_{\alpha o}$ deletions did not allow a clearly VNO-dependent phenotype to be unambiguously attributed [30.90, 91].

Despite the fact that genetic deletion studies are also lacking, greater consensus is achieved on a critical function of phospholipase C (PLC) in vomeronasal signal transduction [30.13]. PLC activity promotes turnover of phosphatidylinositol-4,5-bisphosphate (PIP_2), resulting in elevated concentrations of the soluble messenger molecule inositol-1,4,5-trisphosphate (IP_3) and membrane-bound diacylglycerol (DAG). While DAG either directly targets membrane proteins or is metabolized to tertiary polyunsaturated fatty acid signals, IP_3 triggers massive Ca^{2+} release from intracellular storage organelles. Over the years, different functions for either or all products of PLC-dependent lipid turnover have been proposed [30.73, 77, 80, 92, 93]. The common denominator of all such models is both a central role of cytosolic Ca^{2+} elevations and an important, though not indispensable function of the *transient receptor potential (TRP) channel* TRPC2 [30.94] that

is abundantly expressed in VNO sensory microvilli. Without a doubt, *TrpC2*$^{-/-}$ mice display severe defects in various social and sexual behaviors [30.95, 96]. There are, however, phenotypic differences when *TrpC2* deletion models are compared to surgical VNO ablation [30.97, 98]. Moreover, evidence for (residual) urine-evoked activity in *TrpC2*$^{-/-}$ VSNs is accumulating [30.73, 93, 97], adding another layer of complexity to our current concept of vomeronasal signaling. Interestingly, a recent study documents two previously unrecognized types of *Trpc2*$^{+}$ neurons in the MOE of mice of various ages, thus, challenging the conventional VNO-centric interpretation of the behavioral phenotypes of *Trpc2* knockout mice.

A variety of signaling cascade proteins are, either directly or indirectly, subject to Ca^{2+}-dependent modulation. Therefore, transient or sustained cytosolic Ca^{2+} elevation, resulting from either TRPC2-dependent influx [30.77] and/or IP_3-mediated store depletion [30.73, 93], exerts both negative and positive feedback regulation in VSNs [30.7]. VSN sensory adaptation and gain control was shown to depend on down-regulation of TRPC2 by Ca^{2+}/calmodulin [30.99]. By contrast, a substantial portion of stimulus-evoked vomeronasal activity seems to be carried by a Ca^{2+} -activated chloride current (I_{Cl}) [30.73, 92, 93], resembling OSN transduction mechanisms [30.100, 101]. Vomeronasal I_{Cl} might be mediated by members of the *anoctamin* family of Ca^{2+} -activated chloride channels [30.102–104]. Microvillar colocalization with TRPC2 suggests that either anoctamin1 or anoctamin2, or both, could represent the vomeronasal Ca^{2+}-activated chloride channel(s) [30.92, 105–107]. However, caution should be exerted when interpreting signaling cascade similarities between VSNs and OSNs. Whether I_{Cl}, analogous to OSNs, contributes to depolarization or, by contrast, induces membrane hyperpolarization depends solely on the chloride equilibrium potential at the VSN microvillar membrane in vivo. This physiological parameter, however, is currently unknown.

30.2.2 Anatomy of the Accessory Olfactory System

Compared to many other sensory systems, both the anatomy and function of VNO-dependent neural circuits is poorly investigated. In general, sensory information in mammals is primarily processed by cortical neurons that allow for associative learning and enable a plastic stimulus response, both within and across individuals. By contrast, semiochemical-dependent behavior seems invariant and highly conserved among individuals. Accordingly, the VNO primarily activates noncortical circuits. Specifically, semiochemicals predominantly activate limbic networks that utilize more hardwired mechanisms than the cortex [30.14, 18]. Furthermore, vomeronasal stimulus processing is considered to follow a relatively *simple* logic. Between sensory input and output command, the circuits activated by VNO stimulation consist of three primary processing relays: the AOB, *amygdala*, and *hypothalamus* [30.108]. Whether and, if so, how circuit processing of vomeronasal stimuli is controlled by modulatory feedback mechanisms remains to be determined.

The Accessory Olfactory Bulb

After they passed through the *cribriform plate*, VSN axon bundles target the AOB, a distinct region at the dorso-caudal end of the olfactory bulb (Fig. 30.1). The two functional subsets of VSN axons project into two distinct AOB regions, thus, maintaining the anatomical dichotomy of the accessory olfactory system [30.7]. Apical V1R-positive neurons express the olfactory cell adhesion molecule (OCAM) but project to OCAM-negative second-order output neurons, mitral cells, in the rostral AOB, whereas basal V2R-positive VSNs lack OCAM expression and synapse with OCAM-positive mitral cells in the caudal AOB [30.109, 110]. Similar inverse OCAM expression has been reported in subsets of OSN axons and main bulb mitral/tufted cells [30.111].

VSNs with a distinct vomeronasal receptor (VR) chemodetector *morph* [30.43] converge onto mitral cell dendrites in 6–10 *glomeruli* that are scattered across broad AOB regions, but restricted to either the rostral or caudal part depending on receptor identity [30.112]. While some glomeruli appear exclusively innervated by neurons of a single VR *morph* [30.112], others were found to coalesce with different, but closely related, VSN axons [30.113]. While the jury is still out on the exact organizational rules that govern VSN-to-mitral cell connectivity in the AOB, the general glomerular organization likely serves to integrate incoming sensory information [30.43, 46].

As correctly noted by *Dulac* and *Wagner*, most basic properties of AOB biology remain almost unexplored. Thus, functional analogies with described main bulb circuitry is mostly speculative [30.114]. Unfortunately, premature extrapolation of anatomical principles and physiological mechanisms from the main to the AOB has seriously hampered an unbiased assessment of AOB neurobiology. Already in 1901, Ramon y Cajal pointed out several fundamental differences between the main to the accessory bulb [30.115]:

1. AOB glomeruli are relatively small, rather confluent and less defined.

2. The *external plexifom layer* in the AOB is, at best, rudimentary.
3. Projecting neuron somata in the AOB are rarely mitral shaped.

Conventional plexifom layers are, in fact, largely missing [30.116]. Instead, an internal and external cellular layer are separated by fiber bundles of the lateral olfactory tract [30.115]. Furthermore, AOB glomeruli are indeed small, though highly variable in size (10–30 µm diameter [30.117]). They are tightly clustered, surrounded by only a few periglomerular cells and innervated by a few hundred glutamatergic VSN axons terminals [30.118].

To date, two basic mitral cell connectivity models for glomerular information processing are discussed. The homotypic connectivity model suggests exclusive targeting of glomeruli that are innervated by VSN populations that share a VR *morph*, effectively rendering a divergent pattern of sensory axonal projections convergent by second-order AOB neurons [30.30, 112]. According to the heterotypic wiring scheme, mitral cell primary dendrites specifically target glomeruli that are innervated by VSNs expressing receptors of the same subfamily [30.113]; thus forming a glomerular map of subfamily-specific domains [30.114].

While lacking an extensive lateral dendritic tree, AOB mitral cells elaborate extensively branched primary dendrites. These dendrites terminate as multiple tufts in up to 12 different glomeruli [30.19, 113, 119–121] although the tufts' size and shape are anything but uniform. Interestingly, local regenerative *tuft spikes* were observed independently of somatic spikes, suggesting modes of local nonlinear synaptic input integration [30.121]. In addition, dendritic transmitter release in response to both subthreshold and suprathreshold excitation [30.122] as well as active backpropagation of sodium spikes from the soma [30.123] increase the computational power of individual mitral cells within the AOB network. Considering their output, AOB mitral cells are relatively slow with maximum firing frequencies < 50 Hz [30.124]. While this upper limit somewhat restricts the dynamic output range, the high density of axon collaterals that project to both the anterior and posterior AOB [30.115] ensures that activity will be transmitted throughout the network, providing an ideal circuit for synchronized output.

Similar to the main olfactory bulb, GABAergic granule cells build the main type of AOB *interneurons* [30.125]. They form synapses with mitral cells both within the glomerular layer and along proximal parts of mitral cell primary dendrites [30.126].

While thoroughly analyzed in the main olfactory bulb [30.127, 128], the reciprocal *dendrodendritic synapse* in the AOB has received considerably less attention [30.129–131]. Located along the basal segments of mitral cell dendrites, dendrodendritic AOB synapses primarily seem to provide relay-like recursive self-inhibitory feedback which sharpens *projection neuron* response selectivity [30.132] and might, thus, function as a sensory *gate* during learning and olfactory memory formation [30.129, 133–135].

30.2.3 Pheromones

Pheromones (*pherin* = to transfer and *hormone* = to excite), per definition, are conspecific chemosignals that elicit a specialized behavior or neuroendocrine response upon detection by members of the same species [30.136]. When the term was coined, Karlson and Lüscher most likely did not foresee that more than 50 years later there would still be a lively and controversial debate about the biological role and even the existence of pheromones [30.137–139]. Concerning the vomeronasal system, it is clear that VSNs detect ethologically relevant odors that other animals emit. These include pheromones and other semiochemicals as well as *kairomones*, heterospecific ligands that upon detection convey an ethological benefit to the receiver. In addition, in vitro studies also showed VSN responses to odors of unknown biological function that are not secreted by other animals [30.7, 140, 141].

While the chemical identity of vomeronasal stimuli, to a large extend, remains to be uncovered, different putative semiochemicals have been identified and can be roughly categorized as follows:

1. Major urinary proteins (MUPs)
2. MHC class I peptide ligands
3. Exocrine gland-secreting peptides (ESPs)
4. Sulfated steroids
5. Formylated peptides
6. Volatile small molecules.

MUPs, MHCs, and ESPs are peptides/small proteins that evolved by species-specific gene duplication events in the rodent lineage [30.142, 143]. For members of each peptide/protein class recognition by basal V2R-positive VSNs has been reported [30.69, 76, 142, 144, 145]. Sulfated steroids and other secreted small molecules appear to signal state- or gender-specific internal physiology [30.78, 146]. These stimuli are predominantly detected by apical neurons expressing members of the V1R receptor family [30.75, 147]. Formylated peptides as well as other pro- or anti-inflammatory disease-associated compounds were proposed to activate FPR-rs receptors [30.63]. In-

deed, these ligands activate both apical and basal VSNs [30.68].

Twenty-one species-specific genes constitute the mouse MUP family [30.143, 148]. MUPs are produced in the liver in a testosterone- and growth hormone-dependent manner primarily by adult males [30.149, 150]. As members of the lipocalin protein family, MUPs fold into a β-barrel that creates an internal binding pocket, which efficiently binds small molecules [30.151]. Therefore, MUPs have initially been attributed a carrier function for small volatile ligands to facilitate their transmission to the VNO lumen and delay their release after environmental excretion [30.151]. Today, it has become increasingly clear that a *tailored* urinary MUP excretion of only 4–12 protein types per individual carries some identity information. Moreover, MUPs alone, without associated small molecules, stimulate basal V2R-positive VSNs [30.69, 79, 145], thereby promoting such different behaviors as male territorial aggression [30.69, 145], female conditioned place preference [30.152, 153], or, if emitted by heterospecifics, innate fear [30.79].

Somewhat similar to formylated peptides, MHC class I peptide ligands are another example of chemosensory signaling components with a prominent immune system function [30.154]. These relatively small peptides are detected by both V2R-positive basal VSNs as well as neurons in the MOE [30.60, 76, 155]. MHC peptides are part of the urinary *peptidome* [30.156] and, as such, they are stably secreted throughout life, providing a potential means for representation of individuality. Strikingly, MHC class I peptide ligands were the first and so far the only molecularly defined chemosignals shown to mediate selective pregnancy block (or *Bruce effect*), which is associated with the formation and maintenance of an olfactory recognition memory by the vomeronasal system [30.76, 157].

A total of 38 mouse genes encode ESPs [30.74]. These peptides are emitted in lacrimal, Harderian, and submaxillary gland secretions and differentially expressed between genders and across development [30.74, 142]. Two ESPs were studied in detail, ESP1 and ESP22. Both are found in male tear fluid and activate basal VSNs with high selectivity [30.142, 144]. While ESP1 expression is male-specific, expression of ESP22 is age-dependent. Both peptides induce robust, though very different behavioral responses in females and males, respectively: ESP1 stimulates lordosis, a receptive female mating posture [30.59], whereas ESP22 is a juvenile mouse pheromone that inhibits sexual behavior in adult male conspecifics [30.144].

Volatile urine-enriched gender-specific bioactive ligands were identified by means of gas chromatography-mass spectrometry. Among the described urinary constituents were several vomeronasal in vitro ligands such as 2-sec-butyl-4,5-dihydrothiazole, 3,4-dehydro-exo-brevicomin, 6-hydroxy-6-methyl-3-heptanone, α/β-farnesene, 2-heptanone, isoamylamine, and others [30.146, 158, 159]. Interestingly, these compounds bind MUPs [30.160], are able to directly activate VSNs at extraordinarily low concentrations [30.75], and promote behavioral responses such as puberty acceleration or delay in females [30.161, 162].

30.3 The Septal Organ

The septal organ of Masera and the Grüneberg ganglion (Sect. 30.4) are undoubtedly the most enigmatic olfactory subsystems in the mammalian nose. First described in detail in 1943 by the Italian anatomist *Rodolfo-Masera* [30.163], chemosensory research had neglected the *Organ of Masera* for a long time. Accordingly, few aspects of the SO physiology have been elucidated and various basic questions about the organ and its functional relevance remain unresolved.

In mice, the SO is a small, isolated patch of sensory neuroepithelium that, surrounded by respiratory epithelium, is bilaterally located near the base of the nasal septum close to the nasopalatine duct openings (Fig. 30.1). The SO epithelial organization closely resembles the structure of the MOE. However, the generally thinner neuroepithelium is composed of only up to three layers of ciliated bipolar OMP-expressing OSNs (compared to six to eight layers in most regions of the MOE) [30.3]. Entering the brain through a defined perforation of the cribriform plate, axons of SO neurons project exclusively to a small subset of glomeruli in the main olfactory bulb [30.1]. Approximately 30 glomeruli, clustered at the ventromedial aspect of the bulb, receive substantial synaptic input from the SO and a few of these glomeruli might actually be exclusively innervated by SO neurons [30.164].

Gene expression profiling in the SO provided evidence for a canonical signaling machinery expressed in SO neurons. While a very small subset of cells appear to express both GC-D and PDE2 (Sect. 30.5.2), the majority SO sensory neurons express canonical odorant receptors, $G_{\alpha olf}$ and adenylate cyclase III [30.165, 166]. Results from microarray analysis and large-scale reverse transcription polymerase chain reaction exper-

iments suggest that not the entire repertoire of ORs is present in SO neurons. Rather a distinct group of 50–80 OR genes, all class II receptors of various families, are expressed in both the MOE and SO [30.167, 168]. Interestingly, OR expression in the SO is not randomly distributed. While apparently abiding by the *one neuron – one receptor* rule, > 90% of SO neurons express one of only nine ORs [30.168]. SR1 (also known as *mOR256-3*) is by far the most abundant receptor found in ≈ 50% of all SO neurons. OSNs that express SR1 respond to many, structurally unrelated odorants over a wide concentration range, whereas OSNs expressing a gene-targeted SR1 locus that lacks the SR1 coding sequence do not show this broad responsiveness [30.169]. The *broad tuning profile* of this specific, most abundant SO receptor likely reflects early reports of broad general odor sensitivity of the SO [30.170].

What functional role is played by the SO? Its unique strategic location in the nasal cavity has prompted speculation about a function as an alert sensor during quiet respiration, when air flow does not reach the entire MOE [30.165]. Others have suggested that the SO may detect compounds of low volatility [30.24]. Whatever its exact role(s), the SO has evolved as a unique *outpost* of the olfactory system that is likely involved in a specific chemosensory function.

30.4 The Grüneberg Ganglion

In 1973, *Grüneberg* discovered an arrow-shaped *ganglion of unknown function* [30.171] at the anterior end of the nasal cavity [30.3]. It bilaterally lines the rostro-dorsal nasal septum close to the opening of the naris [30.172] (Fig. 30.1). Originally considered to constitute a part of the *Nervus terminalis*, the GG was ignored for decades. However, the lack of terminal nerve marker expression and more recent anatomical and functional evidence suggest that the GG represents an independent chemosensory subsystem. Since its *rediscovery* a few years back, the ganglion's peculiar anatomy and elusive physiology have sparked new interest within the field of chemosensory neuroscientists.

GG neurons express OMP and project axonal processes to glomeruli in defined olfactory bulb areas [30.172, 173]. However, GG neurons are devoid of cilia or microvilli that represent hallmarks of other chemosensory neurons in the nose. Moreover, light and scanning electron microscopy show that the GG lacks direct access to the nasal cavity [30.1, 174]. This has fueled speculation about a function as a detector of gaseous or other highly membrane permeant stimuli [30.1]. By contrast, studies have reported expression of several chemosensory receptors in GG cells, including V2Rs and TAARs [30.175, 176] as well as elements of a cyclic guanosine 3',5'-monophosphate (cGMP) pathway [30.177]. GG neurons project along the dorsal nasal septum and medial olfactory bulb surface to dorso-caudal regions near the AOB [30.172, 178] that somewhat overlap with the area occupied by the necklace glomeruli (see below).

A sensory function of the GG subsystem has been proposed on the basis of behavioral data [30.174] and later demonstrated by physiological recordings from GG cells [30.179–181] as well as immediate early gene activity assays [30.182, 183]. So far, however, different studies/approaches have yield rather controversial results. Thus, further physiological investigation is required to unify our concept of GG chemosignaling.

30.5 Noncanonical Olfactory Signaling Pathways in the Main Olfactory Epithelium

More than two decades ago, the discovery of the rodent OR gene family by *Linda B. Buck* and *Richard Axel* marked the beginning of the molecular era of chemosensory research [30.10]. As such, this landmark finding was a watershed event for understanding olfaction. Since 1991, however, a number of additional studies have greatly expanded the classes of receptor genes and proteins implicated in chemosensory signaling. Compared to major advances in understanding canonical olfactory signaling, our current concept(s) of chemodetection mechanisms, signaling pathways, and information coding principles in OR-independent olfaction are still in their infancy. We are only just beginning to unravel the secrets of noncanonical chemosignaling.

30.5.1 Trace Amine-Associated Receptor (TAAR)-Expressing Neurons

Given the vast dimensionality in *odor space* [30.184, 185], the growing appreciation for parallel processing of socially relevant odors by both the main and accessory olfactory system [30.13], as well as the

discovery of different chemoreceptor families in the VNO [30.38, 49–51], it was tempting to speculate that canonical ORs might not represent the only GPCR family serving as MOE chemosensory receptors. In 2006, *Liberles* and *Buck* identified expression of members of the trace amine-associated receptor (TAAR) family in OSN-enriched mouse cDNA samples [30.186]. Like ORs, TAARs appear to be monoallelically expressed in sparse, nonoverlapping subsets of mouse OSNs and localized both in cilia, the site of odor detection, and in axons, where they may serve a guidance function [30.186, 187]. Early on, these properties strongly suggested an olfactory role. Phylogenetically, *Taar* genes are related to other aminergic GPCRs such as metabotropic dopamine and serotonin receptors [30.188]. While $\approx 25\%$ of ORs are suspected pseudogenes [30.189], only one *Taar* gene, *Taar7c*, appears pseudogenized, suggesting that the burden of pseudogene selection is significantly lower in TAAR-expressing neurons [30.187]. Moreover, the *Taar* repertoire is evolutionarily retained in mammals and many intact *Taar* genes are also found across diverse vertebrate genomes, from zebrafish to humans [30.186, 190] further substantiating a common, rather than species-specific olfactory role that is not met by the much larger repertoire of canonical ORs.

Apparently, the expression of ORs and TAARs is mutually exclusive since fluorescence in-situ hybridization studies did not identify OSNs coexpressing both receptor types [30.186]. The regulatory logic of *Taar* expression, however, is different from OR gene choice. *Taar* genes lack the epigenetic signature of OR selection. Moreover, knockout of a specific *Taar* allele resulted in frequent expression of a second *Taar* without silencing the deleted allele [30.187]. These differences are further substantiated by recent findings indicating that TAAR neurons form a sensory neuron population restricted to *Taar* expression prior to initial receptor gene choice [30.191].

Both ORs and TAARs transduce chemostimuli through a signaling mechanism that employs G proteins and an increase in cAMP [30.1]. As initially predicted [30.188], mouse TAARs respond to biogenic amines such as isoamylamine, 2-phenylethylamine and trimethylamine (activating mTAAR3, mTAAR4, and mTAAR5 proteins, respectively). TAAR5 was shown to mediate a species-specific attraction response to trimethylamine. Interestingly, phylogenetic analysis of related rodents suggests that synchronized evolution of trimethylamine biosynthesis pathways and odor-evoked behavioral responses could ensure species-appropriate social interactions [30.192]. Further evidence for a selective function of olfactory TAARs as detectors of socially and/or behaviorally relevant odors has been provided by Ferrero and coworkers [30.193]. Heterologously expressed TAAR4 is activated by carnivore urine and the TAAR4 ligand 2-phenylethylamine enriched in urine samples from carnivore species. Furthermore, this predator-derived cue promotes innate avoidance behavior and increases stress hormone release [30.193]. The exquisite sensitivity of TAAR4 for 2-phenylethylamine gave rise to an alternate (though by no means mutually exclusive) interpretation – that this phylogenetically distinct class of aminergic receptors is simply required for high-sensitivity detection of amines: innately aversive odours [30.191].

The majority of TAAR neurons project to a discrete cluster of glomeruli in a confined bulb region between the previously characterized DI and DII domains in the dorsal olfactory bulb of the mouse [30.191]. In vivo glomerular imaging in this region confirmed that dorsal TAAR glomeruli are selectively activated by volatile amines at low concentrations and further revealed that aversive amines are represented in a nonredundant fashion [30.194].

Together, these recent findings all strongly suggest that TAAR neurons constitute a distinct olfactory subsystem with unique molecular and anatomical features. The TAAR subsystem may thus provide a hard-wired, genetically and anatomically distinct, parallel input stream in the main olfactory pathway that is specialized for the detection of volatile amines [30.191].

30.5.2 Receptor Guanylyl Cyclase-D (GC-D)-Expressing Neurons

The repertoire of olfactory receptors is not restricted to GPCRs. A small percentage of OSNs express neither ORs nor TAARs, but a type-D receptor guanylyl cyclase (GC-D) [30.195]. Receptor GCs are expressed in many tissues of numerous species. They include both orphan and peptide receptors [30.196]. Initially isolated from sea urchin sperm, receptor GCs serve diverse functions including marine invertebrate sperm chemotaxis, regulation of diuresis, transduction in mammalian photoreceptor cells as well as nematode chemosensation [30.196–198]. Receptor GCs share an evolutionary conserved structure: an extracellular receptor domain is coupled by a single transmembrane helix to an intracellular regulatory (kinase homology) and a catalytic domain [30.196, 197]. Ligand binding to the extracellular receptor domain – in mammals, these ligands are mostly natriuretic peptides [30.197] – triggers cyclase domain activity and, consequently, the elevation of intracellular cGMP.

For years, the functional role of GC-D in olfaction has remained mysterious. OSNs that express the GC-D protein also express a variety of putative signaling pro-

teins that could be involved in a cGMP-dependent transduction cascade, for example, the cGMP-gated cyclic nucleotide-dependent channel subunit CNGA3 or the cGMP-stimulated phosphodiesterase PDE2 [30.199, 200]. By contrast, GC-D$^+$ neurons lack many components of the canonical olfactory signaling pathway [30.198, 201]. The phylogenetic conservation of *Gucy2d*, the GC-D encoding gene, across many mammalian species suggests a common olfactory function. As for most vomeronasal receptors, however, humans and other primates are a notable exception.

Do GC-D neurons respond to *conventional* odors or other chemostimuli? And, if so, is GC-D itself the cellular receptor? Studies in gene-targeted mice and heterologous expression systems strongly suggest that GC-D neurons are critical for the detection of natriuretic peptides and/or gaseous stimuli [30.202–205]. As regulators of fluid balance in the kidney and intestine, uroguanylin and guanylin activate enterocytes via guanylyl cyclase C (GC-C), which leads to cGMP-dependent inhibition of Na^+/H^+ exchange and activation of the cystic fibrosis transmembrane regulator [30.206]. Both peptide hormones induce responses in the MOE, even in recordings from *Cnga2* null mice (which are often referred to as *anosmic* as these animals lack a CNG channel subunit essential for canonical OSN signaling). This peptide-induced activity, however, is completely abolished when mice lack either *Gucy2d* or *Cnga3*, the gene encoding the cGMP-dependent CNG channel subunit [30.204]. Single cell recordings from identified GC-D neurons confirmed their sensitivity to uroguanylin and guanylin as well as to diluted urine samples. Strikingly, GC-D neurons showed exquisite peptide sensitivity with half-maximal neural activity stimulated by peptide concentrations as low as 66 pM.

Small gaseous molecules have also been shown to stimulate GC-D neurons. Initially, the small population of *Gucy2d*-expressing olfactory neurons was proposed to function as a CO_2 sensor [30.203]. Coexpression of GC-D and carbonic anhydrase type II indicates that enzymatic catalysis of CO_2 by carbonic anhydrase might characterize this noncanonical olfactory signaling pathway. A few years later, another gaseous molecule, carbon disulfide (CS_2), was found to stimulate GC-D$^+$ OSNs. Using gene-targeted mice, *Munger* and coworkers showed that both chemosensory responses to CS_2 and CS_2-dependent socially transmitted food preferences are drastically reduced in mice lacking GC-D, CNGA3, or carbonic anhydrase type II [30.205]. Their findings indicate that GC-D$^+$ OSNs detect chemosignals that facilitate food-related social interactions via associative olfactory learning.

GC-D neurons not only display unique signaling protein expression, but are also distinct in their glomerular projection pattern. Necklace glomeruli, a peculiar and still poorly defined chain-like band of glomeruli that run between the main and the accessory bulb, are the sole olfactory bulb targets of GC-D$^+$ OSNs [30.207–209]. In contrast to the apparently homogeneous innervation of glomeruli from canonical OSNs, each necklace glomerulus receives heterogeneous innervation from at least two sensory neuron populations: GC-D neurons and an OMP$^+$/GC-D$^-$ neuron population [30.207]. This disparate functional topology underscores the notion that very different coding principles describe both subsystems.

30.6 Olfactory Subsystems in Humans?

Do functional olfactory subsystems exist in humans? Especially the role of a human VNO, with respect to a potential biological significance of proposed human pheromones, has been controversially discussed both within the field as well as in a more popular scientific context. So far, not a single human pheromone has been chemically identified [30.137, 139]. Among the many reasons for that are the inherent difficulties to identify robust and reproducible effects when working with human subjects [30.210]. As recently pointed out by Peter Brennan, this does not necessarily mean that human pheromones do not exist, but complexities of modern human society may diminish their biological significance and make it difficult to identify consistent effects [30.211]. So, may human pheromones yet be found in the future? Candidate sources of such chemosignals are axillary sweat, areolar secretion of lactating women, and tear fluid [30.212–214]. The existence of human pheromones as constituents of such complex bodily secretions is, for instance, suggested by odor-mediated menstrual synchrony in female roommates which was observed in some studies, but not in others [30.4]. Whatever the molecular nature and physiological function of any such behaviorally relevant chemical, designation as a *pheromone* might be particularly problematic in humans. Probably, as has been proposed by Wyatt, the term *signature odor(s)* might be a more useful classification [30.138, 139].

If human signature odors exist, they are not detected and processed by an accessory olfactory system. While an embryonic structure that somewhat resembles a VNO is present early in human fetal devel-

opment, anatomical evidence shows that any residual structure that has been proposed as the adult human VNO – the *vomeronasal pit* – is clearly nonfunctional [30.215–217]. The vomeronasal pit is an epithelial diverticulum in the adult human nasal septum that does not have a similar structure or function to the rodent VNO [30.157]. Cells in the pit do not express OMP [30.218], the signature protein of mature olfactory/vomeronasal neurons in other mammals [30.219]. Moreover, while vomeronasal nerves appear to play a vital role during human fetal development in guiding LHRH neuron migration to the hypothalamus [30.157], no axonal connections to higher brain centers remain in the adult [30.215, 216]. These anatomical findings are corroborated by an overwhelming amount of molecular evidence. Almost all genes encoding for rodent vomeronasal receptors and VNO-specific transduction proteins are pseudogenes in humans. All but five *V1r* orthologs [30.220], all *V2r*s, vomeronasal *Fpr*s, and *H2-Mv* genes, as well as *TrpC2* [30.221] are nonfunctional in the genomes of both humans and Old World monkeys. The relaxation of selective pressure on the *TrpC2* gene, and probably other VNO-specific genes, occurred approximately 23 million years ago, coincident with the acquisition of trichromatic color vision in the common ancestor of Old World monkeys and apes [30.222]. Moreover, gene families that encode important vomeronasal peptide/protein stimuli in rodents, such as MUPs and ESPs, are also absent from the human genome.

More evidence for the lack of an accessory olfactory system in humans comes from anatomical studies in the central nervous system. While an AOB of different size and degree of differentiation is found in most adult nonaquatic mammals, this structure is absent in Old World monkeys, apes, and humans [30.223]. Quite similar to the transient embryonic development of a fetal VNO, a well-developed fetal AOB that regresses in later stages of development has been reported in humans and apes [30.32].

While we know little about human signature odors and remnants of a VNO, hardly any evidence is currently available for (or against) the existence of other olfactory subsystems in humans. Although Grüneberg reported in his original publication that a Grüneberg ganglion is found in human embryos [30.171], the organ likely regresses during fetal development [30.210]. Likewise, a human septal organ has not been found. For noncanonical subpopulations of OSNs, it is clear that at least five members of the *Taar* gene family are potentially functional in humans [30.4]. Whether TAARs are expressed in neurons of the human olfactory epithelium, however, is not clear. For the other main class of noncanonical OSNs, the GC-D neurons, it has been shown that GC-D is also pseudogenized in primates, and, in addition, human necklace glomeruli have not been described [30.210].

30.7 Glossary

- *Accessory olfactory bulb (AOB)*: Structure of the olfactory forebrain in the dorsal posterior region of the olfactory bulb. All VSN axons terminate in the AOB where they form synapses with second-order mitral cells.
- *Action potential*: Short-term change in membrane potential in response to stimulation; also known as *nerve impulse* or *spike*; a neuron that emits an action potential (or *trains* of action potentials) is often said to *fire*.
- *Amygdala*: Structure in the forebrain that is an important component of the limbic system and plays a central role in emotional learning.
- *Anoctamins*: The anoctamin (TMEM16) family of membrane proteins are, at least in part, Ca^{2+}-activated Cl^{-} channels; the term *anoctamin* was coined as these channels are anion selective and have eight transmembrane segments.
- *Broad/Narrow tuning profile*: The sensitivity range of a sensory neuron that significantly changes its action potential discharge in response to either a wide range of different stimuli (broad tuning) or a very precise subset of stimuli (narrow tuning).
- *Bruce effect*: Termination of pregnancy by chemosensory cues (pregnancy block); occurs when a recently impregnated mouse aborts her litter in response to chemosensory cues from an unfamiliar male.
- *Chemotopy*: Physical distribution of neurons/glomeruli on the surface of the olfactory bulb with respect to their individual receptive fields for odorants.
- *Cribriform plate*: A sieve-like structure between the anterior cranial fossa and the nasal cavity; part of ethmoid bone that supports the olfactory bulb; perforated by foramina for the passage of olfactory nerve fibers.
- *Dendrodendritic synapses (in MOB/AOB)*: Reciprocal synapses between two dendrites (here, from a mitral and granule cell); in contrast to axodendritic

synapses that are polarized from an axonal bouton onto a dendrite.

- *Deorphanization*: The process, which results in the identification of a natural ligand acting on an orphan receptor.
- *Diencephalon (Diencephalic, adj.)*: Posterior part of the forebrain that connects midbrain with cerebral hemispheres; encloses the third ventricle, and contains the thalamus and hypothalamus, and associated areas.
- *G Protein-coupled receptor (GPCR)*: Large superfamily of receptors with a characteristic seven-transmembrane topology; binding of extracellular ligands (or activation by light in cases of *opsins*) activates heterotrimeric intracellular G protein signaling cascades; typical GPCRs are receptors for hormones, neurotransmitters, visual, and chemosensory (olfaction and taste) stimuli.
- *Ganglion*: A nerve cell cluster or a group of nerve cell bodies located in the peripheral nervous system.
- *Gene cluster*: Groups (clusters) of two to several dozens of genes belonging to the same gene family; the vast majority of OR, V1R, and V2R genes in mice are found in clusters scattered throughout the genome.
- *Glomerulus (Glomeruli, pl)*: Specific structure/functional unit of spherical neuropil in the outer layers of both the main and accessory olfactory bulb; glomeruli consist of synapses of OSN/VSN axons with apical dendrites of mitral, tufted, and periglomerular cells; they segregate and organize synaptic inputs and, thus, form an olfactory topographic map (*chemotopy*) that allows the interpretation of transmitted chemical signals to the brain.
- *Heterologous expression*: Expression of a gene (or part of a gene) in a host cell or organism, which does not endogenously express this gene or gene fragment.
- *Hypothalamus*: Complex brain structure composed of many nuclei with various functions; regulator of internal organ activities by monitoring information from the autonomic nervous system, controlling the pituitary gland, and regulating sleep and appetite.
- *Input resistance*: The input resistance of a neuron reflects the extent to which membrane channels are open; it is defined as the change in voltage associated with injection of a current (divided by the input current); an increase in input resistance means a greater change in membrane potential in response to a current, thus rendering a neuron more excitable.
- *Interneurons (in MOB/AOB)*: Periglomerular and granule cells, inhibitory neurons of both the main and accessory olfactory bulb; both function to inhibit mitral cells via feed-forward and feedback reciprocal dendrodendritic synapses.
- *Kairomones*: Chemosignals transmitted between species (interspecific chemical cues) that benefit a member of another species without benefitting the emitter. For example, the presence of a predator might be signaled by kairomones.
- *Major histocompatibility complex (MHC)*: Group of genes that code for cell surface proteins that *present* both endogenous and exogenous protein fragments to cells of the immune system; recognition of foreign substances triggers an immune response; MHC proteins are found in all higher vertebrates; the human MHC is frequently referred to as the human leukocyte antigen (HLA) system.
- *Monoallelic expression*: By default, both alleles of a gene are actively transcribed (biallelic expression); in few cases, however, a single allele of a given gene is expressed (X-linked genes in females as a result of X chromosome inactivation).
- *Monogenic expression*: Exclusive expression of a single gene (or pair of allelic genes) from a family of related genes.
- *Olfactory placode*: Thickening of ectoderm that arise through cell division during neural tube formation; the olfactory system is one peripheral nervous system component that arises from paired sensory placodes during development; olfactory placodes give rise to OSNs, supporting and basal cells of the olfactory epithelium [30.224].
- *Ontogeny*: Origin and development of an individual organism from embryo to adult.
- *Orphan receptors*: Receptors for which no ligand is known.
- *Pheromones*: Molecules used for conspecific chemical communication (intraspecific chemical cues). Originally defined by Karlson and Lüscher as chemicals that are released by one member of a species causing specific reactions in other members of the same species [30.136].
- *Plexiform layer*: Meticular layer (of the retina or the olfactory bulb) mostly consisting of nerve cell processes (neuropil) and situated between layers of cell bodies.
- *Projection neuron*: Mitral cell in the MOB and AOB (and/or tufted cell in the case of the main olfactory bulb); these neurons receive information from OSNs or VSNs and relay or *project* this information to higher order brain nuclei.
- *Telencephalon (telencephalic, adj.)*: Anterior part of the forebrain that constitutes the cerebral hemispheres and related structures.

- *Tetrodotoxin (TTX)*: A potent neurotoxin found in pufferfish; inhibits action potential firing by binding to and blocking voltage-gated sodium channels.
- *Transient receptor potential (TRP) channels*: Ion channels named after the role of the channels in *Drosophila* phototransduction; mammalian *Trp* genes are encoded by at least 28 channel subunit genes; channels form six protein families; primary structures predict six transmembrane domains with a pore domain between the fifth and sixth segments and both C and N termini presumably located intracellularly [30.225].
- *Vomeronasal sensory neurons (VSNs)*: Bipolar sensory neurons that reside in the sensory epithelium in the VNO. VSN dendrites end in microvilli, which represent the site of chemosensory transduction. Accordingly, all relevant transduction molecules, such as V1/2R and FPR receptors as well as the transient receptor potential channel 2 (TRPC2) are located in the microvilli. The axons of VSNs terminate at glomeruli in the AOB, where they form synapses with second-order projection neurons (mitral cells).

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31. Disrupted Odor Perception

Thomas Hummel, Basile N. Landis, Philippe Rombaux

Olfactory loss is frequent. However, in public not many people complain of that, or they are even not (fully) aware of it. This indicates that it is possible to live a life without a sense of smell, albeit it is more dangerous, less pleasant, and food tastes much less interesting. Most common causes for smell loss are sinunasal disease (chronic rhinosinusitis with and without nasal polyps), acute infections of the upper airways, head trauma, and neurodegenerative disorders. In many people smell loss seems to be due to the aging process. Before treatment olfactory disorders are diagnosed according to cause with the medical history being a big portion of the diagnostic process. Olfactory disorders are in principle reversible, with a relatively high degree of spontaneous improvement in olfactory loss following infections of the upper respiratory tract. Medical treatment is according to cause. It also involves surgical approaches as well as conservative treatments including the use of corticosteroids, antibiotics, or *smell training*. Because today olfactory dysfunction seems to receive more attention than in previous years it can be expected that *tomorrow* we will have more specific and effective treatment options available.

31.1 Epidemiology of Olfactory Loss

Population-based studies of olfactory loss show a prevalence of olfactory impairment of 22% (25–75 years; [31.1]), 19% (≥ 20 years; [31.2]), or 24% (≥ 53 years; [31.3]), with the highest prevalence in older men. However, unawareness of olfactory loss is common [31.3–5]. It also has to be kept in mind that total loss of the sense of smell is seen much less frequently (3–10%) [31.6].

With regard to patients presenting themselves to specialized clinical centers, the most common etiologies of smell loss are post viral upper respiratory tract infection (URTI) (18–45%) and sinunasal disease (SND) (7–56%), followed by head trauma (8–20%), exposure to toxins/drugs (2–6%), and congenital anosmia (0–4%) [31.7–15]. A survey in Germany, Austria, and Switzerland shows higher results for SND [31.16].

For qualitative disorders, the prevalence is considerably lower. In the general population, the prevalence of phantosmia is estimated between 0.8 and 2.1% [31.17] and parosmia to about 4% [31.18]. Among patients with olfactory disorders, parosmia frequency ranges from 10 to 60% [31.7, 19, 20] – possibly indicating that the detection of parosmia is critically dependent on how the investigator asks for parosmia.

31.2 Definitions of Olfactory Dysfunction

31.2.1 Quantitative Olfactory Disorders

Normosmia indicates a normal sense of smell; *hyposmia* indicates a decrease in olfactory function and *anosmia* indicates the loss of olfactory function. Next to general anosmia, specific anosmias have been described, where only certain odors cannot be perceived whereas most odors are [31.21]. The term functional anosmia was chosen since many subjects with severe olfactory loss appear to be able to still perceive a few single odors. Nevertheless, those rare and weak olfactory impressions are too poor to be of any help to these patients in daily life.

31.2.2 Qualitative Olfactory Disorders

The term *qualitative olfactory disorder* reflects the qualitatively changed perception of odorous sensation [31.19, 20, 22, 23]. Their diagnosis relies on the patients' complaints [31.23]. They can be graded in four grades 0–III (frequency of occurrence: daily = 1 point; intensity: very intense = 1 point; social/important other consequences (weight loss, change of daily activities)= 1 point; the degree of parosmia/phantosmia is the sum of points).

Qualitative olfactory disorders are frequently, but not necessarily associated with quantitative olfactory disorders. *Parosmia* describes the distorted perception of smells in the presence of an odor source; parosmias are triggered by odors. They occur particularly often after infections of the URTI or head trauma [31.24]. The distorted odors are mostly perceived as unpleasant (although some exceptions seem to exist [31.25]). Parosmias are thought to be the result of changes at a peripheral or central-nervous level [31.26, 27]; at the moment it is unclear how they are generated. The diagnosis *qualitative olfactory disorder* can be supported by relatively lower scores in an odor identification test [31.19] or the presence of relatively small olfactory bulbs (OB) compared to patients without parosmia/phantosmia [31.28].

Clinically important, most parosmic impressions tend to weaken over months and finally disappear after years [31.24]. Currently it is also not entirely clear whether parosmias are a positive sign in terms of the prognosis of post-URTI and post-traumatic olfactory loss [31.29]. *Phantosmia* describes the distorted perception of smells in the absence of an odor source. Most often phantosmias occur after trauma or URTI [31.24]. Stress-related phantosmias have also been reported [31.30]. Similar to parosmia, the exact explanation of the molecular modifications leading to phantosmia is yet unknown and also the site of its generation remains unclear. Phantosmias also have a tendency to disappear over the course of years.

31.3 Otorhinolaryngological Examination

Evaluation of a patient starts with a thorough history [31.31]. This should include demographics, eating, drinking or smoking habits, listing of major illnesses and injuries, medications taken in relation to symptom onset, history of present illness, endocrine information (thyroid gland, diabetes), general nasal health including obstruction, rhinorrhea, and changes of the sense of smell (Fig. 31.1). Physical examination should include at least the patient's head and neck. Sometimes a neurological examination may be needed. Specific nasal examination should include nasal endoscopy. Radiological evaluation is helpful to rule out the presence of tumors or vascular malformations [31.32, 33], to judge the volume of the OB [31.34], measure the depth of the olfactory sulcus [31.35, 36], and to investigate the paranasal sinuses. Especially the volume of the OB seems to carry also prognostic information – the larger its volume the more likely recovery [31.37].

Additional diagnostic tests may include the search for other underlying causes of the olfactory disorders, deficiency of vitamin A or B12, or hypothyroidism. Finally, biopsies of the olfactory epithelium may be helpful in the diagnosis of olfactory disorders [31.27, 38, 39].

Questionnaire: History of smell/taste disorder

(self-adhesive label)

Phone (home):

Phone (other):

What kind of problem do you have? *You may check more than one box*	☐ a smell problem ☐ a taste problem concerning aromas (subtle taste perception) ☐ a taste problem concerning the perception of sweet, sour, bitter, or salty
When was the onset of your problem?	☐ less than 3 months ago ☐ 3 to 24 months ago ☐ more than 2 years ago ☐ it has been there as long as I can remember ☐ I dont't know
How did the problem start?	☐ slowly ☐ suddenly ☐ I never could smell in all my life ☐ I don't know
How has the situation changed?	☐ there was an improvement ☐ the situation is unchanged ☐ it has become worse
What could have been the cause of your problem?	☐ accident ☐ cold/infection ☐ medication ☐ surgery ☐ breathing through the nose/nasal polyps/sinusitis ☐ dry mouth ☐ dentures ☐ others (please name)
Do you have chronic nasal problems?	☐ no ☐ yes – please indicate: running nose, nasal obstruction, sneezing, allergy, polyps, facial pain ....
Is your condition fluctuating or constant?	☐ fluctuating ☐ constant ☐ I don't know ☐ if fluctuations are dependent on certain circumstances: please describe:
How badly does the problem affect you?	☐ very badly ☐ badly ☐ medium badly ☐ mildly ☐ hardly at all ☐ not at all
How would you describe your nasal patency?	☐ very good ☐ good ☐ bad ☐ very bad ☐ I cannot breathe through the nose at all
The following questions concern **taste** disorders only	
The taste disorder mainly concerns the perception of:	☐ sweet ☐ sour ☐ salty ☐ bitter ☐ spicy ☐ none of these
Do you suffer from any constant oral sensation?	burning mouth ☐ yes ☐ no bitter taste ☐ yes ☐ no salty taste ☐ yes ☐ no sour taste ☐ yes ☐ no dry mouth ☐ yes ☐ no foreign body sensation ☐ yes ☐ no

Fig. 31.1 History questionnaire

To be completed by the **physician**	
Weight loss due to problem?	☐ no ☐ yes .....kg/.....years
Medication?	☐ no ☐ yes – which?
Chronic diseases?	☐ no ☐ yes – which? ☐ diabetes ☐ high blood pressure ☐ neoplasia ☐ others: .....
Head surgery?	☐ no ☐ yes – which? ☐ sinuses ☐ nasal septum ☐ nasal polyps ☐ nasal turbinates ☐ palatal tonsils ☐ adenoids ☐ middle ear ☐ left ☐ right ☐ dental surgery: ..... ☐ others .....
Flue shots?	☐ no ☐ yes – when?
Smoker?	☐ no ☐ yes – extent?
Alcohol?	☐ no ☐ yes – ☐ occasionally ☐ regularly
Diagnostic imaging?	**CAT** ☐ no ☐ yes **rad.-sinuses** ☐ no ☐ yes **MRI** ☐ no ☐ yes Findings: .....
Profession?	Specific exposure to gaseous, powdered, or other chemicals? ☐ no ☐ yes If **YES**, which? ..... duration (years)? ..... hours per day? .....
If idiopathic etiology is suspected:	Parkinson's disease among relatives ☐ no ☐ yes Alzheimer's disease among relatives ☐ no ☐ yes
Parosmia ☐ no ☐ yes ☐ left ☐ right	☐ daily ☐ not daily ☐ very strong ☐ mild ☐ weight loss due to parosmia ☐ no weight loss
Phantosmia ☐ no ☐ yes ☐ left ☐ right	☐ daily ☐ not daily ☐ very strong ☐ mild ☐ weight loss due to phantosmia ☐ no weight loss

Test results

"Sniffin sticks" T: D: I:

Taste strips (x out of 32): .....

Taste sprays (4 Sprays): .....

Retronasal (x out of 20): .....

Nasal findings

Septal deviation ☐ left ☐ right ☐ none

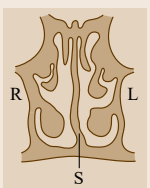
Olfactory cleft visible ☐ left ☐ right

Polyps left: ☐ 0 ☐ I ☐ II ☐ III

right: ☐ 0 ☐ I ☐ II ☐ III

Suspected etiology:

☐ post traumatic ☐ post-infectious
☐ sinunasal ☐ idiopathic
☐ toxic ☐ congenital
☐ neurodegenerative ☐ others



Examiner (name/signature)

Fig. 31.1 (continued)

31.4 Questionnaires

To detect changes related to olfactory loss, several questionnaires have been developed [31.40]. The sinonasal outcome test-16 specifically addresses nasal dysfunction [31.41] (see also [31.42–46]; it is a 16-item measure that assesses the degree of rhinosinusitis based on the presence of symptoms associated with sinusitis. The relatively elaborate questionnaire of olfactory disorders (QOD) [31.47, 48] was designed to asses daily life problems associated with olfactory loss and has been used in a number of studies [31.49, 50]. It consists of 26 items that can be divided into three domains: *negative* statements indicating patients suffering from olfactory impairment and distorted odorous perceptions (parosmia or phantosmia), *positive* statements about coping with the disorder, and statements of social desirability, for control. Another questionnaire asks about the importance of olfaction in daily life [31.51]. This questionnaire does not focus on impairments, but asks how often and in which circumstances people use their sense of smell.

In addition, questionnaires are available to measure mood states or quality of life (QoL) [31.52], with the short form-36 health survey being the standard QoL questionnaire [31.53]. However, there is a choice of questionnaires that allows the selection of the best suited tool [31.54, 55]. Depressive symptoms are often assessed with the Beck depression inventory (BDI) questionnaire [31.56], or its more modern version [31.57]. However, it has to be kept in mind that olfactory loss is often confounded with comorbidity. In fact, in patients with chronic rhinosinusitis the additional effect of olfactory loss on general QoL seems to be not very high [31.58]. In order to be able to track the patients' ability to cope with the olfactory disorder, *Nordin* et al. introduced an 11-question instrument [31.59].

31.5 Psychophysical Methods of Olfactory Testing

The basic principle of psychophysical testing of olfaction is to expose a patient to an odor and to collect responses to that exposure. These procedures are easy to understand by the patient, but, importantly, they are also easily understood by the investigator. Asking the patient about his/her chemosensory function does not appear to be useful, at least not in all patients [31.60–62]; in addition, may patients confuse taste and retronasal olfactory function (*flavor*).

Numerous tests for olfactory function are available many of which are based on odor identification (for review, see [31.63]). In daily clinical life, these tests serve as quick screening tools for olfactory dysfunction [31.64]. All olfactory tests should be reliable and valid. Tests apart from screening tools should distinguish between anosmic, hyposmic, and normosmic subjects, respectively, which requires availability of normative data acquired and validated on large samples of healthy and diseased subjects, respectively. In addition, it should be known which change of the test score indicates a clinically significant change of function [31.65]. Most of these requirements apply only to a few olfactory tests [31.66–71]. The best-validated olfactory tests include the University of Pennsylvania smell identification test (UPSIT) [31.67], the Connecticut Chemosensory Clinical Research Center test (CCCRC) [31.72], and the *Sniffin' Sticks* [31.68, 69] (Fig. 31.2).

Fig. 31.2 *Sniffin' Sticks* and how they are presented to the patient (courtesy of T. Hummel)

Most odor identification tests are presented within a forced choice paradigm. The subjects have to identify odors at supra-threshold concentrations from a list of descriptors [31.73, 74]. For example, subjects receive a rose odor, and they are asked whether the odor was *banana*, *fish*, *rose*, or *coffee*; such tasks are no problem for healthy people, but they are very difficult for people with smell loss. The forced-choice procedure controls the patients' response bias. The result of the test corresponds to the sum of the correctly identified items. *Smell identification tests* are most widely used [31.66–71, 75]. The more odors the test contains the more reliable it is [31.76, 77]. Identification tests have to be

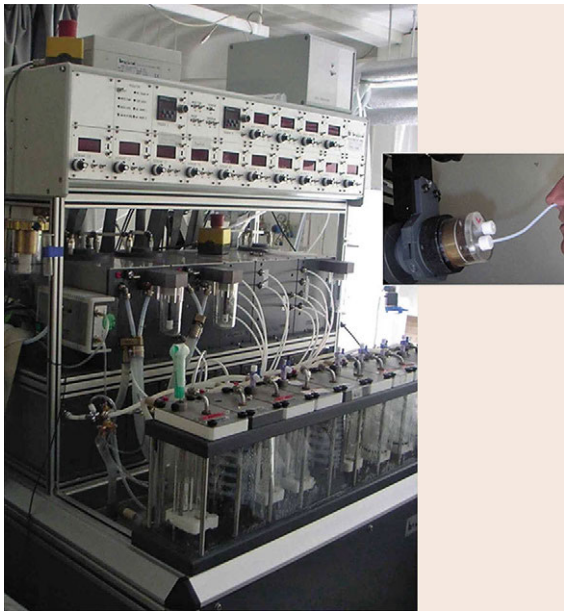


Fig. 31.3 Olfactometer *OMb6* (Burghart, Wedel, Germany) and how stimuli are presented intranasally (courtesy of T. Hummel)

adjusted to the various cultures [31.78, 79], simply because not all odors are known everywhere; for example, many Europeans do not know the smell of *wintergreen* or *root beer*, whereas this is well known in the United States.

Two other widely used test designs are threshold tests and tests of odor discrimination. The idea of threshold tests is to expose a subject repeatedly to ascending and descending concentrations of the same odorant and to identify the least detectable concentration for this individual odor [31.80–82]. Other designs are based on logistic regression [31.83, 84]. Discrimination tasks mainly consist of a three-alternative forced choice technique [31.68, 85]. Two of the administered odors are identical, one is different. The subjects' task is to find out the different one. Tests for odor threshold/odor discrimination can be easily used repetitively, which is more difficult with odor identification tests.

Generally, identification and discrimination tests are believed to reflect central olfactory processing while thresholds are thought to reflect peripheral olfactory function to a stronger degree [31.86–88]. For example, in patients with chronic rhinosinusitis a low threshold score and normal identification and discrimination are frequently seen [31.89].

In order to investigate retronasal function, simple and inexpensive flavor identification tests have been investigated for their test–retest reliability and their validity [31.90–92]. One test, the *taste powders* (*Schmeckpulver*), is also validated on a multinational level [31.93]. Other tests of olfactory function include the investigation of pupillary reflexes [31.94, 95], blinking reflexes [31.96], psychogalvanic skin reactions [31.97], or changes in respiratory/sniffing pattern [31.98, 99].

31.6 Electrophysiological/Imaging Techniques

31.6.1 Electroolfactogram (EOG)

Electroolfactograms (EOG) are electrical potentials of the olfactory epithelium that occur in response to olfactory stimulation. The EOG represents the sum of generator potentials of olfactory receptor neurons (ORNs) [31.100]. Although this technique appears to be attractive [31.101], for example, for the functional characterization of the human olfactory epithelium [31.102], there are only a handful of reports utilizing the human EOG [31.103–108]. Notably there are no published investigations in patients.

31.6.2 Chemosensory Event-Related Potentials (CSERP)

Event-related potentials are EEG-derived signals. They are due to the activation of cortical neurons, which generate electromagnetic fields [31.109]. To extract event-related potentials (ERP) from the background electroencephalogram (EEG) activity, stimuli are presented repeatedly and the individual recordings are then averaged, which improves the signal-to-noise ratio (random activity would cancel itself out; nonrandom activation would remain). In addition, stimuli are presented with a steep onset (< 20 ms) in a monotonous environment such that stimulus onset synchronizes the activity of as many cortical neurons as possible. Olfactory ERP are direct correlates of neuronal activation; they have a high temporal resolution in the range of microseconds, and they can be obtained independently of the subject's response bias, they are well-suited for medico-legal investigations [31.110].

Based on a system developed by *Kobal* [31.104, 111], odors are applied intranasally (Fig. 31.3). Presentation of odorous stimuli does not produce mechanical or thermal sensations because odor pulses are embed-

ded in a constantly flowing air stream [31.104, 112, 113]. ERP peaks are either early or late. Earlier peaks like N1 encode exogenous stimulus characteristics to a larger extent (*What is the nature of this stimulus?*) than later, so-called endogenous components (*What is the meaning of this stimulus?*) [31.114, 115]. Although there is a very clear concept available as to how to record and analyze olfactory ERP [31.116, 117] recently there have been a number of attempts to improve the signal-to-noise ratio [31.118–120] and to extract additional information from the stimulus-related EEG [31.121, 122].

Using magneto-encephalographic techniques [31.123] cortical generators of the responses to trigeminal stimulation with CO_2 were localized in the secondary somato-sensory cortex [31.124], sources in response to olfactory stimulation were found in the anterior-central parts of the insula, the para-insular cortex, and the superior temporal sulcus [31.125–127]. More recent work based on EEG [31.128] suggests that olfactory information in humans is processed first ipsilaterally to the stimulated nostril and then sequentially activates the major relays in olfactory information processing in both hemispheres.

Other EEG-related tests are based on the contingent negative variation (CNV), which occurs in response to an expectation [31.129, 130] or more general EEG changes [31.131] (Fig. 31.4).

31.6.3 Functional Magnetic Resonance Imaging (fMRI), Positron Emission Tomography (PET)

Brain imaging allows to study the human olfactory system in detail [31.132–139]. In contrast to electroencephalogram (EEG) and magneto-encephalogram (MEG), PET and fMRI largely reflect blood-flow related changes. In addition, PET and fMRI have a relatively low temporal resolution whereas they exhibit a better spatial resolution than EEG and MEG. Although there are some data available on fMRI and PET in patients with olfactory loss [31.140–142], it seems that the use of these techniques in individual patients with smell or taste disorders is difficult.

31.7 Causes and Symptoms of Smell Disorders

31.7.1 Most Common Causes

Olfactory Loss Following Infections of the URTI

The patients' history typically starts with an episode of a cold during, which they lose their sense of smell [31.20, 143–147]. Some authors claim viral (influenza, parainfluenza viruses type III, rhinovirus, coronavirus, and Epstein Barr) rather than bacterial infections to be responsible for olfactory disturbances [31.148, 149] and observed a higher incidence of dysosmias after spring and summer URTI [31.144]. Furthermore, women above 45 years of age seem to be affected at a higher percentage than men [31.7, 10, 144] – which brings up the potential olfactory protective effect of estrogens [31.150]. Nevertheless, the effect of estrogen on olfactory function remains an open debate [31.17, 151]. It is important to inform patients with post-URTI olfactory loss about the possibility of parosmia (see above). It tends to occur 1−3 months after the URTI, although it appears sometimes to occur directly after the URTI. The frequency of parosmia is in the range of 25% [31.152, 153].

Posttraumatic Olfactory Disorders

Posttraumatic olfactory disorders are said to occur after occipital trauma. The current explanation is that *coup-contre-coup* lesions or tearing of the filae olfactoriae leads to anosmia or hyposmia. Although the entity of posttraumatic olfactory loss has already been described at the end of the last century it has received little systematic attention, like most olfactory disorders [31.154]. Olfactory loss seems to correlate with the severity of the trauma [31.155–157], although several authors pointed out the fact that there is considerable individual variability in terms of the vulnerability of olfactory structures [31.157, 158]. The injured parts of the olfactory system are most often the filae olfactoriae which cross the cribriform plate. However, central structures such as the orbitofrontal cortex and gyrus rectus have also been found to be affected from head trauma [31.147, 155]. Similar to post-URTI olfactory impairment, these patients are prone to develop parosmia and phantosmia several months after the trauma. Clinical experience shows that most patients with posttraumatic olfactory disturbance realize the alteration with some latency [31.159].

Sinunasal Disorders

Sinunasal disorders constitute the most frequent causes of olfactory loss [31.16]. This is due to mechanical obstruction of nasal cavity (septal deviation, nasal polyposis, congested mucosa) and/or the inflammatory component of chronic rhinosinusitis [31.160–165]. Mild

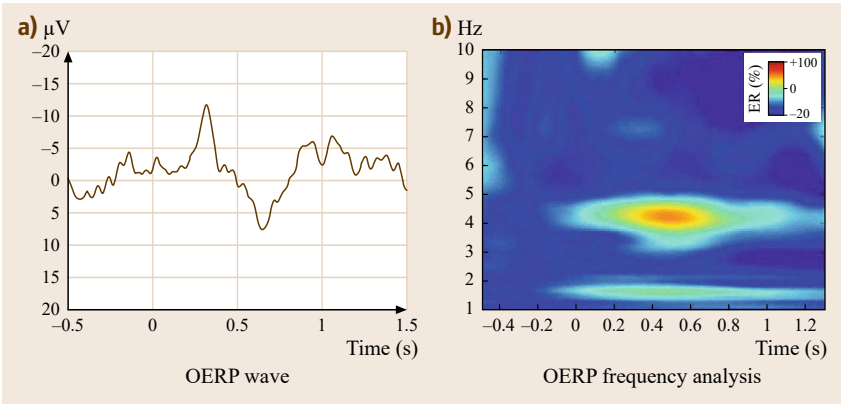


Fig. 31.4a,b Olfactory event-related activity shown in the time domain **(a)**: negativity upward or the frequency domain **(b)**. Stimulus presentation (phenylethylalcohol: PEA) started at time 0 for 200 ms

olfactory impairments could also be identified in other groups of patients with SNDs such as allergic and uncomplicated chronic rhinosinusitis [31.147, 166, 167]. In contrast to posttraumatic and post-URTI olfactory dysfunctions, these patients rarely exhibit parosmia or phantosmia.

Neurodegenerative Causes

Olfactory loss is common in patients with idiopathic Parkinson's disease (IPD) [31.168–170]. While a decreased sniff volume seems to add to the decrease in olfactory function [31.171], electrophysiological recordings in response to passive olfactory stimulation clearly established the presence of olfactory impairment in IPD [31.172, 173]. This olfactory deficit is so reliable that it can be used as a marker of IPD [31.170, 174]. In other words: If a patient with normal olfactory function presents with IPD symptoms the diagnosis should be re-investigated. Olfactory loss precedes the onset of motor symptoms by 4–6 years [31.175–177]. Olfactory loss is also observed regularly in Alzheimer's disease, and at a much lower frequency/less pronounced in multiple system atrophy, Huntington's disease, and motor neuron disease [31.178].

Idiopathic Olfactory Loss

Idiopathic olfactory loss seems to reflect the poor understanding of factors interfering with olfaction [31.147]. With further insight and research this percentage should decrease. A considerable number of these idiopathic causes might be due to SND, post-URTI dysosmias following an almost undetected URTI, or neurodegenerative diseases [31.165].

31.7.2 Less Frequent Causes

Diabetes is probably among the best investigated endocrine diseases concerning olfactory disorders [31.2, 179, 180]. Most studies reveal slight olfactory deficiencies in diabetic patients especially at threshold levels indicating a peripheral patho-mechanism compatible with a possible diabetic micro-angiopathy or peripheral polyneuropathy. However, olfactory impairment in diabetes is relatively mild. Two recent studies conducted with identification tests in large study samples could not find diabetic patients to exhibit a decreased ability to identify odors compared to healthy controls [31.2, 17]; there is evidence showing that olfactory loss is most prominent in complicated diabetes II [31.181]. Several other endocrine diseases like hypothyroidism [31.182] or adrenocortical insufficiency (Addison's disease) [31.183] have been reported to cause olfactory loss.

Affections of the kidney [31.184] and liver [31.17, 185, 186] have been associated with decreased olfactory function. Olfactory disturbances in those pa-

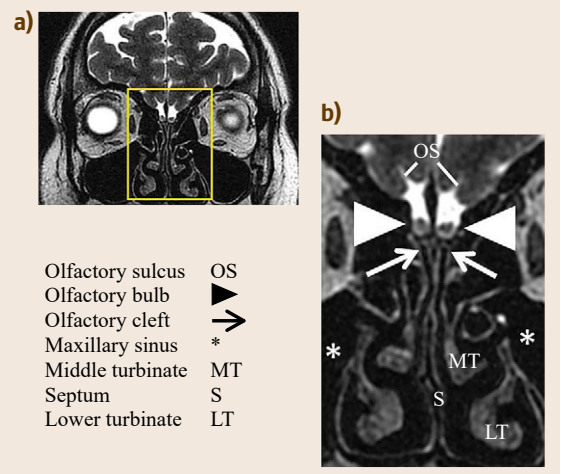


Fig. 31.5a,b Coronal sections (MR-scans) of the head including olfactory bulbs: **(a)** section through whole head; **(b)** magnification of rectangular section indicated in the whole head section

tients are of special importance because they are discussed as a potential cause of malnutrition [31.187]. Olfactory loss may be induced by drugs [31.188]. Among these cardiovascular drugs [31.189], anti-hypertensive drugs [31.190, 191], antibiotics [31.192], and chemotherapeutic agents [31.193] are frequently mentioned. However, most information has been accumulated on the basis of case reports [31.194, 195]. Typically, the chemosensory side effects disappear when the medication is discontinued.

Isolated congenital anosmia seems to appear at a frequency of approximately 1 : 8000. Only magnetic resonance (MR) imaging leads to a more definitive diagnosis [31.35, 36, 196]. In the frontal imaging planes just tangential to the eye bulbs hypoplastic or aplastic olfactory bulbs (OLB) can be visualized (Fig. 31.5). This plane also allows an evaluation of the olfactory sulcus which is flattened in case the OLB is absent or hypoplastic. This is a useful indicator of congenital anosmia, especially because the bulb is not always easy to identify. Congenital/inborn anosmia as part of a syndrome is the Kallmann syndrome [31.197, 198] – here decreased olfactory function is associated with hypogonadotropic hypogonadism meaning the missing/slowed development of the gonads because of decreased levels of hormones, so-called gonadotropins [31.199].

31.8 Symptoms/Quality of Life

Patients with olfactory disorders are impaired in areas of food intake, safety, personal hygiene, and in their sexual life [31.200, 201]. Most often, difficulties related to eating are reported [31.202], also reduced appetite [31.8, 59] and difficulties in preparing food/cooking [31.40]. Many patients have problems detecting spoiled food [31.203]. Interestingly, however, these eating problems do not lead to a general pattern of reduced food intake [31.204]. In a study by *Ferris* and *Duffy*, 18% of the smell patients described an increase in food consumption, 20% a decrease, and the majority reported no change in food consumption [31.202].

No significant weight difference and no difference in food preferences was found in patients who were born without a sense of smell in comparison to an age-matched control group [31.198]. This is also supported from observations indicating that congenital absence of olfaction does not result in markedly aberrant food preferences [31.205]. As patients with acquired olfactory disorders, congenitally anosmic people report enhanced problems with burning food and detecting spoiled food [31.198].

Another common problem is worry about failure to detect fire, gas, or smoke [31.40]. The failure to detect fire or smoke is a main risk associated with olfactory disorder [31.59, 206]. Patients also express problems related to personal hygiene and also social relations are reported to be affected by olfactory disorders [31.59]. Impaired sex life has been reported inconsistently [31.42, 207–209]. Problems in working life have been reported to various degrees [31.62].

Olfactory dysfunction also affects QoL [31.210]. However, in SND, for example, it is difficult to separate the effects of olfactory disorders from those of decreased nasal patency. A similar situation is present in posttraumatic olfactory loss, where patients not only exhibit olfactory loss, but often more severe trauma-related disorders.

Finally, most patients also suffer from a feeling of not being recognized as being sensory disabled since both healthcare professionals and peers often lack any knowledge about smell disorders. This non recognition of the smell-impaired person is reported to generate a lot of frustration and has been overlooked in the past [31.211, 212].

31.9 Spontaneous Recovery of Smell Disorders

Age-related anosmia does not seem to exhibit spontaneous recovery. Sinunasal smell disorders have a tendency to become worse over time – typical is a gradual loss of the sense of smell. This leads to a situation that the entire process may not be noticed or that patients only complain of orthonasal loss but not of loss of retronasal olfactory function. Toxic and drug-induced smell disorders may recover once the drug intake is interrupted [31.192, 193].

Several authors described recovery rates for post-URTI and posttraumatic disorders to be highest within the first year [31.20, 213–215]. This is probably due to the ability of olfactory neurons to regenerate [31.216, 217]. Post-URTI disorders have a better prognosis com-

pared to posttraumatic disorders, probably (at least in part) owing to the fact that they often cause hyposmia rather than anosmia [31.146]. In approximately 5% of the cases total recovery can be observed, while up to 60% of all patients experience partial recovery of some olfactory function over the following years [31.145].

Positive predictors of spontaneous recovery include: short duration of disease, young age, viral cause of olfactory loss better than trauma, presence of parosmia, women better than men, higher olfactory function (also indicated in the presence of olfactory/trigeminal ERP and larger volume of the OLB), and nonsmoking [31.29, 34, 146, 218]. In contrast to quantitative olfactory disorders, qualitative disorders seem to have a better prognosis of spontaneous disappearance. Parosmias tend to decrease to a bearable level after approximately one year [31.152]. However, recent work revealed that more than 50% of the parosmias still are present after 2 years [31.210]. Over time, parosmia seems to lose its devastating effect on QoL. To summarize, the best current therapeutic attitude toward post-URTI and posttraumatic olfactory disorders is to correctly inform the patient, without taking any hope of recovery nor promising quick and complete recovery. The patients should receive satisfactory olfactory testing. Follow-up investigations give both the physician and the patient the possibility to observe improvements.

31.10 Treatment of Olfactory Disorders

31.10.1 Surgical Therapy of SND-Related Olfactory Loss

Most of the patients undergo surgery to remedy decreased nasal patency, a feeling of pressure or recurrent infections of the nasal sinuses. Surgery is rarely performed to specifically treat olfactory dysfunction. However, when asked, postoperative improvement of olfactory function is reported by a majority of the patients [31.219–221]. When olfactory function is measured, a different picture emerges with 25% of patients with preoperative hyposmia and 5% with preoperative anosmia [31.222] (compare [31.223]). In terms of the sense of smell, nasal surgery produced the highest success rates in patients with eosinophilia and a high degree of polyposis [31.223]; in addition, higher success rates were found in women, and patients with aspirin-intolerance [31.224]. Neither age, presence of asthma, nor the number of preoperative surgical interventions had a major impact on the outcome of surgery [31.223, 224]. Findings of endoscopical investigations do not correlate with improvement of the sense of smell [31.225]. While beneficial in many cases, surgery may also pose a certain, albeit low risk to olfactory function [31.223, 226].

31.10.2 Conservative Therapy of SND-Related Olfactory Loss

Antibiotics

In the chronic form of putrid sinusitis *Staphylococcus aureus* and *Pseudomonas aeruginosa* are of high significance. Whenever possible antibiotic therapy should only be started after the bacteria have been identified and tested for resistances to antibiotics. It is important to note that in chronic putrid sinusitis, antibiotic treatment is not always successful.

Steroids

Among many other effects, corticosteroids act as anti-inflammatory drugs, the effects of which are produced via a number of different pathways including inhibition of phospholipase A2 through induction of lipocortin [31.227]. They reduce submucosal edema and mucosal hypersecretion and thereby increase nasal patency. Systemically administered steroids are of help in many SND patients [31.9, 228–232]. In addition to the anti-inflammatory activity it has been postulated that corticosteroids directly improve olfactory function [31.233, 234] by modulating the function of ORN through effects on olfactory Na, K-ATPase [31.227]. In fact, systemic steroids are often helpful even in patients without nasal obstruction due to polyps or obvious inflammatory changes [31.165, 231, 235].

Steroids may be administered systemically or topically. With regard to idiopathic olfactory dysfunction systemic administration is often applied for diagnostic purposes [31.165]. If systemic steroids improve olfactory function treatment is typically continued with locally administered steroids [31.167, 228, 230, 236]; however, the role of topical steroids in the treatment of SND-related olfactory loss has been questioned [31.165, 237, 238]. One reason why systemic steroids have a higher therapeutic efficacy compared to topical steroids [31.9, 239] may relate to the deposition of the spray in the nasal cavity with only few droplets reaching the olfactory cleft [31.238, 240]. In fact, it has been shown that only a small amount of nasally applied drugs reaches the olfactory epithelium which is situated in an effectively protected area of

the nasal cavity [31.241–243]. This situation can be remedied by the use of longer applicators [31.244], which allow to reach further into the nasal cavity so that the spray can reach the olfactory epithelium more effectively.

Other Treatments

Other treatments include the use of antileukotrienes [31.245, 246], saline lavages [31.247], dietary changes [31.248], acupuncture [31.249], antiallergy immunotherapy [31.250], or herbal treatments [31.251].

31.10.3 Conservative Therapy of Post-URTI/ Posttraumatic Olfactory Loss

Post-URTI smell dysfunction seems to be due to an impairment of ORN, both in function and in numbers [31.252, 253]. While numerous treatments have been tried in post-URTI anosmia no pharmacological therapy has been clearly established so far [31.254–257].

However, there are numerous candidates for the conservative treatment of olfactory dysfunction. One of them is alpha-lipoic acid (ALA) which is used in the treatment of diabetic neuropathy [31.258]. The effect of ALA is well described both in experimental animals and in humans (for review see [31.259]). It is known to stimulate the expression of nerve growth factor, substance P, and neuropeptide Y [31.260, 261]. It enhances motor nerve conduction velocity as well as microcirculation [31.262]. Further, ALA also has neuroprotective capabilities [31.263]. Preliminary work indicated that it may be useful in post-URTI olfactory loss [31.264]. Other encouraging pilot studies have been performed with the NMDA-antagonist *caroverine* (NMDA: *N*-methyl-D-aspartate) [31.265]. Potential mechanisms for the hypothesized effect included both reduced feedback inhibition in the OLB as a consequence of NMDA-antagonistic actions or antagonism of an excitotoxic action of glutamate.

Although frequently mentioned as a therapeutic option, studies on olfactory dysfunction with *zinc* have produced negative results [31.254, 266]. It may, however, be of therapeutic value in patients with severe zinc deficiency, in hemodialysis. In studies in postmenopausal women, *estrogens* have been reported to provide a certain protection against olfactory disturbances [31.7]. However, as mentioned above, recent studies [31.151] indicate that estrogens are probably ineffective in the treatment of olfactory loss. Finally, although discussed frequently, the potential therapeutic use of orally administered *vitamin A* [31.255, 267] is questionable; at least doses as high as 10 000 IU do not seem to be effective [31.268].

A different approach to the treatment of olfactory disorders is the detection and treatment of underlying causes. This approach may also involve the replacement of drugs suspect to affect the sense of smell [31.194, 269–271]. Among the nonpharmacological treatments acupuncture has been mentioned frequently [31.249, 272, 273] although its effectivity is a matter of discussion [31.274].

The use of phophodiesterase inhibitors has been described in several studies, none of which was double-blinded, throwing some doubt on the results [31.275–280]. In addition, animal studies indicated a decreased amplitude of responses recorded from the olfactory epithelium after topical administration [31.281].

Numerous studies indicate the usefulness of *olfactory training*, which is typically performed over a period of 12 or more weeks [31.282]. Patients expose themselves twice daily to four intense odors (phenyl ethyl alcohol: *rose*, eucalyptol: *eucalyptus*, citronellal: *lemon*, eugenol: *cloves*). A number of studies, from different laboratories [31.283–285], one of them performed in a blinded way [31.286], indicate that patients training with odors experience a significant improvement in olfactory function over patients who do not perform such a training. One idea about the effectivity of that training relates to the possible stimulation of regenerative capacities of the olfactory epithelium [31.287].

31.11 Concluding Remarks and Outlook

Olfactory dysfunction receives more attention than in previous years probably because modern societies not only care about simple survival but much about QoL. It can be expected that soon we will (1) have a better understanding of the processes leading to smell loss and (2) have more specific and effective treatment options available for people with olfactory disorders.

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32. Human and Animal Olfactory Capabilities Compared

Matthias Laska

Humans are traditionally considered to have a poorly developed sense of smell that is clearly inferior to that of nonhuman animals. This view, however, is mainly based on an interpretation of neuroanatomical and recent genetic findings, and not on physiological or behavioral evidence. An increasing number of studies now suggest that the human sense of smell is much better than previously thought and that olfaction plays a significant role in regulating a wide variety of human behaviors. This chapter, therefore, aims at summarizing the current knowledge about human olfactory capabilities and compares them to those of animals.

Comparing olfactory capabilities between species is not a trivial task. Several potentially confounding factors have to be taken into consideration when trying to make statements as to differences or similarities concerning the efficiency of the sense of smell between species.

First, we find a high variability in results between human studies. With regard to olfactory sensitivity, for example, published mean threshold values for a given odorant may vary up to 6 orders of magnitude [32.1]. This is probably, at least in part, due to different methods used to determine such thresholds. However, there is a clear trend that more sophisticated psychophysical procedures employing signal detection methods and rigorous stimulus control yield lower threshold values than more simple procedures [32.2].

Second, the vast majority of psychophysical studies on the human sense of smell only report mean values of performance (usually plus a measure of variation), but not the distribution of values or their range. This is all the more problematic as measures of olfactory performance are notorious for a high interindividual variability. This is true for both studies on olfactory sensitivity in which individual threshold values with a given odorant have been reported to commonly vary up to 3 orders of magnitude within a study population [32.3], and for studies on olfactory discrimination performance in which subjects have been found to vary in their ability to distinguish between a given pair of odorants from chance to perfect discrimination [32.4]. However, here too, more recent studies using state-of-the-art psychophysical methods (pioneered by *Cain* and coworkers) generally report a considerably lower range between the most and least sensitive subjects within a study population [32.5].

Third, it is inevitable to use different methods for assessing olfactory performance with animals and humans, and also with different species of animals. This may affect the comparability of results. However, differences between methods employed with different species of animals are usually necessary adaptations for meeting the physiological, anatomical, and behavioral needs and limitations of the study species to successfully cooperate in a behavioral test. (In other words, even if it was possible to use the same method with different species, this would very likely put one species at an advantage over another species, thus invalidating any comparisons. Therefore, it is better to aim for optimizing a testing method for each study species.) It is commonly agreed that operant conditioning procedures are the gold standard among the methods employed with animals for assessing sensory performance [32.6], and therefore only studies based on such procedures are considered in this chapter. Figures 32.1–32.5 illustrate operant conditioning procedures for assessing

Fig. 32.1a–d Olfactory conditioning method used with spider monkeys. **(a)** Portrait of a spider monkey (*Ateles geoffroyi*). **(b)** The two-choice apparatus used, viewed from the animal's side. It consists of two manipulation boxes of which one is baited with a Kellogg's honeyloop while the other is empty, depending on the odorant applied on the absorbent paper strip attached to the box. **(d)** A spider monkey smelling at one of the absorbent paper strips bearing an odorant used either as a rewarded stimulus or as an unrewarded stimulus. **(c)** A spider monkey indicating its decision for one of the two simultaneously presented odorants by opening the corresponding manipulation box (courtesy of M. Laska)

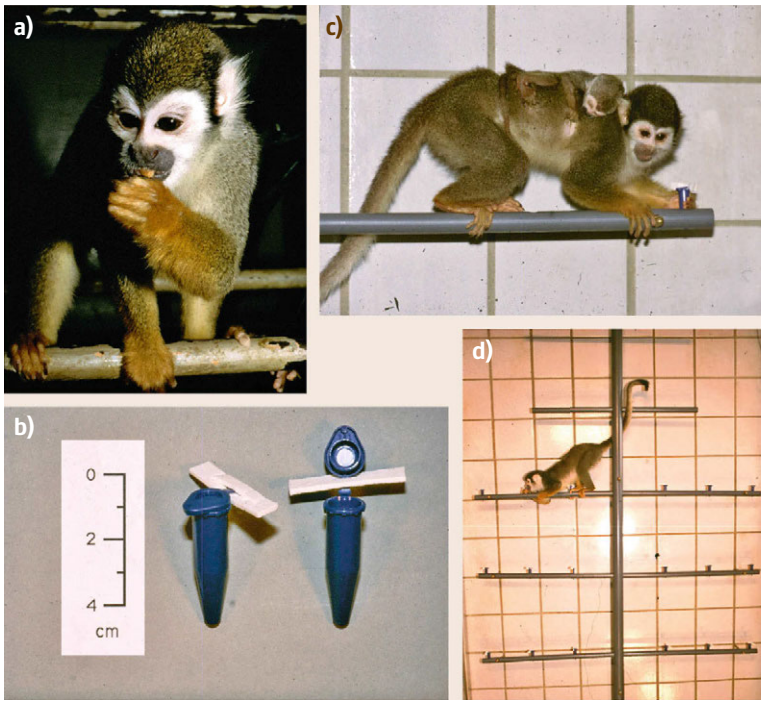

Fig. 32.2a–d Olfactory conditioning method used with squirrel monkeys. **(a)** Portrait of a squirrel monkey (*Saimiri sciureus*). **(b)** Eppendorf cups equipped with absorbent paper strips. The Eppendorf cups serve as manipulation objects (*artificial nuts*) that are either baited with a piece of peanut or not, depending on the odorant applied on the absorbent paper strip. **(d)** Experimental setup. An *artificial nut tree* is used to present numerous *artificial nuts*, half of them baited with a piece of peanut and bearing an odorant used as rewarded stimulus, and half of them empty and bearing an odorant used as unrewarded stimulus. **(c)** A squirrel monkey inspecting an *artificial nut* on a branch of the *artificial nut tree* (courtesy of M. Laska)

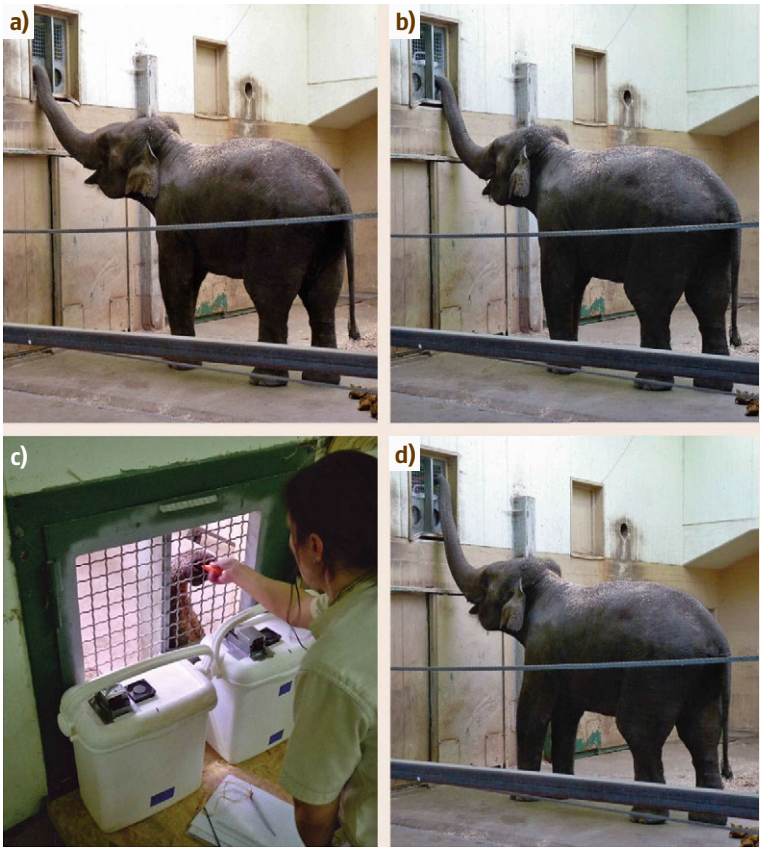

Fig. 32.3a–d Olfactory conditioning method used with Asian elephants (*Elephas maximus*). **(a)** Elephant sniffing at the left odor port. **(b)** Elephant sniffing at the right odor port. **(d)** Elephant indicating its decision for one of the two simultaneously presented odorants by placing her trunk onto the grid on top of the corresponding odor port. **(c)** Elephant receiving a carrot as a food reward after a correct choice (courtesy of M. Laska)

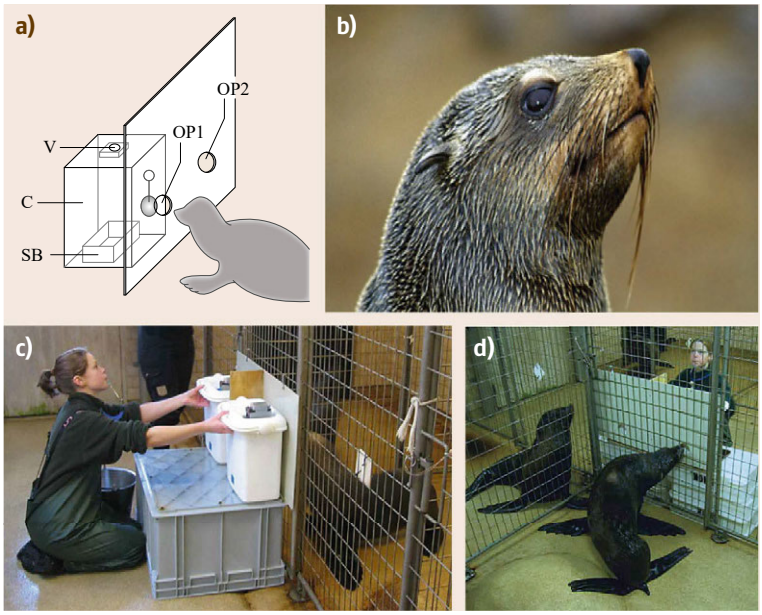

Fig. 32.4a–d Olfactory conditioning method used with South African fur seals. **(a)** Schematic drawing of the experimental set up. C: container bearing an odor stimulus; V: ventilator for ingoing airflow; O: outlet for outgoing airflow; *SB*: stimulus box; OP1: odor port 1; OP2: odor port 2. **(b)** Portrait of a South African fur seal (*Arctocephalus pusillus*). **(c)** Simultaneous presentation of two odor stimuli to a fur seal. **(d)** A fur seal sniffing at one of the two odor ports (courtesy of M. Laska)

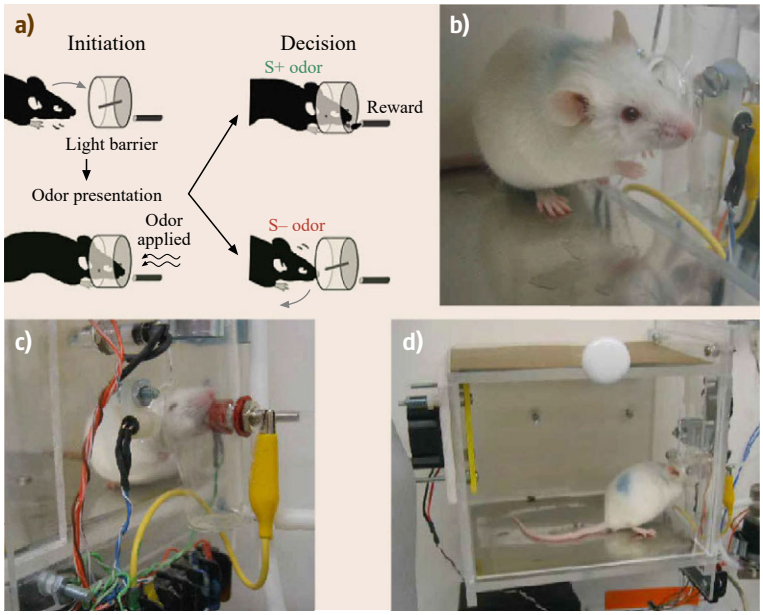

Fig. 32.5a–d Olfactory conditioning procedure used with mice. **(a)** Schematic drawing of the experimental setup. **(b)** A mouse (*Mus musculus*) in front of the odor port. **(d)** A mouse poking its head into the odor port. **(c)** A mouse licking at the water spout after a correct choice (courtesy of M. Laska)

olfactory performance in different species of mammals.

Finally, animal studies usually only employ a low number of individuals, sometimes only one or two animals per species, thus making the use of mean values arguable, and statements as to how representative the findings are for the whole species difficult.

Despite all these difficulties, there are several good reasons that make it worthwhile to compare olfactory capabilities between humans and animals: first, such comparisons allow us to study the neural and/or genetic mechanisms underlying possible differences or similarities in olfactory efficiency between species [32.7]. Second, between-species comparisons of olfactory performance allow us to test hypotheses about the evolution of sensory systems and the selective pressures acting on them [32.8]. Finally, the integration of animal and human studies on olfactory performance may help us to better understand medically relevant phenomena such as aging processes or neurodegenerative diseases, which are often accompanied by a loss of olfactory function [32.9, 10].

32.1 Olfactory Sensitivity

The sensitivity of the sense of smell is usually assessed by determining olfactory detection thresholds, that is, the lowest concentration of a given odorant that a human subject or an animal is able to detect. Human olfactory detection thresholds have been reported for a total of ≈ 3300 odorants [32.1]. In contrast, the total number of odorants tested in animals is much lower. Table 32.1 summarizes the species of mammals and the number of odorants for which olfactory detection thresholds using operant conditioning procedures have been published. The total number of species is 17 (please note that there exist about ≈ 5500 species of mammals), and the highest number of odorants tested with a given species is 81. These 17 species represent 7 of the 29 orders of mammals.

With the exception of four odorants tested with rats (one explosive substance, and three *explosive taggants*, that is, substances added to explosives to identify their sources) and one odorant tested with mice (pyridazine), all 138 odorants for which olfactory detection thresholds have been determined with nonhuman mammals have also been tested with human subjects, allowing for direct comparisons of their performance.

In the following figures (as well as in all other comparisons that follow), I compare the lowest mean threshold values reported in human subjects to the lowest individual threshold values reported in a given animal species. The rationale for this is as follows:

1. Human studies only rarely report the range of threshold values, but usually a mean value.

Table 32.1 Animal species and number of odorants for which olfactory detection threshold values using operant conditioning procedures have been published

No.	Common name	Scientific name	Mammalian order	Number of odorants tested
1	Spider monkey	*Ateles geoffroyi*	Primates	81
2	Mouse	*Mus musculus*	Rodentia	72
3	Squirrel monkey	*Saimiri sciureus*	Primates	61
4	Pigtail macaque	*Macaca nemestrina*	Primates	60
5	Rat	*Rattus norvegicus*	Rodentia	45
6	Short-tailed fruit bat	*Carollia perspicillata*	Chiroptera	18
7	Dog	*Canis lupus familiaris*	Carnivora	15
8	Common vampire bat	*Desmodus rotundus*	Chiroptera	15
9	Common mouse-eared bat	*Myotis myotis*	Chiroptera	13
10	Sea otter	*Enhydra lutris*	Carnivora	7
11	Pig	*Sus scrofa domestica*	Artiodactyla	5
12	Hedgehog	*Erinaceus europaeus*	Insectivora	4
13	Great fruit-eating bat	*Artibeus lituratus*	Chiroptera	3
14	Pale spear-nosed bat	*Phyllostomus discolor*	Chiroptera	3
15	Common shrew	*Sorex araneus*	Insectivora	3
16	Rabbit	*Oryctolagus cuniculus*	Lagomorpha	1
17	Harbor seal	*Phoca vitulina*	Carnivora	1

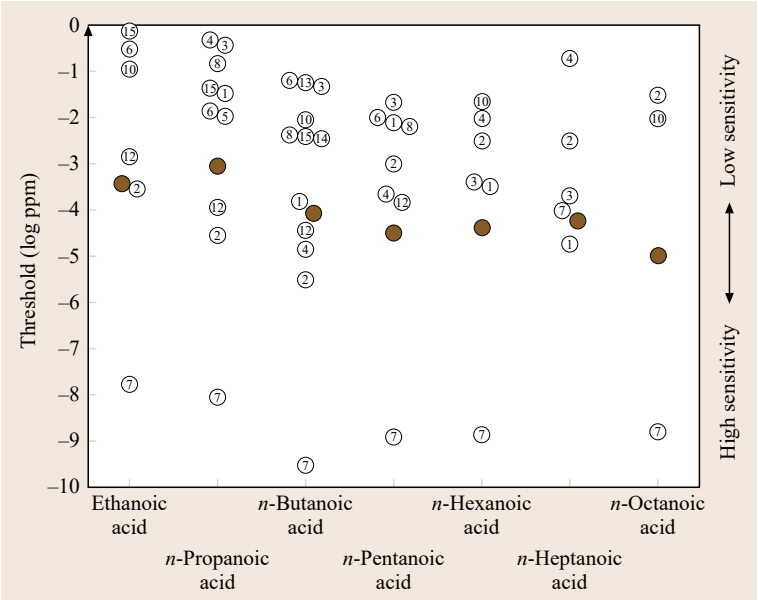

Fig. 32.6 Comparison of the olfactory detection threshold values (expressed as vapor phase concentrations) of human subjects for aliphatic *n*-carboxylic acids and those of other mammalian species. Data points of the human subjects (*brown circles*) represent the lowest mean threshold values reported in the literature, and data points of all animal species (*numbered circles*) represent the lowest threshold values of individual animals reported in the literature. *Numbers in circles* refer to the numbers assigned to each species in Table 32.1 (*human data*: [32.1]; *spider monkey data*: [32.11]; *mouse data*: [32.12]; *squirrel monkey data*: [32.13]; *pigtail macaque data*: [32.11]; *rat data*: [32.14]; *short-tailed fruit bat data*: [32.15]; *dog data*: [32.16, 17]; *common vampire bat data*: [32.18]; *sea otter data*: [32.19]; *hedgehog data*: [32.20]; *great fruit-eating bat data*: [32.18]; *pale spear-nosed bat data*: [32.18]; *common shrew data*: [32.21])

2. Animal studies usually only employ a low number of individuals (in some cases only one animal), making the use of mean values arguable.
3. Comparing the threshold value of the very best individual animal with the mean threshold value of a group of human subjects minimizes the risk of unintentionally favoring humans over animals.

Figure 32.6 compares human olfactory detection threshold values for aliphatic *n*-carboxylic acids to those from other mammalian species. With the notable exception of the dog, human subjects have a higher sensitivity, that is, lower olfactory detection thresholds, than the majority of mammalian species tested with this class of odorants. (Please note that all data points for the dog, except the one for *n*-heptanoic acid, are from a study by *Neuhaus* [32.16] who employed only one animal. Later studies by other researchers [32.17, 57] employed several dogs as well as more rigorous stimulus control, and, interestingly,

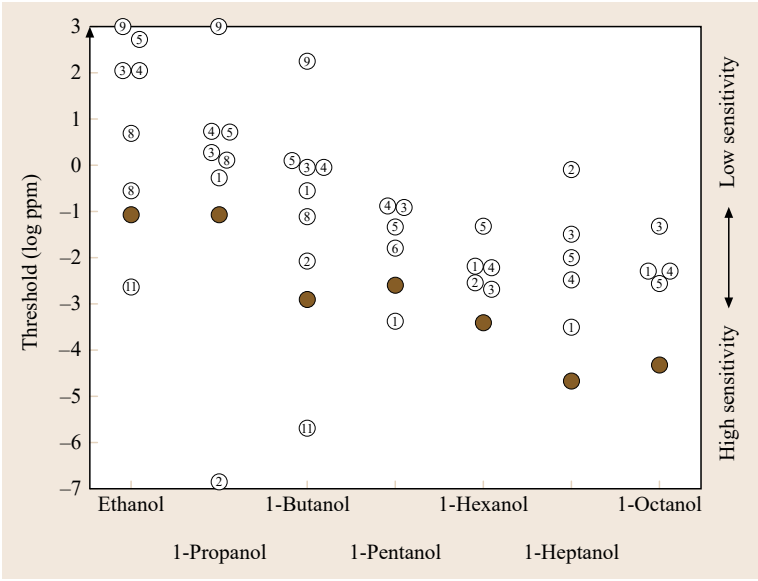

Fig. 32.7 Comparison of the olfactory detection threshold values (expressed as vapor phase concentrations) of human subjects for aliphatic alcohols and those of other mammalian species. Data points of the human subjects (*brown circles*) represent the lowest mean threshold values reported in the literature, and data points of all animal species (*numbered circles*) represent the lowest threshold values of individual animals reported in the literature. *Numbers in circles* refer to the numbers assigned to each species in Table 32.1 (*human data*: [32.1]; *spider monkey data*: [32.22]; *mouse data*: [32.23–25]; *squirrel monkey data*: [32.26]; *pigtail macaque data*: [32.26]; *rat data*: [32.27, 28]; *short-tailed fruit bat data*: [32.15]; *common vampire bat data*: [32.18]; *common mouse-eared bat data*: [32.29]; *pig data*: [32.30, 31])

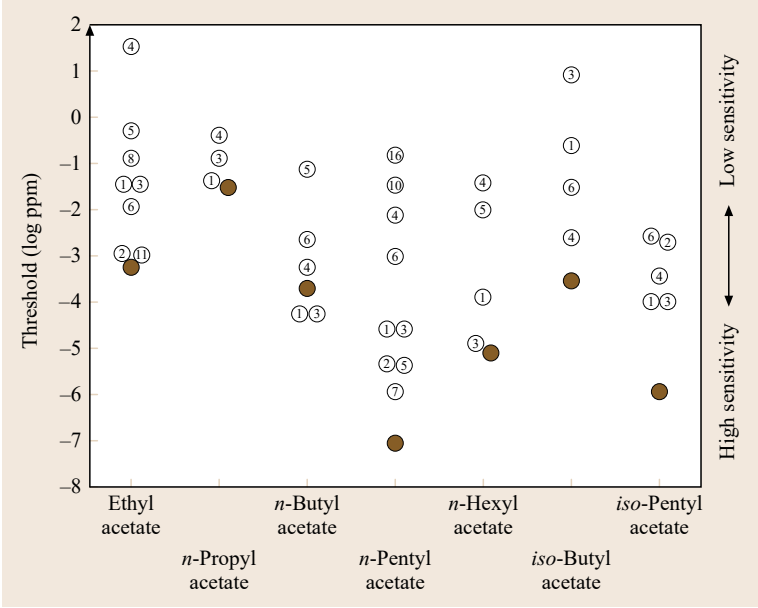

Fig. 32.8 Comparison of the olfactory detection threshold values (expressed as vapor phase concentrations) of human subjects for aliphatic acetic esters and those of other mammalian species. Data points of the human subjects (*brown circles*) represent the lowest mean threshold values reported in the literature, and data points of all animal species (*numbered circles*) represent the lowest threshold values of individual animals reported in the literature. *Numbers in circles* refer to the numbers assigned to each species in Table 32.1 (*human data*: [32.1]; *spider monkey data*: [32.32]; *mouse data*: [32.33–35]; *squirrel monkey data*: [32.36]; *pigtail macaque data*: [32.36]; *rat data*: [32.37–39]; *short-tailed fruit bat data*: [32.15]; *dog data*: [32.40]; *common vampire bat data*: [32.18]; *sea otter data*: [32.19]; *pig data*: [32.31]; *rabbit data*: [32.41])

obtained markedly higher threshold values with these odorants compared to those reported by Neuhaus.) The mouse, another mammal with a reputation for a keen sense of smell, is more sensitive than humans with only three of the seven *n*-carboxylic acids whereas humans outperform this rodent with four of these seven odorants.

Figure 32.7 compares human olfactory detection threshold values for aliphatic 1-alcohols to those from other mammalian species. Here, too, human subjects have a higher sensitivity, that is, lower olfactory detection thresholds, than most of the other species tested. It is interesting to note that humans outperform the rat, another mammal believed to have a highly developed sense of smell, with all seven 1-alcohols. Similarly, humans generally have lower olfactory detection thresholds than the bats and nonhuman primates tested with these odorants. The pig, in contrast, is clearly more sensitive than humans with the two alcohols tested in this species.

Figure 32.8 compares human olfactory detection threshold values for aliphatic acetic esters to those from

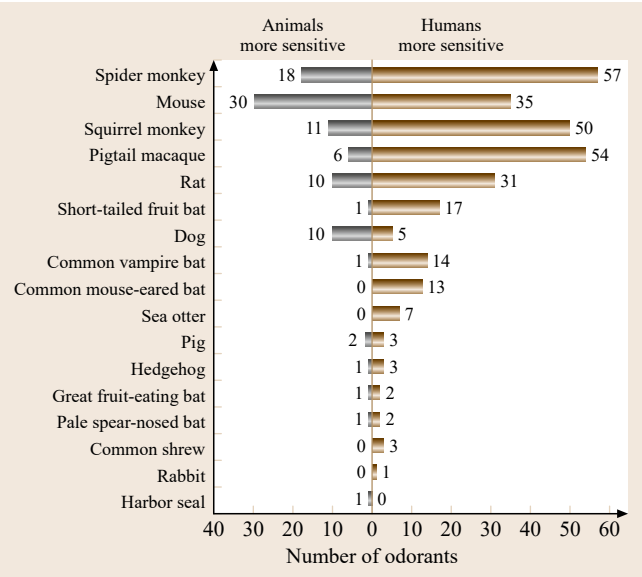

Fig. 32.9 Comparison of all olfactory detection threshold values between human subjects and animal species. Depicted are the number of odorants for which either human subjects or a given species of mammal are more sensitive (*human data*: [32.1]; *spider monkey data*: [32.11, 22, 25, 32, 42–49]; *mouse data*: [32.12, 23, 25, 33–35, 42–44, 50, 51]; *squirrel monkey data*: [32.13, 26, 36, 45–48]; *pigtail macaque data*: [32.11, 26, 36, 45–48]; *rat data*: [32.14, 27, 28, 37–39, 45]; *short-tailed fruit bat data*: [32.15]; *dog data*: [32.16, 17, 40, 52–54]; *common vampire bat data*: [32.18]; *common mouse-eared bat data*: [32.29]; *sea otter data*: [32.19]; *pig data*: [32.30, 31, 55]; *hedgehog data*: [32.20]; *great fruit-eating bat data*: [32.18]; *pale spear-nosed bat data*: [32.18]; *common shrew data*: [32.21]; *rabbit data*: [32.41]; *harbor seal data*: [32.56])

other mammalian species. With only two exceptions (spider monkeys and squirrel monkeys with *n*-butyl acetate), human subjects have lower olfactory detection thresholds, that is, a higher sensitivity for these odorants than all other mammal species tested, including dogs, mice, and rats. With only few exceptions, humans are also more sensitive for aliphatic acetic esters than spider monkeys, squirrel monkeys, and pigtail macaques. This is remarkable given that these nonhuman primates are highly frugivorous suggesting that a high olfactory sensitivity for fruit-associated odorants such as acetic esters should be adaptive for these species. However, both human and nonhuman primates generally outperform granivorous species, such as rats, insectivorous species such as bats, and herbivorous species such as the rabbit with this class of odorants.

Figure 32.9 summarizes all comparisons of olfactory detection thresholds between human subjects and other mammal species. Depicted are the number of odorants for which either human subjects or a given species of mammal are more sensitive. With the exception of the dog (and the harbor seal, which has been tested with only one odorant), human subjects have lower olfactory detection thresholds, that is, a higher sensitivity with the majority of odorants tested so far compared to all other mammal species tested so far. This includes species traditionally considered to have a highly developed sense of smell, such as mice, rats, hedgehogs, shrews, pigs, and rabbits.

It is interesting to note that humans outperform even the dog, often considered as the undisputed supernose of the animal kingdom, with 5 of the 15 odorants tested with both species. The fact that these 5 odorants comprise plant odor components such as β-ionone and *n*-pentyl acetate suggests that the behavioral relevance of odorants rather than neuroanatomical or genetic features may strongly affect a species' olfactory sensitivity. This idea is further supported by the fact that 7 of the 10 odorants for which the dog has been reported to be more sensitive than humans comprise carboxylic acids and thus typical components of the odor of prey species of the dog.

Thus, based on these comparisons, and contrary to traditional textbook wisdom, humans are not generally inferior in their olfactory sensitivity compared to animals.

32.2 Olfactory Discrimination Ability

Olfactory discrimination can be defined as the ability to reliably respond differently to the successive presentation of two nonidentical odorants. Thus, olfactory discrimination is usually assessed by determining the proportion of correct responses with repeated presentations of a given pair of odorants. A statistical criterion can then be used to decide whether a human subject or an animal is able to discriminate between the two odorants in question or not.

Human studies on olfactory discrimination capabilities usually either employ structurally related

Table 32.2 Between-species comparison of olfactory discrimination performance with aliphatic odorants sharing the same functional group but differing in carbon chain length

	Human subjects	Squirrel monkeys	Asian elephants	Fur seals	CD-1 mice	Honey bees
1-Alcohols	+++++−	−øø−+ø	øø +ø++	+++ø++	++++++	+++++−
n-Aldehydes	−++−++	øø+ø++	øø+ø++	+++ø++	++++++	++++++
2-Ketones	−+++++	øø+ø++	øø+ø++	+++ø++	++++++	++++++
Acetic esters	−+++++	øø+ø++	øø+ø++	+++ø−+	++++++	øøøøøø
n-Carboxylic acids	++++++	+øø++ø	øø+ø++	+++ø++	++++++	øøøøøø
Success rate	**25/30**	**13/15**	**15/15**	**24/25**	**30/30**	**17/18**

A "+" symbol indicates that the group of human subjects or animals succeeded in discriminating a given aliphatic odor pair, a "−" symbol indicates failure to do so, and a ø symbol indicates that this odor pair was not tested. The six symbols in each table cell refer to the discrimination of carbon chain lengths C_4 versus C_5, C_4 versus C_6, C_4 versus C_7, C_5 versus C_6, C_5 versus C_7, and C_6 versus C_7, respectively. *Human data*: [32.58–60]; *squirrel monkey data*: [32.59, 61, 62]; *Asian elephant data*: [32.63, 64]; *fur seal data*: [32.65]; *mouse data*: [32.66]; *honeybee data*: [32.67]

Table 32.3 Between-species comparison of olfactory discrimination performance with aliphatic odorants sharing the same carbon length but differing in functional group

	Human subjects	Squirrel monkeys	CD-1 mice	Honey bees
1-Alcohols versus *n*-aldehydes	+++	+++	+++	+++
1-Alcohols versus 2-ketones	+++	+++	+++	+++
1-Alcohols versus *n*-carboxylic acids	+++	+++	+++	øøø
n-Aldehydes versus 2-ketones	+++	+++	+++	+++
n-Aldehydes versus *n*-carboxylic acids	+++	+++	+++	øøø
2-Ketones versus *n*-carboxylic acids	+++	+++	+++	øøø
Success rate	**18/18**	**18/18**	**18/18**	**9/9**

A "+" symbol indicates that the group of human subjects or animals succeeded in discriminating a given aliphatic odor pair, a "−" symbol indicates failure to do so, and a ø symbol indicates that this odor pair was not tested. The three symbols in each table cell refer to the discrimination of odorants that share chain lengths of either 4, or 6, or 8 carbon atoms, respectively. *Human data*: [32.68]; *squirrel monkey data*: [32.69]; *mouse data*: [32.66]; *honeybee data*: [32.67]

monomolecular odorants (to investigate correlations between structure and perceived quality of odorants), or complex odor mixtures of commercial use such as fragrances (perfumes, body care products) or food odors (wines, coffees, artificial flavors). In contrast, the vast majority of animal studies assessing olfactory discrimination capabilities employ naturally occurring complex odor mixtures that are behaviorally relevant for the species under study such as conspecific body odors, species-typical food odors, or predator odors. As a consequence, there is only little overlap in the stimuli used between human and animal studies on olfactory discrimination. However, at least a few animal species have also been studied for their ability to distinguish between some of the structurally related monomolecular odorants tested with humans.

Table 32.2 compares the ability of humans and several species of animals to discriminate between members of homologous series of aliphatic odorants. These are odorants sharing the same oxygen-containing functional group (e.g., an alcohol- or an aldehyde group) but differing in carbon chain length. With this set of odorants, the proportion of successfully discriminated odor pairs is slightly lower in humans compared to squirrel monkeys, fur seals, honey bees, Asian elephants, and mice. Nevertheless, humans succeeded with more than 80% of these odor pairs, which are both structurally and qualitatively similar to each other.

Table 32.3 compares the ability of humans and several species of animals to discriminate between aliphatic odorants sharing the same carbon chain length but differing in functional group. With this set of odorants, not only squirrel monkeys, mice, and honey bees, but also human subjects successfully discriminated between all odor pairs tested.

Table 32.4 compares the ability of humans and several species of animals to discriminate between enantiomers. These are pairs of molecules with mirror-image structures that exhibit identical chemical and physical properties except for their optical activity, that is, rotation of polarized light. They are particularly useful for assessing how molecular structure is encoded by olfactory systems as perceptual differences between enantiomers cannot be caused by differing

Table 32.4 Between-species comparison of olfactory discrimination performance with enantiomers

	Human subjects	Squirrel monkeys	Asian elephants	Fur seals	CD-1 mice	Honey bees	Pigtail macaques	SD/LE rats
Carvone	+	+	+	+	+	+	+	+
Dihydrocarvone	+	+	+	+	+		+	
Dihydrocarveol	+	+	+	+	+		+	
Limonene	+	+	+	−	+	+	+	+
α-Pinene	+	+	+	+		+		
Isopulegol	−	−	+	−	+		+	
Menthol	−	−	+	+	+	+		
β-Citronellol	−	−	+	+	+	+		
Rose oxide	−	−	+	−	+	−		
Fenchone	−	+	+	+	+	−		+
Limonene oxide	−	−	+	+	+		−	
Camphor	−	−	+	−	+	−		
Success rate	**5/12**	**6/12**	**12/12**	**8/12**	**11/11**	**5/8**	**5/6**	**3/3**

A "+" symbol indicates that the group of animals or subjects succeeded in discriminating a given enantiomeric odor pair, and a "−" symbol indicates failure to do so. *Human data*: [32.70, 71]; *squirrel monkey data*: [32.72, 73]; *Asian elephant data*: [32.63]; *fur seal data*: [32.74]; *mouse data*: [32.75]; *honeybee data*: [32.76]; *pigtail macaque data*: [32.73]; *rat data*: [32.77, 78]

diffusion rates in the mucus covering the olfactory epithelium or differing air–mucus partition coefficients, but must originate from chiral selectivity at the receptor level [32.79].

Human subjects, as a group, are only able to discriminate between 5 of the 12 enantiomeric odor pairs tested and thus perform similar to squirrel monkeys, which succeeded with 6 out of 12 pairs. Asian elephants, mice, and rats, in contrast, succeeded with 12 out of 12, 11 out of 11, and 3 out of 3 of these odor pairs. South African fur seals, pigtail macaques, and honeybees are able to discriminate between the majority, but not all enantiomeric odor pairs tested with these species (Table 32.4).

Based on these comparisons, humans appear to have a slightly inferior olfactory discrimination ability compared to some species such as mice and Asian elephants. However, their performance in discriminating between structurally related monomolecular odorants appears to be comparable to that of squirrel monkeys, fur seals, and honeybees.

A special, and only rarely investigated, aspect of olfactory discrimination performance is the ability to distinguish between different concentrations of a given odorant. The smallest concentration difference that a nose can reliably detect is often referred to as *just noticeable difference* (JND) and is usually expressed as a so-called Weber fraction (according to Weber's law, which states that the JND between two stimuli is proportional to the magnitude of the stimuli: $\Delta I/I =$ constant). A Weber fraction of 0.3, for example, indicates that a stimulus has to be presented at a 30% higher intensity, relative to a standard stimulus, in order to be reliably perceived as *stronger* than the standard.

The human JND has been found to be odorant-dependent and Weber fractions have been reported to range from 0.09 for *n*-pentyl butyrate, 0.16 for *n*-pentanol, 0.30 for pyridine, 0.35 for *n*-butanol, to 0.47 for phenylethanol [32.80–82]. The only study that directly compared JNDs between species found the Weber fraction for *n*-pentyl acetate to be 0.041 in rats and thus considerably lower than the 0.315 found with this odorant in humans [32.83]. However, a later study reported the rat's Weber fraction for the same odorant to be 0.28, and thus comparable to that of humans [32.84].

A possible explanation for the extreme paucity of animal data on olfactory JNDs is their difficulty in learning the concept that two different concentrations of the same odorant should have different reward values. To understand this difficulty, one must know that in operant conditioning procedures an animal learns that an odorant A is rewarded and that an odorant B (or a blank stimulus) is not rewarded. Once an animal has successfully learned the association between odorant A and a reward, it will consider this odorant as a rewarded stimulus irrespective of its concentration. Biologically, this makes perfect sense as odors that are behaviorally relevant for an animal hardly ever change their significance as a function of concentration: a food odor will always be attractive, whether detected at high or low concentrations, and a predator odor will be always avoided, whether at high or low concentrations. Thus, it takes an animal extensive training to overcome this perseverance with regard to the learned reward value of odorants.

32.3 Qualitative Comparisons of Olfactory Capabilities Between Species

In addition to using quantifiable measures of olfactory performance, such as sensitivity and discrimination ability, one can also try to compare the sense of smell between species using qualitative measures. In the simplest case, this means to assess whether a given species possesses a certain ability or not. To this end, it might be useful to take a look at different behavioral contexts in which the sense of smell is known to play a role for animals and then to ask whether human subjects are able to use their noses for the same purpose.

32.3.1 Gathering Information About the Chemical Environment

The ability to gather information about the chemical environment is almost ubiquitous in the animal kingdom. In most species of animals, the detection of chemical hazards, for example, leads to adaptive behavioral responses intended to minimize further exposure. Humans are no exception to this rule and display adaptive behaviors such as head turning, eye closure, apnea (suspension of breathing), sneezing, and coughing when exposed to harmful volatile chemicals. Although some of these reflex-like responses involve the nasal trigeminal system, the significance of the olfactory system in this context becomes apparent when considering anosmic subjects: humans without a functioning sense of smell run a significantly higher risk of suffering from gas poisoning [32.85] and of not detecting fire [32.86] compared to healthy controls.

32.3.2 Foraging and Food Selection

The ability to find and select food using the sense of smell is also widespread among animal species. Human babies, similar to the offspring of other mammals, already display adaptive behavioral responses such as positive head turning and gaping mouth movements when presented with the odor of their mother's breast or the odor of breast milk [32.87]. Although humans nowadays hardly ever need to use their noses to forage, that is, to search for food, they do possess the ability to follow a food odor scent trail [32.88]. The involvement of the sense of smell in human food selection is more obvious: anosmic subjects run a significantly higher risk of suffering from food poisoning compared to healthy controls with an intact sense of smell [32.89]. Similarly, the risk of suffering from malnutrition is markedly higher in anosmic subjects compared to normosmic control subjects [32.90]. Not surprisingly, anosmic subjects – particularly those who lost their sense of smell instantaneously, for example, due to head trauma – often report a considerable loss of quality of life with regard to enjoying food [32.91].

32.3.3 Spatial Orientation

Quite a number of animal species rely on their sense of smell for spatial orientation. This is particularly true for nocturnal and subterranean species. There are two basic mechanisms that allow animals to find their way through their habitat using their sense of smell: they can either use existing landmarks that emit an odor, or they can deposit scent marks themselves at certain points in their habitat. Both types of odor sources serve as navigational landmarks that can be used by animals to recognize their position in space.

Although humans with an intact sense of vision hardly ever need to use their noses for finding their way, they do possess the ability to follow scent trails laid by conspecifics and when blindfolded [32.88]. A recent study suggests the existence of spatial information processing in the human olfactory system and thus of an implicit ability for directional smelling [32.92]. Further, there is anecdotal evidence that blind persons use olfactory landmarks as sensory cues for spatial orientation [32.93, 94].

32.3.4 Social Communication

Many species of animals have been shown to use body-borne odors for social communication. Such odors may convey information about species, social group, sex, age, reproductive status, health status, social rank, individual identity, genetic relatedness, dietary habit, and emotional state of the odor donor.

Psychophysical studies have demonstrated that humans are able to correctly assign body odors to sex [32.95] and to age classes [32.96]. Similarly, it has been shown that humans are able to correctly assign body odors to reproductive status [32.97, 98] and to health status [32.99, 100].

Further, humans are able to distinguish between individual body odors [32.101] and between body odors of kin and nonkin [32.102, 103]. This includes mutual recognition of the body odors between mothers and their babies [32.104].

Humans are also able to distinguish between the body odors of vegetarians and meat eaters [32.105] and

thus they can sniff out the dietary habits of conspecifics. Recent studies suggest that humans are also able to detect emotions via body odors [32.106, 107].

Dogs, mice, and rats are not only able to discriminate between the odors of individual conspecifics, but also between the odors of individuals of other species, for example, of individual humans. However, it has been demonstrated that human subjects are also able to correctly identify the odor of their own pet dog among other dog odor samples [32.108], to correctly assign body odor samples to individual gorillas [32.109], and to correctly discriminate between the body odors of mouse strains that only differ from each other in their major histocompatibility complex (MHC) genes [32.110].

32.3.5 Reproduction

The ability to correctly identify the reproductive status of potential mates using olfactory cues is widespread among mammals. Similarly, mate choice has been shown to be based on or at least influenced by olfactory cues in a number of mammal species. Human males are able to distinguish between female body odors from different phases of the estrous cycle and prefer the body odor of females produced around the time of ovulation [32.97, 98, 111]. Several lines of evidence suggest that human mate choice may be influenced by body odors and that, as is the case in mice, MHC genes are involved in the formation of individual odor signatures [32.112–114]. A recent study found that congenitally anosmic men exhibit a strongly reduced number of sexual relationships compared to normosmic men, and that congenitally anosmic women feel less secure about their romantic partner compared to normosmic women [32.115]. Further, anosmia-related depression has been shown to reduce sexual appetite in humans [32.116].

32.3.6 Learning and Memory

Learning and memory are inevitably linked to sensory input. Many species of animals rely on olfactory cues for learning about their environment or about situational contexts as well as for building and retrieving memories. Humans are no exception to this as they are able to rapidly and robustly learn long-lasting associations between food odors and positive or negative physiological consequences of ingestion [32.117]. Similarly, humans are able to rapidly and robustly learn appetitive and aversive associations between odors and visual stimuli [32.118]. Several studies reported human long-term memory for odors to be outstanding and superior to that for other sensory modalities [32.119]. The longest interval tested so far with humans for successful odor recognition was 1 year [32.120], and for above-chance level retention of odor–name associations even 9 years [32.121].

From these qualitative comparisons of olfactory capabilities, one must conclude that humans have at least the basic ability of using their sense of smell in all behavioral contexts in which animals are known to use their noses.

32.4 General Conclusions

The amount of published data on quantifiable measures of olfactory performance, which allow for direct comparisons between humans and animals, is rather limited. However, based on this limited set of data the following conclusions can be drawn:

Human subjects have lower olfactory detection thresholds, that is, a higher sensitivity with the majority of odorants tested so far compared to most of the mammal species tested so far. This includes species traditionally considered to have a highly developed sense of smell such as mice, rats, hedgehogs, shrews, pigs, and rabbits.

Human subjects appear to have a slightly inferior olfactory discrimination ability compared to species such as mice and Asian elephants. However, their performance in discriminating between structurally related monomolecular odorants appears to be comparable to that of species such as squirrel monkeys, fur seals, and honeybees.

Qualitative comparisons of olfactory capabilities suggest that human subjects have at least the basic ability of using their sense of smell in all behavioral contexts in which animals are known to use their noses. This includes gathering of information about the chemical environment, food selection, spatial orientation, social communication, reproduction, as well as learning and memory.

Taken together, these findings suggest that the human sense of smell is not generally inferior compared to that of animals and much better than traditionally thought.

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33. Ectopic Expression of Mammalian Olfactory Receptors

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Olfactory receptors (ORs) are not exclusively detectable in the olfactory epithelium but are ectopically expressed in all other body tissues tested so far such as brain, heart, lung, testis, intestine, and skin. Within these tissues, a specific subset of ORs can be found with some of the ORs being exclusively expressed in only one specific nonolfactory tissue and other OR subsets being more widely distributed throughout different tissues of the body. It is assumed that ectopically expressed ORs, which are nothing but highly specific chemosensors, play a role in the regulation of cell–cell recognition, migration, and pathfinding processes. Additionally, they are attributed to have potential as diagnostical and therapeutical tools as ORs are differentially expressed in pathological tissues (e.g., cancer tissue). Besides the canonical signaling pathways of ORs, as found in the olfactory tissue, alternative pathways are activated in the diverse nonolfactory tissues. In this chapter, the expression and function of ORs outside the olfactory epithelium of the nose will be highlighted.

One of the criteria that led to the initial identification of olfactory receptors (ORs) was that they seemed to be exclusively expressed in the olfactory epithelium [33.1]. However, only one year after their discovery, the first OR genes were shown to be expressed ectopically in the testis of dogs [33.2]. Within the last two decades ORs were found to be ectopically expressed in every tissue investigated so far [33.3–7]. The number and types of present ORs seems to be specific to the respective tissues. However, the function of only a minor portion of ectopically expressed ORs could be revealed to date. Evidence accumulates that ORs take over specific tasks in nonolfactory cell types which might be explained by the enormous discriminating capacity of this huge G-protein coupled receptor (GPCR) family. Actually, this subcategory of ORs that are found to be ectopically expressed is considerably higher conserved between primate species than those receptors that are exclusively expressed in the olfactory epithelium. This fact points to a positive selection pressure and crucial functions in non-neuronal tissues [33.5].

33.1 Discovery of Ectopically Expressed Olfactory Receptors

Only one year after their discovery in the olfactory epithelium [33.1], the first study revealed the expression of OR genes in the dog and human testis [33.2]. Using degenerate primers, Parmentier and his coworkers

identified 3 and 21 OR transcripts that are expressed in dog and human testis, respectively. Within the following decade, numerous studies corroborated this finding and identified OR transcripts in the testis of various species, including human, dog, mouse, hamster, and rat [33.8–18]. Using antibody stainings in in situ hybridization approaches, ORs were located to late round and elongated spermatids and the midpiece of mature spermatozoa. However, although it was speculated that ORs might participate in germ cell development, in the detection of HLA peptides, and in sperm chemotaxis, none of these early studies could experimentally support a functional necessity for the ectopic expression of ORs in testicular tissue.

With the improvement of transcriptome analysis, microarrays were conducted to investigate comparative OR expression patterns revealing the presence of OR transcripts in numerous tissues of several rodents, humans, and chimpanzees [33.3, 5–7, 19]. Recently, next generation sequencing (NGS) technique was employed to operate the first comprehensive RNA-Seq expression analysis of ectopically expressed ORs in multiple human tissues [33.4]. Taken together, about 80 tissues and cell types have been investigated in several species and all of them with no exception expressed at least one OR (Table 33.1). However, the number of ORs found to be expressed in the distinct tissues varies between studies and also depends on the screening approach and the stringency of the analysis, the highest number of expressed ORs being either testis [33.3, 4] or heart and lung [33.7] or brain and pancreas [33.19].

The expression rate for the majority of ORs in nonolfactory tissue is moderate to low [33.8]. However, it is not clear whether the overall expression in the respective tissues is generally low or if only a distinct subtype of cells expresses the particular OR. Besides intact ORs, a multitude of pseudogenes are also expressed in nonolfactory tissues, in the majority of tissues exceeding the number of intact genes [33.4]. In each tissue, specific ORs showed a high expression [33.3] and in most cases, one or more OR genes were exclusively expressed in the respective nonolfactory tissue [33.3]. In contrast to the olfactory sensory neurons, which are believed to express only one type of OR [33.20], cells in nonolfactory tissues tend to express more than one individual OR gene [33.21–27].

The ectopic expression of OR genes is not correlated to their belonging to a specific structural subgroup, or to a specific gene cluster or chromosomal segment [33.3–6]. However, OR genes located adjacent to non-OR genes have a higher tendency to be expressed in nonolfactory tissues than others [33.3, 4]. This suggests that the genomic environment has an influence on the OR expression. It was also shown that OR genes are post-translational modified by alternative splicing in the 5′-untranslated region (5′-UTR) [33.4, 17, 28]. In some cases, OR genes might even be differentially processed in the olfactory epithelium and in the nonolfactory tissue [33.8, 15]. However, this is not a general phenomenon as other ORs were found to utilize the same initiation site for transcripts in the olfactory epithelium (OE) and other tissues [33.17, 29].

33.2 Functionality of Ectopically Expressed ORs

Since their first discovery, considerable controversy exists about the functionality of ectopically expressed ORs. One point that led to questioning the functionality of ORs in nonolfactory tissues in the past was that orthologous expression profiles did not always correlate well in contrast to that of other gene families. In one study, 64 OR pairs of human and mouse were compared in two tissues and only few orthologous OR pairs were found to be expressed in the same tissues [33.3]. The authors claim that this could argue in favor of neutral or nearly neutral transcription mechanisms, e.g., small DNA sequence changes in regulatory regions, fixed in the population by random drift and not necessarily related to function or fitness. An additional possibility would be that ectopic OR transcription is due to leaky promoters [33.3, 7] as gene activity is not necessarily related to gene function [33.30]. In a few hundred million years, a relatively short time on the evolutionary scale, humans silenced two-thirds of all the genes for ORs that are present in other higher mammals, and converted them to nonfunctioning pseudogenes. Having this rapid evolvement in mind, the genetic distance between rodents and humans might be too big. In contrast, orthologous OR genes of humans and chimpanzees are expressed in the same nonolfactory tissues more often than expected by chance alone [33.5], which underlines the notion that ectopically expressed ORs have additional functions. Furthermore, some orthologous genes that exhibited conserved patterns in nonolfactory tissues evolved under stronger evolutionary constraint than those that were expressed exclusively in the nose [33.5]. This again favors the assumption that ectopically expressed OR genes are functional. Regarding the numerous studies that revealed several distinct functions of ectopically expressed ORs in the past years, the question about their functionality seems to be clarified.

33.3 Olfactory Receptors as Cell–Cell Recognition Molecules

Among the proposed functions for ectopically expressed ORs is the cell–cell recognition and organ construction during development [33.31, 32]. In line with this, ORs are expressed in the developing heart [33.33] and spleen [33.34] and in the chicken notochord where they were suggested to play a role in proper positioning of the neural tube and the somatic mesoderm [33.35]. This led to the speculation that ORs may be the *last digits in a cell surface code for assembling embryos* [33.36, 37]. According to this theory, it is assumed that the precision of cell migration and tissue assembly requires a complex addressing system. Each cell would be equipped with a hierarchic repertoire of recognition molecules encoding the cell identity that enables the cells to find the appropriate position and interaction partners. As this task would require an enormous number of similar molecules, the large superfamily of ORs has been considered as possible candidates serving this function [33.36].

33.4 Olfactory Receptors in Migration and Pathfinding Processes

Another proposed function for ectopically expressed OR genes is that in migration and pathfinding processes. It has been shown that axonal OR expression is crucial for the axon guidance and correct wiring of the glomeruli in the olfactory bulb [33.38, 39]. Spermatozoa swim up a gradient of OR ligands (bourgeonal or lyral) which might be of importance for the localization of the oocyte in the female genital tract [33.23, 25, 26, 40]. Moreover, myocytes' migration during muscle regeneration seems to be influenced by the MOR23 ligand lyral [33.41]. Similar, it was suggested that ORs play a role in the migration of primordial germ cells to the developing gonad [33.42] and participate in proper positioning of the chicken neural tube and somatic mesoderm [33.35].

33.5 Diagnostical and Therapeutical Potential of Olfactory Receptors

Multiple studies about ectopically expressed ORs suggest a significant diagnostical and therapeutical potential for this receptor family that has been pharmacologically underestimated so far. Activation of ORs in enterochromaffin (EC) cells of the human intestine leads to food-induced release of serotonin and regulation of the cytosolic calcium balance [33.21, 43]. Serotonin secretion is implicated in pathologic conditions such as vomiting, diarrhea, and irritable bowel syndrome. Thus, targeting ORs of the EC cells might serve as pharmacologic therapy of irritable bowel syndrome, of conditions such as diarrhea with vomiting caused by anticancer therapy, and of other gastrointestinal disorders [33.21]. Moreover, ORs expressed in the rat colon have been implicated in anion secretion of the mucosa and the increase of the permeability to substances such as dextran [33.44]. ORs present in the rat duodenal enterocytes seem to be significantly upregulated in obesity when a high fat diet is consumed [33.45]. These receptors may play a role in the sensing and regulation of dietary fat, and may be important for the individual susceptibility to obesity. Altogether, ORs in the gastrointestinal tract could be relatively accessible targets for future therapeutics for obesity, diabetes, and malabsorption syndromes.

A further functional relevance of ORs has been suggested in human blood cells. Down-regulation of OR expression in peripheral blood mononuclear cells (PBMC) has been correlated with traumatic brain injury [33.27]. The degree of downregulation is tightly correlated with the severity of the brain injury. The combinatorial expression pattern of two ORs (OR4M1 and OR11H1) in PBMC provides a reliable criterion for segregating traumatic brain injury patients from control cases with 90% accuracy and 100% specificity [33.27].

Furthermore, also in human skin ORs seem to play a significant role. Activation of OR2AT4 promotes cell proliferation and migration and as a result accelerates wound healing in human keratinocytes [33.22]. Odorant stimulation of human keratinocytes also induces adenosine triphosphate (ATP) and cytokine release [33.22, 46]. Javanol-induced ATP release was shown to activate trigeminal neurons via Pannexins [33.46].

In the murine kidney, ORs play a role in the regulation of renin secretion and the glomerular filtration rate [33.47–49]. Olfr78 (the mouse ortholog of OR51E2/PSGR) activation by the short-chain fatty acid

propionate was shown to cause a dose-dependent drop in blood pressure and *Olfr78* knockout mice have significantly lower plasma renin levels and baseline blood pressure compared with wild-type littermates [33.49]. However, the propionate-mediated blood-pressure drop is intensified in *Olfr78* knockout mice indicating that Olfr78 activation antagonizes (rather than mediates) the acute hypotensive effects of propionate [33.49].

Interestingly, a lot of ORs were also found in carcinoma tissue in which they usually seem to be higher expressed compared to the corresponding normal healthy tissue [33.50–57]. Class I receptor OR51E2 is probably the receptor that is investigated the most. It was first discovered to be highly expressed in the prostate and thus initially named prostate-specific G-protein coupled receptor (PSGR). Later it was found to belong to the superfamily of ORs. The expression of OR51E2 and the closely related OR51E1 (PSGR2) is upregulated in prostate carcinoma cells in comparison to normal prostate epithelial cells. Consequently, these ORs have been discussed as potential biomarkers for identification of prostate tumors [33.54–59]. Matsueda and coworkers developed an immunotherapeutic concept for cancer treatment based on these findings: imprinting of CD8+T cells on OR51E2 peptide sequence recognition allows targeted elimination of OR51E2-expressing tumor cells [33.60]. Latest studies reveal that overexpression of OR51E2 in the xenograft mouse model promotes the development of prostate neoplasia and tumors [33.61]. Activation of OR51E2 by its specific ligands β-ionone and the steroid hormone 1,4,6-androstatriene-3,17-dione (ADT) inhibited proliferation of human prostate cancer cells in vitro [33.58]. ADT inhibits aromatization of androgens to estrogens and it has already been used as pharmacological treatment of prostate and other carcinoma [33.62]. Moreover, it has been known for years that the isoprenoid β-ionone has an anti-proliferative effect on carcinoma cells [33.63–66] and it seems very likely that this effect is at least partially mediated via OR51E2. However, the terpenoid β-ionone might have additional roles in vivo as application of β-ionone seems to promote metastases formation of induced prostate tumors in xenograft mouse models [33.67]. OR51E2 is not exclusively expressed in the prostate but was identified also in various other human tissues [33.3, 4, 60, 68, 69], although generally at lower expression rates. Nonetheless, the widespread expression of OR51E2 might hamper the idea to establish an anti-cancer therapy using OR51E2 as a target. OR51E1 was also suggested as a biomarker for small intestine neuroendocrine carcinoma (SI-NEC) cells and lung carcinoma cells [33.50, 52, 70]. Furthermore, OR1A2 and OR1A1 are expressed in hepatocellular carcinoma cells [33.24, 71]. Again, proliferation of carcinoma cells was inhibited by activation of OR1A2 by its ligand (-)-citronellol [33.24]. In a genome-wide association study, OR4F15 was found to be among the top genes associated with salivary gland carcinoma in humans [33.72]. Despite the known overexpression of ORs in different kinds of carcinoma cells, data on their functional role remain sparse. OR2A4 and OR1A2 have been shown to be expressed in human cervical carcinoma cells (HeLa cells). Here they seem to play a role in cytokinesis as shown by RNAi studies [33.73]. OR2A4 knockdown resulted in 4–10-fold increase of bi- or multinucleated cells, possibly by exerting a regulatory role on the actin cytoskeleton [33.73].

GPCRs and their downstream signaling cascades play a pivotal role in the pharmacological treatment of diseases such as cancer [33.74]. Multiple studies demonstrate an overexpression and a functional role of GPCRs in human carcinoma of lung, skin, liver, and the intestine [33.75–77]. Strikingly, despite the fact that more than 25% of pharmacological tools target GPCRs [33.76], relatively few cancer treatments target GPCRs [33.75]. In the mammalian genome, ORs by far represent the largest group within the GPCR gene superfamily. This in combination with the known dysregulation of certain ORs in malignant tissues, renders ORs as promising markers and potential therapeutical targets in cancer treatment.

Besides the known connection to cancer, ectopically expressed ORs have also been implicated in the development and progression of neurodegenerative diseases such as Alzheimer's disease, Creutzfeldt–Jakob, progressive supranuclear palsy and Parkinson's disease [33.78, 79]. Four of eight ORs that are expressed in the frontal and entorhinal cortex have been shown to be dysregulated in Alzheimer's disease and the degree of dysregulation increased with the progression of the disease [33.78]. In Parkinson's disease (PD), OR expression is altered (mainly downregulated) even at premotor stages indicating that OR expression may serve as an early indicator of PD [33.79].

Despite the obvious potential of ectopically expressed ORs in development of medical treatment, knowledge about the function of ORs in both normal and pathological tissues remains sparse. The few existing studies point to involvement of ectopically expressed ORs in the regulation of growth processes, being particularly relevant in tumor formation. Recently, ORs were identified as specific targets for the anesthetic ketamine [33.80]. This again underlines the role of ORs as promising drug targets.

Table 33.1 Olfactory receptors expressed in diverse nonolfactory tissues

Tissue/cell type Reproductive system	Species	Olfactory receptors	Putative function/signaling	Detection method	References
Testis	Dog	DTMT (OR1E2)	Not determined	RT-PCR, NB RNase protection assay, ICC	*Parmentier* et al. [33.2], *Vanderhaeghen* et al. [33.12]
Testis	Rat	Olr825, Olr1696	Not determined	RT-PCR, WB, ICC	*Walensky* et al. [33.16]
Testis	Mouse	MOR23 (OR10J5)		RT-PCR, RNase protection assay	*Asai* et al. [33.8]
Testis	Human, dog, rat, mouse	Various ORs	Not determined	RT-PCR, RNase protection assay	*Vanderhaeghen* et al. [33.13, 14]
Testis	Human	Various ORs	Not determined	RT-PCR, ICC	*Ziegler* et al. [33.18]
Testis/spermatozoa	Human	OR1D2	Chemotaxis	RT-PCR, Ca^{2+} imaging, chemotaxis assay	*Spehr* et al. [33.40]
Testis	Human	Various HLA-linked ORs	Not determined	RT-PCR	*Volz* et al. [33.15]
Testis/spermatozoa	Mouse	MOR23 (OR10J5)	Chemotaxis	RT-PCR, ISH, Ca^{2+} imaging, TG mouse model	*Fukuda* et al. [33.23]
Testis	Mouse	Various ORs	Not determined	Microarray	*Zhang* et al. [33.6]
Testis (spermatids & spermatocytes)	Mouse	Various ORs	Not determined	RT-PCR, ISH	*Fukuda* and *Touhara* [33.28]
Testis	Human, mouse	Various ORs	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]
Testis	Human	Various ORs (83)	Not determined	Microarray	*Zhang* et al. [33.7]
Testis	Chimpanzee	Various ORs (11)	Not determined	Microarray	*De La Cruz* et al. [33.5]
Testis/spermatozoa	Human	OR4D1, OR7A5		RT-PCR, Ca^{2+} imaging	*Veitinger* et al. [33.25, 26]
Testis	Human	Various ORs (55)	Not determined	NGS	*Flegel* et al. [33.4]
Prostate, prostate carcinoma	Human	OR51E2 (PSGR)	Not determined	NB, RT-PCR, ISH	*Xu* et al. [33.56]
Prostate, prostate carcinoma	Human	OR51E2 (PSGR)	Not determined	NB, RT-PCR	*Xia* et al. [33.55]
Prostate, prostate carcinoma	Human, mouse, rat	OR51E2 (PSGR)/ Olfr78 (MOL2.3)/ Ra1c	Not determined	NB, RT-PCR	*Yuan* et al. [33.69]
Prostate, prostate carcinoma	Human	OR51E2 (PSGR)	Not determined	RT-PCR, ISH	*Weng* et al. [33.81, 82]
Prostate, prostate carcinoma	Human	OR51E1 (PSGR2)	Not determined	RT-PCR, ISH, NB	*Weng* et al. [33.54]
Prostate, prostate carcinoma	Human	OR51E2 (PSGR)	Not determined	RT-PCR	*Cunha* et al. [33.68]
Prostate carcinoma, LNCaP	Human	OR51E2 (PSGR)	Inhibition of proliferation via MAPK pathway	Ca^{2+} imaging, RT-PCR, WB, ICC, siRNA,	*Neuhaus* et al. [33.58]
Prostate carcinoma, LNCaP	Human	OR51E2 (PSGR)	Src kinase-dependent influx of Ca^{2+} via TRPV6	Patch-clamp, Ca^{2+} imaging, siRNA	*Spehr* et al. [33.83]
Prostate	Human	OR51E2 (PSGR)	Not determined	NGS	*Flegel* et al. [33.4]

Table 33.1 (continued)

Tissue/cell type	Species	Olfactory receptors	Putative function/signaling	Detection method	References
Reproductive system					
Prostate carcinoma, LNCaP	Human	OR51E2 (PSGR)	Phosphorylation of Pyk2, p38 & NDGR1	Phosphoproteomics, mass spectrometry	*Wiese* et al. [33.84]
Primordial germ cells	Human	HT2, OR1D4, OR7E24	Not determined	RT-PCR	*Goto* et al. [33.42]
Placenta	Rat	Olr1513, Olr1571, Olr1687, Olr1767	Not determined	RT-PCR	*Itakura* et al. [33.32]
Placenta	Mouse	Olfr154, Olfr433, Olfr520, Olfr1381	Not determined	Microarray	*Mao* et al. [33.85]
Uterus	Human, mouse	Various ORs	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]
Ovary	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]
Breast	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]
Digestive system					
Enterochromaffin cells	Human	OR1A1; OR1G1; OR1E3; OR5D18	Serotonin secretion	RT-PCR, Ca^{2+} imaging, ELISA, amperometry	*Braun* et al. [33.21]
Enterochromaffin cells	Human	OR1G1	Serotonin secretion	RT-PCR, Ca^{2+} imaging, ELISA	*Kidd* et al. [33.43]
Colon, small intestine	Human	Various ORs	Not determined	Analysis of microarray data	*Ichimura* et al. [33.19]
Small intestine neuroendocrine carcinoma	Human	Various ORs	Not determined	Microarray, RT-PCR	*Leja* et al. [33.70]
Colon	Human, rat	OR1G1	TRPA1-mediated Ca^{2+} influx	RT-PCR, short circuit current measurements	*Kaji* et al. [33.44]
Small intestine neuroendocrine carcinoma	Human	OR51E1	Not determined	RT-PCR, ICC	*Cui* et al. [33.50]
Colon	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]
Duodenal enterocytes	Rat	Olr1744, Olr50, Olr124, Olr1507	Not determined	Microarray	*Primeaux* et al. [33.45]
Pancreas	Human, mouse	Various Ors	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]
α-cells of pancreatic islets	Mouse	Various ORs (15)	Regulation of glucagon secretion	Microarray, RT-PCR	*Kang* et al. [33.86]
Insulin-secreting cell line (MIN6), spleen	Mouse	OL2	Not determined	RT-PCR	*Blache* et al. [33.34]
Spleen	Rat	Ra1c	Not determined	RT-PCR	*Raming* et al. [33.87]
Tongue	Rat	Olr1867	Not determined	RT-PCR	*Abe* et al. [33.88]

Table 33.1 (continued)

Tissue/cell type	Species	Olfactory receptors	Putative function/signaling	Detection method	References
Digestive system					
Tongue	Human	OR1E1; OR8B8; OR5P3; OR8D1; OR8D2; OR10A5	Not determined	RT-PCR	*Gaudin* et al. [33.89]
Tongue	Human	OR6Q1; OR10A4; OR7A5; OR2K2; OR5P2	Not determined	RT-PCR	*Durzyński* et al. [33.31]
Tongue	Mouse	Olfr20	Not determined	RT-PCR	*Gaudin* et al. [33.90]
Glands					
Thyroid	Human, mouse	Various ORs	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]
Thyroid	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]
Parafollicular cells of the thyroid	Mouse	Olfr544, olfr558, olfr1386, olfr1392, Olfr78, Olfr181, Olfr288	Not determined	RT-PCR, ICC, IP, WB	*Kang* et al. [33.91]
Thymus, adrenal gland	Human	Various ORs	Not determined	Analysis of microarray data, RT-PCR	*Ichimura* et al. [33.19]
Medullary thymic epithelial cells of the thymus	Mouse	Olfr39, olfr181, Olfr325, Olfr378	Not determined	RT-PCR, ICC, IP, WB	*Kang* et al. [33.91]
Adrenal gland, lymph node	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]
Salivary gland	Human, mouse	Various ORs	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]
Salivary gland	Human	OR4F15	5 SNPs associated with risk of SGC	Genotyping	*Xu* et al. [33.72]
Cardiovascular system					
Heart	Rat	Olr1654	Not determined	RT-PCR, ISH	*Drutel* et al. [33.33]
Heart	Rat	Olr1654	Not determined	RT-PCR	*Ferrand* et al. [33.92]
Heart	Mouse	MOR2.3	Not determined	TG mouse MOL2.3-IGITL	*Weber* et al. [33.93]
Heart	Mouse	Various ORs	Not determined	Microarray	*Zhang* et al. [33.6]
Heart, atrioventricular node	Human	Various ORs	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]

Table 33.1 (continued)

Tissue/cell type	Species	Olfactory receptors	Putative function/signaling	Detection method	References
Cardiovascular system					
Heart	Human	Various ORs (108)	Not determined	Microarray	*Zhang* et al. [33.7]
Heart	Chimpanzee	Various ORs (24)	Not determined	Microarray	*De La Cruz* et al. [33.5]
Heart	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]
Aorta, coronary artery, umbilical vein endothelial cells (HUVEC)	Human	OR10J5	Migration of HUVEC	Ca^{2+} imaging, WB, cell migration assay	*Kim* et al. [33.94]
Erythroid cells	Human, mouse	OR52A1 / murine ortholog	Not determined	RT-PCR, RNase protection assay	*Feingold* et al. [33.95]
Peripheral blood mononuclear cells (PBMC)	Human	OR52N5, OR11H1, OR4D10, OR2M1P, OR51L1, OR2J3, OR4M1, OR4Q3	Downregulation in traumatic brain injury	Microarray, RT-PCR	*Zhao* et al. [33.27]
Leukocytes	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]
Leukocytes	Human	Various ORs	Not determined	RT-PCR	*Malki* et al. [33.96]
Pulmonary system					
Lung	Mouse	MOL2.3	Not determined	TG mouse MOL2.3-IGITL	*Weber* et al. [33.93]
Lung	Mouse	Mouse ortholog of S25/mJCG1	Not determined	RT-PCR	*Gaudin* et al. [33.90]
Lung	Human, mouse	Various ORs	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]
Lung	Human	Various ORs	Not determined	Analysis of microarray data, RT-PCR	*Ichimura* et al. [33.19]
Lung	Human	Various ORs (93)	Not determined	Microarray	*Zhang* et al. [33.7]
Lung	Chimpanzee	Various ORs (6)	Not determined	Microarray	*De La Cruz* et al. [33.5]
Lung cancer cells	Human	OR51E1	Not determined	RT-PCR, ICC	*Giandomenico* et al. [33.52]
Airway tissue and pulmonary macrophages	Mouse	OR1014, OR657, OR622, OR568, OR446, OR352, OR272, OR65	Microarray, RT-PCR, ICC, cell migration assay	Microarray, RT-PCR, ICC, cell migration assay	*Li* et al. [33.97]
Lung	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]

Table 33.1 (continued)

Tissue/cell type	Species	Olfactory receptors	Putative function/signaling	Detection method	References
Kidney					
Pulmonary neuroendocrine cells (PNECs); human tracheobronchial epithelial cells (hTECs)	Human	OR2F1, OR2W1, OR2H3 (32 ORs)	Decrease in serotonin secretion, release of CGRP	Microarray, ICC	*Gu* et al. [33.98]
Kidney	Human, mouse	Various ORs	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]
Kidney	Human	Various ORs	Not determined	Analysis of microarray data, RT-PCR	*Ichimura* et al. [33.19]
Kidney (distal nephrons)	Mouse	Olfr78, Olfr90, Olfr1373, Olfr1392, Olfr1393	Modulation of renin secretion and glomerular filtration rate	RT-PCR, WB, ICC	*Pluznick* et al. [33.48]
Kidney (renal juxtaglomerular apparatus)	Mouse	Olfr78 (ortholog of OR1E2)	Induction of renin secretion, blood pressure control	Olfr78^{-1-} mouse, RT-PCR	*Pluznick* et al. [33.49]
Kidney	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]
Liver					
Liver	Mouse	Various ORs	Not determined	Microarray	*Zhang* et al. [33.6]
Liver	Human, mouse	Various ORs	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]
Liver	Human	Various ORs	Not determined	Analysis of microarray data, RT-PCR	*Ichimura* et al. [33.19]
Liver	Human	Various ORs (58)	Not determined	Microarray	*Zhang* et al. [33.7]
Liver	Chimpanzee	Various ORs (74)	Not determined	Microarray	*De La Cruz* et al. [33.5]
Liver	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]
Hepatocellular carcinoma cell line	Human	OR1A2, OR8B3	Inhibition of proliferation (MAPK pathway, cAMP-dependent Ca^{2+} influx)	Ca^{2+} imaging, ICC, cAMP assay, Proliferation assay, PI staining, WB, siRNA, RT PCR	*Maßberg* et al. [33.24]
Hepatocellular carcinoma cell line	Human	OR1A1	cAMP, accumulation, PKA activation	RT-PCR, ICC, Ca^{2+} imaging, cAMP/PKA assay, lipid analysis, siRNA	*Wu* et al. [33.71]
Nervous system					
Brain	Rat	Ra1c (OR51E2)	Not determined	ISH, NB	*Raming* et al. [33.87]
Medulla oblongata	Mouse	MOL2.3, MOL8.17, MOL10.8	Not determined	TG mouse MOL2.3-IGITL	*Conzelmann* et al. [33.99] 2000)
Autonomic ns	Mouse	MOL2.3	Not determined	TG mouse MOL2.3-IGITL	*Weber* et al. [33.93]
Cortex	Mouse	Olfr151, Olfr49, Olfr15	Not determined	RT-PCR, ISH	*Otaki* et al. [33.29]

Table 33.1 (continued)

Tissue/cell type	Species	Olfactory receptors	Putative function/signaling	Detection method	References
Nervous system					
Cerebellum	Mouse	Various ORs	Not determined	Microarray	*Zhang* et al. [33.6]
Brain, spinal cord	Human, mouse	Various ORs	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]
Brain	Human	Various ORs	Not determined	Analysis of microarray data, RT-PCR	*Ichimura* et al. [33.19]
Brain	Human	OR11H1, OR4M1, OR52N5	OR4M1: Attenuation of abnormal tau phosphorylation after traumatic brain injury	Microarray, RT-PCR, WB	*Zhao* et al. [33.27]
Brain	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]
Trigeminal ganglia, dorsal root ganglia	Mouse	Various ORs (98 and 33)	Not determined	NGS	*Manteniotis* et al. [33.100]
Cortex	Human	OR2L13, OR1E1, OR2J3, OR52L1, OR11H1	Dysregulation in Parkinson's Disease	RT-PCR, microarray, ICC, WB, Lipid raft isolation	*Garcia-Esparcia* et al. [33.79]
Entorhinal and frontal cortex, cerebellum	Human, mouse	Olfr110	Dysregulation in several neurodegenerarive diseases	RT-PCR	*Ansoleaga* et al. [33.78]
Muscle					
Skeletal muscle	Mouse	Various ORs	Not determined	Microarray	*Zhang* et al. [33.6]
Skeletal muscle	Human, mouse	Various ORs	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]
Skeletal muscle	Mouse	MOR23 (12 other ORs)	Myocyte migration, muscle regeneration (cAMP-dependent)	RT-PCR, ICC, cAMP-Assay, cell migration and adhesion assay	*Griffin* et al. [33.41]
Skeletal muscle	Human	Various ORs	Not determined	NGS	*Flegel* et al. [33.4]
Skin					
Skin	Human, mouse	Various ORs	Not determined	Analysis of microarray and EST data	*Feldmesser* et al. [33.3]
Keratinocytes, HaCaT	Human	OR2AT4 (OR6M1, OR5V1, OR11A1, OR6V1)	Proliferation, migration and wound healing (cAMP-dependent)	RT-PCR, ICC, Ca2+ imaging, wound scratch assay, proliferation assay, siRNA	*Busse* et al. [33.22]
Bladder					
Bladder	Mouse	Olfr544, olfr558, olfr1386, olfr1392, Olfr78, Olfr181, Olfr288	Not determined	RT-PCR, ICC, IP, WB	*Kang* et al. [33.91]

33.6 Endogenous Ligands for Ectopically Expressed Olfactory Receptors

Most groups that have identified OR–ligand pairs so far, have used chemical libraries for deorphanization [33.40, 67, 101–103]. A great proportion of these ligands are synthetically produced and do not occur in body fluids. When it comes to ectopically expressed ORs inside our body, the question arises what are the ligands of these receptors and how do they reach their respective receptors. A lot of odorants that are also detected by the OE are included in our food, especially in plant products. The known OR ligand, β-ionone, for example, is an isoprenoid widely found in plants and plant products as a degradation product of carotenoids [33.104]. Ingredients of spices known to activate specific ORs are, for example, thymol from thyme, eucalyptol from basil, rosemary, ginger and other herbs and eugenol, a chemical component from clove and nutmeg. Eugenol and other odorants are not only present in spices but also are ingredients of most cosmetics, perfumes, detergents, deodorants, cigarettes, and others and we are constantly exposed not only via the nose but also directly through food ingestion and via skin and lung. It is known that odorants enter the blood stream within minutes [33.105] and thus, are transported to their site of action in the various tissues. Around 20 min after percutaneous exposure the plasma concentration of linalool and linalylacetat was 121 and 100 ng ml^{-1}, respectively [33.105]. Roughly 1–5 h after ingestion of an eucalyptol capsule, which is commonly prescribed as an expectorant, eucalyptol can be detected in breath gas [33.106], demonstrating the wide distribution of ingested odorants throughout the whole body. Furthermore, metabolites of eucalyptol were identified in human breast milk in concentrations of up to 100–250 µg kg^{-1} milk [33.107], indicating that odorants from the mother's food and their metabolites are passed on to the breastfed child.

After consumption of asparagus, a variety of odorous molecules can be identified in the urine [33.108]. Interestingly, the number and identity of odorants present in human urine seems to depend on the individual's health status. Fourteen different odorants were found in the urine of healthy human beings, whereas 24 could be detected in glucuronidase-treated samples showing that further phase II metabolization is common for odorant derivatives [33.109]. This finding holds the potential of being used as a future diagnostic tool [33.110]. Short-chain fatty acids (SCFA) such as propionate are also known ligands for human OR51E2 and mouse Olfr78 [33.47, 102]. Interestingly, SCFAs are primarily produced by gut microbiota [33.111] and reach concentrations of up to 100 mM in the colon. It is thus conceivable that sufficient amounts of SCFA enter the blood stream and are transported to their target receptors in various tissues. And indeed it has been shown that the plasma concentration of SCFAs ranges from 0.1–10 mM [33.112]. The physiological effects mediated by propionate via ORs were observed with similar concentrations [33.47, 48, 102]. In line with this finding, diaminopimelic acid is a component of Gram-negative bacterial cell walls and an intermediate in the bacterial biosynthetic pathways for lysine and peptidoglycans [33.113], which has recently been identified to act as an OR agonist [33.114]. This again corroborates the thought that some endogenous OR ligands are gut microbiota-derived.

Besides the digestive system, also fluids of the reproductive system seem to contain endogenous OR ligands. In an attempt to identify the active substances that attract spermatozoa to follicular fluid using gas chromatography-olfactometry, 5α-androst-16-en-3-one and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone were identified as endogenously produced OR ligands and were shown to elicit Ca^{2+} signals in human spermatozoa [33.115].

Moreover, some ORs such as the OR51E2 and OR7D4 are also activated by endogenously produced steroid hormones [33.58, 116]. The steroid androstenone has been detected in several bodily fluids such as sweat [33.117, 118], saliva [33.119], blood [33.117], breast milk [33.120], and seminal fluid [33.121].

33.7 Signaling Pathways of Olfactory Receptors in Non-Olfactory Tissues

Several studies investigated the expression of canonical olfactory signaling molecules in diverse nonolfactory tissues [33.4, 122]. Signaling components such as olfactory marker protein (OMP), adenylate cyclase III, and $G_{\alpha olf}$ (*GnaI*) were shown to be expressed in numerous tissues such as testis, ovary, colon, brain, white blood cells, kidney, heart, liver, lung, skeletal muscle, skin, and thyroid [33.4, 22, 41, 48, 78, 79, 91] to name only a few (for detailed information see Table 33.2). However, despite the widespread expression of canonical olfactory signaling components, some of the signaling pathways in nonolfactory tis-

Table 33.2 Canonical signaling molecules of ORs expressed in diverse nonolfactory tissues

Tissue/cell type	Species	Signaling molecules	Detection methods	References
Reproductive system				
Testis	Rat, hamster, human	β-arrestin2	WB, ICC	*Walensky* et al. [33.16]
Testis	Mouse	OMP	RT-PCR, ICC, WB	*Kang* et al. [33.91]
Testis, ovary, breast	Human	ACIII, Gαolf, CNGA2	NGS	*Flegel* et al. [33.4]
Testis	Mouse	ACIII, OMP, CNGA2	NGS	*Kanageswaran* et al. [33.122]
Placenta	Rat	Gαolf	RT-PCR	*Itakura* et al. [33.32]
Digestive system				
Colon	Human	ACIII, Gαolf	NGS	*Flegel* et al. [33.4]
Stomach, duodenum, spleen	Mouse	OMP	RT-PCR, WB	*Kang* et al. [33.91]
Glands				
Thyroid, adrenal gland	Human	ACIII, Gαolf	NGS	*Flegel* et al. [33.4]
Thyroid	Mouse	ACIII, OMP	RT-PCR, ICC, WB	*Kang* et al. [33.91]
Thymus	Mouse	ACIII, Gαolf, OMP	RT-PCR, ICC, WB	*Kang* et al. [33.91]
Pancreas (β-cells)	Rat	ACIII, Gαolf	RT-PCR	*Frayon* et al. [33.123]
Pancreas	Mouse	OMP	RT-PCR, WB	*Kang* et al. [33.91]
Cardiovascular system				
Heart	Rat	ACIII, Gαolf	RT-PCR	*Ferrand* et al. [33.92]
Heart, white blood cells	Human	ACIII, Gαolf	NGS	*Flegel* et al. [33.4]
Heart	Mouse	ACIII, Gαolf, OMP	RT-PCR, ICC, WB	*Kang* et al. [33.91]
Pulmonary system				
Lung	Human	ACIII, Gαolf	NGS	*Flegel* et al. [33.4]
Lung	Mouse	OMP	RT-PCR, WB	*Kang* et al. [33.91]
Kidney				
Distal nephron/macula densa	Mouse	ACIII, Gαolf	ICC	*Pluznick* et al. [33.48]
Kidney	Human	ACIII, Gαolf	NGS	*Flegel* et al. [33.4]
Liver				
Liver	Human	ACIII, Gαolf	NGS	*Flegel* et al. [33.4]
Liver	Mouse	OMP	RT-PCR, WB	*Kang* et al. [33.91]
Hepatocarcinoma cells (Huh7)	Human	ACIII, Gαolf, CNGA1	RT-PCR	*Maßberg* et al. [33.24]
Liver	Mouse	ACIII, OMP	NGS	*Kanageswaran* et al. [33.122]
Nervous System				
Basal ganglia	Rat	Gαolf	WB	*Hervé* et al. [33.124]
Striatum	Mouse	Gαolf	WB	*Corvol* et al. [33.125]
Cortex, hippocampus, thalamus, cerebellum, substantia nigra	Human	ACIII, Gαolf, REEP1, UGT1A6	ICC	*Garcia-Esparcia* et al. [33.79]
Cerebral cortex	Mouse	ACIII, Gαolf	RT-PCR	*Ansoleaga* et al. [33.78]
Brain	Mouse	ACIII, Gαolf, OMP	NGS	*Kanageswaran* et al. [33.122]
Muscle				
Skeletal muscle	Mouse	ACIII, Gαolf	WB, ICC	*Griffin* et al. [33.41]
Skeletal muscle	Human	ACIII, Gαolf	NGS	*Flegel* et al. [33.4]
Skeletal muscle	Mouse	OMP	RT-PCR	*Kang* et al. [33.91]
Skeletal muscle	Mouse	ACIII, OMP	NGS	*Kanageswaran* et al. [33.122]
Pulmonary artery smooth muscle cells (PASMC)	Rat	ACIII	WB	*Jourdan* et al. [33.126]
Skin				
Adipose	Human	ACIII, Gαolf	NGS	*Flegel* et al. [33.4]
Lymph node	Human	ACIII, Gαolf	NGS	*Flegel* et al. [33.4]
Bladder	Mouse	ACIII, Gαolf, OMP	RT-PCR, ICC, WB	*Kang* et al. [33.91]

sues seem to involve completely different constituents. The well-characterized signaling pathway induced by PSGR1 activation in prostate cells, for example, includes the activation of *Sarcoma* (Src) kinase followed by a *transient receptor potential vanilloid type 6* (TRPV6)-mediated Ca^{2+} influx [33.83]. Thereby, nonreceptor protein tyrosine kinase 2 (Pyk2) is phosphorylated which in turn activates p38 kinase and inhibits the tumor suppressor *N-myc downstream regulated gene 1* (NDRG1) [33.84]. In parallel, PSGR1 also activates the proton transporter *sodium/hydrogen exchanger 1* (NHE1) in the plasma membrane by a yet unknown mechanism, leading to an increase in intracellular pH [33.84]. OR1G1 was shown to increase intracellular Ca^{2+} levels through L-type Ca^{2+} channels in a PLC- and IP3 receptor-dependent manner in enterochromaffin cells [33.21]. OR1A1 does not evoke an intracellular Ca^{2+} increase in a human hepatocellular carcinoma cell line. Instead, it induces a change in gene expression and thereby modulates hepatic triglyceride metabolism. In detail, accumulated cAMP leads to *cAMP response element-binding* (CREB) protein phosphorylation and upregulation of the CREB-responsive gene *hairy and enhancer of split* (HES)-1, a corepressor of peroxisome proliferator-activated receptor-γ (PPAR-γ) [33.71].

33.8 Is it Really *Ectopic* Expression?

The term *ectopic* originates from the Greek word ektopos, which means *out of place* (ex – out and topos – place), so it means that something is occurring in an abnormal position or in an unusual manner or form. This might be a misleading nomenclature when it comes to ORs as they are expressed in the majority of cell types albeit in far less multiplicity compared to the olfactory epithelium. On the other hand, the term *OR* implies specific expression in the nose and exclusive function as odorant sensors in the process of smelling. Maybe, it would facilitate to think of ORs as chemosensors belonging to the GPCRs that mediate the sense of smell as one of their scope of duties but also fulfill a diversity of other tasks in numerous other tissues and cell types.

33.9 Challenges and Future Prospects of Ectopic OR Research

Although in the recent years, more and more studies demonstrated distinct functions of ectopically expressed ORs in various cell types, ectopic OR expression research is still in its infancy. In the past, it was very difficult to detect OR proteins because OR antibodies were not available or poor in quality. This is due to the high homology between ORs (40–90%). However, in recent years, an increasing number of high-quality antibodies became available. This allows more studies with higher through-put based on protein detection [33.127].

Another reason for the slow progression in ectopic OR research is that despite immense effort only a tiny proportion of mammalian ORs has been deorphanized [33.102, 128–130]. Many studies on ectopically expressed ORs use ligands in high concentrations, probably because the known ligands are not the most effective ligands. This raises the possibility to investigate unspecific effects. The discovery of endogenous, presumably more potent ligands would immensely facilitate research on putative OR functions in diverse cells and tissues. In line with the need for agonists, discovery of (endogenous) antagonists would be beneficial. To date, only very few antagonists are published: undecanal for OR1D2 [33.40], hydrocinnamaldehyde, bourgeonal, and methyl cinnamaldehyde for OR3A1 (hOR17-40) [33.131], and α-ionone for OR51E2 [33.58]. Methyl isoeugenol was identified as a partial agonist for mOR-EG [33.132]. Moreover, it was reported that rat OR17 recognizes octanal as primary agonist whereas citral was shown to be a partial agonist or antagonist [33.133]. As known for other GPCRs, OR antagonists seem to be structurally similar to agonists [33.132, 134]. Some odorants have dual functions as an agonist to one and an antagonist to other ORs. This provides complexity in the encoding mechanism of an odorant mixture at the receptor level and, thus, also hampers ligand screening using chemical libraries. Furthermore, it is known that some ORs have a rather broad ligand spectrum whereas others are more narrowly tuned [33.135]. The fact that one receptor may bind multiple ligands and one ligand may activate multiple receptors makes it also more challenging to identify specific OR–ligand pairs.

Moreover, as typical for GPCR research, the establishment of an in vitro assay system to study ORs proved to be difficult. The heterologous expression often is poor due to insufficient intracellular trafficking.

Thus only very few cells express ORs in their plasma membrane and the majority of ORs accumulate intracellularly. The heat shock protein Hsc70t was shown to facilitate the cell surface expression of at least some ORs [33.136]. Matsunami's group established an assay system with co-transfection of numerous cofactors (such as RTP1S and REEP1) known to be important for OR expression in the OE [33.137]. These attempts enhanced plasma membrane localization of ORs. However, despite the clear improvement, this assay system is still far from being optimal.

Another challenge of ectopic OR research are the pronounced species differences in sequence and functional GPCR properties which render basic discoveries in model systems such as rodents only conditionally feasible as the results may not be directly transferable to the human setting. This also applies to knockout models. However, there may be human *knockout* available due to polymorphisms that in some cases result in nonfunctional receptors. ORs belong to one of the most polymorphic gene families. The best known selective anosmia is to isovaleric acid, the ligand of OR11H7, which is one of the main unpleasant component of body odor. About 6% of the human population cannot detect this substance [33.138]. However, the correlation between this anosmia and an actual gene defect has to be confirmed. Another well-described polymorphism is that of OR7D4 which responds to androstenone and its derivatives [33.116]. Different people report a wide range of perceived odors from androstenone and androstadienone, from unpleasant and urinous to sweaty, woody or even pleasantly floral or citrussy, and nearly 30% of the human population claims not to be able to smell these substances at all [33.139, 140]. An increase in knowledge about anosmia and the discovery of more human *knockouts* could immensely contribute to our comprehension about OR expression in nonolfactory tissues.

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34. Spices and Odorants as TRP Channel Activators

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Flavors and odorants are important aspects in our daily life controlling a diversity of selection processes like food intake, social interactions, aversion or love. The detection of flavor and odorant compounds by our sensory organs for taste, olfaction and chemesthesis are important for the detection and discrimination among chemical cues in the environment. Flavors and odorants have impact on food selection as well as social interaction by being influential for feelings of pleasure and discontent, sexuality and mood. Most of flavors and odorants originate from spice plants like capsaicin from chili or vanillin, the odorous principle of vanilla. They activate a large set of receptors and ion channels. In this review, we summarize the recent findings regarding the effects of flavors and odorants on the activity of the transient receptor channel family (TRP) and their potential role in olfaction, taste and chemesthesis. Potential health benefits of spices are discussed in the light of their bioavailability of the flavors and odorants after systemic intake.

Olfaction and gustation, our capability for perception of odors and tastes, depend on detecting and transducing the appearance of chemical structures to electrical activity. The senses are thereby involved in detection of threats, represent lifeguards in the selection of nutrition, but also contribute to social interaction, pleasure and general wellbeing in humans [34.1]. The chemical compounds trigger preference and avoidance reaction by a variety of receptor molecules such as G-protein-coupled receptors, enzyme-coupled receptors or ion channels. G-protein-coupled receptors function as taste and odorant receptors comprising the taste receptors for sweet, umami, or bitter and the canonical odorant receptors, the trace amine-associated receptors, as well as the vomeronasal type 1 and 2 receptors [34.2]. For a comprehensive review summarizing all molecular mechanisms mediating taste, chemesthesis or smell please refer to *Roper* et al. and *Spehr* and *Munger* [34.2, 3].

TRP channels form a large family of nonselective ion channels involved in olfaction, gustation and chemesthesis. Mainly, plant-derived volatile or nonvolatile compounds such as cineol, menthol or capsaicin directly activate TRP channels (Fig. 34.1) contributing to the distinct flavors of individual foods [34.4]. In the following chapter, TRP channels and plant-derived activators, which are involved in taste, chemesthesis and olfaction, are summarized and their potential to cause physiological effects in our body via TRP channel activation beside olfaction, taste and chemosensation is discussed.

34.1 TRP Channels – Olfaction, Chemesthesis and Taste

In the context of olfaction, chemesthesis and gustation mainly, TRP channels of the classical TRP channel subfamily (TRPC) such as TRPC1, TRPC2, TRPC4, TRPC6, of the vanilloid TRP subfamily (TRPV) TRPV1, and TRPV3 of the melastatin subfamily (TRPM) TRPM4, TRPM5 and TRPM8 are dis-

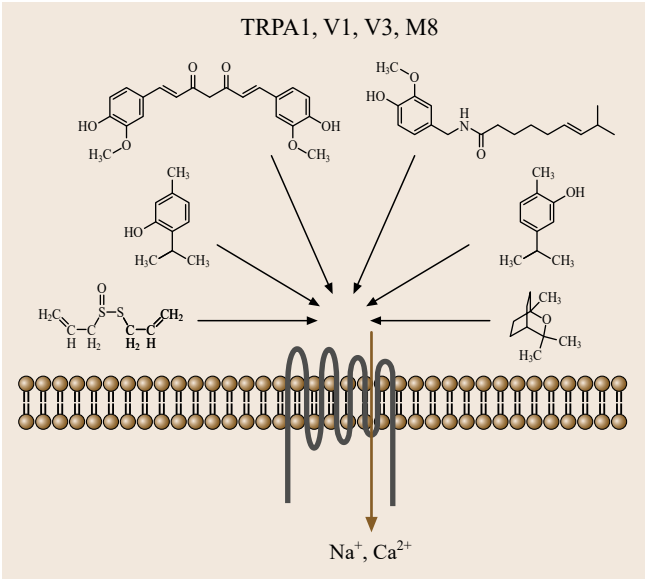

Fig. 34.1 TRP channels in the sensation of plant-derived volatile and nonvolatile compounds

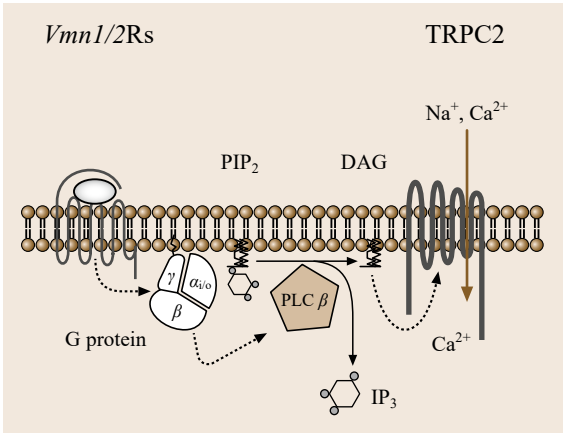

Fig. 34.2 Signal transduction cascade leading to the activation of TRPC2 in the vomeronasal organ

cusses to be involved in the transduction of odor, taste and chemosensation [34.5, 6].

The best characterized TRP channel regarding its role in immediate olfaction is TRPC2 [34.5, 7–11]. In TRPC2-deficient mice, innate sexual and social behavior is altered, comprising impaired sex discrimination, failure to initiate aggressive attacks to intruders, and lowered maternal aggression and lactating behavior [34.7, 8]. In humans, TRPC2 is a pseudogene. TRPC2 is highly expressed in the sensory neurons of the vomeronasal organ responsible for pheromone sensation in rodents modulating innate sexual and social behavior. Pheromones released by the urine of conspecifics are sensed by receptors of the vomeronasal organ *Vmn1r/Vmn2r* transducing the extracellular signal to intracellular processes initiated by G-proteins of the Gi/o family. Subsequent breakdown of phosphoinositides results in the activation of calcium entry mediated by TRPC2 (Fig. 34.2) and subsequent neuronal activity. Other TRP channels belonging to the classic TRP channel subfamily integrated in GPCR signaling are TRPC1 and TRPC4, as well as TRPC6 channels which are expressed in different cells of the olfactory system with a yet unclear role in olfaction [34.5].

From other subfamilies, TRPM5 channels have also been detected in the olfactory systems, in the main olfactory endothelium where pheromones might lead to an activation of TRPM5 channels [34.12].

Like TRPC2, TRPM5 is a downstream target of G-protein-coupled receptors (GPCR). TRPM5 is expressed in taste receptor cells and other chemosensory cells of the taste signaling cascade comprising the large intestine, pancreatic β-cells, duodenum, stomach, lung, brain e.g., brainstem and the vomeronasal organ [34.13]. In enteroendocrine cells of the intestine, TRPM5 regulates the release of the incretine hormons glucagon-like peptide-1 (GLP-1) and gastrin-inhibitory polypeptide as well as the appetite-stimulating hormone ghrelin. GLP-1 upon release reduces food consumption, facilitates gastric emptying and promotes insulin release in response to glucose consumption. In addition, TRPM5 channels directly regulate glucose-mediated insulin secretion in pancreatic β-cells. In enterochromaffine cells of the small intestine, TRPM5 mediates the release of cholecystokinin (CCK), which stimulates gallbladder contraction and suppresses hunger. There might be also a role of TRPM5 in weight control. TRPM5 channels are also expressed in brush cells of the ileum and the colon where they are considered to detect the presence of dietary nutrients.

TRPM5 plays a central role in taste and is required for sweet, amino acid and bitter perception [34.3, 14]. In addition, TRPM5 might also be activated by free fatty acids and may elicit fat taste [34.15]. Ligand-binding to GPCR results in the activation of gustducin and phospholipase Cβ2 mediated breakdown of phosphoinositides (Fig. 34.3). Phospholipase C enzymes generate the intracellular second messenger diacylglycerol and inositoltrisphosphate. The latter being the ligand of IP3 receptors induce the release of calcium from intracellular endoplasmic stores. The increase in intracellular calcium concentration results in activation and membrane depolarization mediated by TRPM5, which is exclusively permeable to monovalent cations.

In contrast to TRP channels of the TRPC family and TRPM5 being integrated in odor and taste signaling cascades, several plant-derived compounds

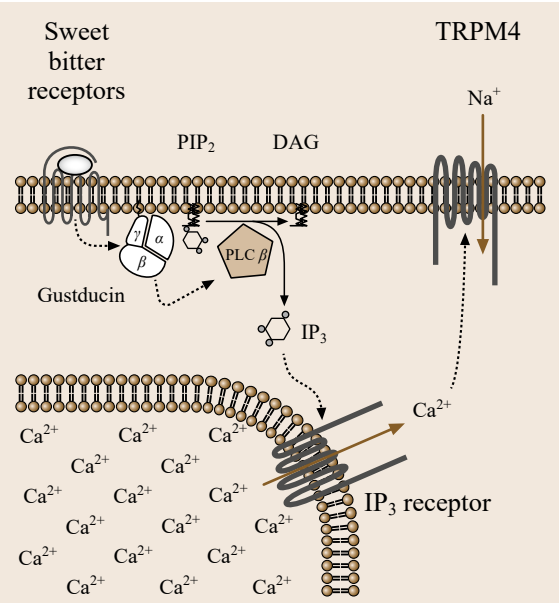

Fig. 34.3 Signal transduction cascade leading to the activation of TRPM5 in taste buds

directly activate TRPA1 (ankyrin), TRPV1, TRPV3 and TRPM8 (Fig. 34.1), which are also involved in mediating trigeminal stimuli and chemesthesis [34.13, 16]. They are activated by a wide range of secondary plant constituents which are summarized in part 2 of this chapter.

The involvement of TRPV1 in salt perception is currently discussed. A polymorphism in the TRPV1 gene (rs8065080, $C > T$, Val585Ile) modifies suprathreshold taste sensitivity where carriers of the T allele were more sensitive to salt solutions [34.17]. However, there are also controversial studies (for an excellent summary please read [34.3]) such as a study in TRPV1 KO (knockout) animals which showed no alteration in the two components, the amiloride-sensitive and amiloride-insensitive salt perception [34.18]. Interestingly, a TRPA1 polymorphism detected in twins was associated with different chemosensory ratings for basil and cilantro [34.19]. Therefore, TRPA1 channels might also be involved in taste perception.

Importantly, these channels are not only expressed in organs involved in olfaction, taste and chemosensation such as nasal trigeminal neurons. Every TRP channel has a specific expression profile. TRPV1 channels, for example, are considered to be expressed in the central nervous system (CNS) comprising the olfactory bulb, the cerebellum as well as the cortex and basal glia [34.13, 20]. However, the exact tissue expression pattern of TRPV1 in the brain is still under discussion because a recent study using a TRPV1 reporter mouse only reported minimal TRPV1 expression in only a few CNS areas. In the periphery, TRPV1 is expressed in kidney, pancreas, testes, uterus, spleen, stomach, small intestine, liver, lung, bladder, skin, skeletal muscle, mast cells, macrophages as well as leucocytes [34.21]. In this chapter, we focus on spices, which activate TRP channels and will discuss their physiological effects in respect to their bioavailability (for a comprehensive review please read the excellent article by [34.13]). The bioavailability of the compounds in food as well as nonfood matrices is questionable. However, these compounds show profound effects on the function of the gastrointestinal and the respiratory system which will be discussed below.

TRPV1, one of the major pain sensing channels for the detection of thermal stimuli and chemical irritants, was also suggested to be involved in visceral pain. TRPV1 channel expression is upregulated in reflux esophagitis and might be involved in heartburn as the major acid sensor [34.22]. TRPV1 immunoreactivity in the gastrointestinal tract is also increased in patients suffering from the irritable bowel syndrome, *Morbus Crohn*, and ulcerative colitis [34.23, 24]. In addition, TRPV1 channels control appetite, satiety, nausea as well as bloating and discomfort [34.25]. However, in TRPV1 KO animals, controversial results were found regarding body weight, therefore its physiological role in metabolism is still under debate [34.25]. Furthermore, several lines of evidence suggest that TRPV1 in rodents regulates the development and maintenance of insulin resistance in Type 2 diabetes [34.13, 26, 27]. TRPV1 activation using acute capsaicin administration by gastric gavage increased GLP-1 and insulin secretion in vivo in TRPV1 wild-type (WT) mice but not in TRPV1 KO mice [34.26]. Furthermore, chronic dietary capsaicin (24 weeks application) not only improved glucose tolerance and increased insulin levels but also lowered daily blood glucose profiles and increased plasma GLP-1 levels in WT mice. In a recent study, TRPV1 KO and WT mice were fed a high-fat diet, and metabolic studies were performed to measure insulin and leptin action [34.28]. The TRPV1 KO mice became more obese than the WT mice after high fat diet, partly attributed to altered energy balance and leptin resistance in the KO mice. In addition, TRPV1 KO mice were more insulin resistant. In the respiratory system, TRPV1-positive nerve fibers innervate the nose, larynx, the trachea of the upper airways, lung parenchyma as well as alveoli and smooth muscle cells and blood vessels [34.29]. However, expression levels in the healthy respiratory system seem to be rather low. In disease-state in patients with emphysema [34.30] or in patients suffering from refractory asthma [34.31], or in other conditions of lung inflam-

mation [34.30], TRPV1 expression is increased. Interestingly, single nucleotide polymorphisms (SNPs) in TRPV1 channels are associated with cough in subjects without asthma and enhanced susceptibility to cough in smokers [34.32]. In childhood asthma, a SNP was described leading to decreased TRPV1 channel activity associated with a lower risk of current wheezing or cough [34.33]. For an excellent review regarding TRP channel function in airway disease, please refer to *Grace* et al. [34.30].

TRPV3 is highly expressed in tongue, testis and skin keratinocytes and is considered to be essential for different skin functions such as skin barrier formation and hair morphogenesis [34.34]. Besides, TRPV3 channels are also involved in heat sensing.

TRPA1 channels are mainly expressed in nociceptive neurons, dental pulp as well as the organ of Corti in the mammalian cochlea [34.35–39]. Furthermore, these channels are also widely expressed in brain, heart, small intestine, lung, skin, skeletal muscle, bladder, prostate, vascular endothelial cells, pancreas and taste cells [34.13]. TRPA1 channels are essential in sensing irritant compounds of our food and harmful airborne irritants [34.30, 40]. TRPA1 are important nociceptors and are involved in epigastric pain in functional dyspepsia [34.41, 42]. They might also be involved in the regulation of gastric emptying (delaying gastric emptying) [34.43], promotion of satiety and suppression of food intake via the release of the gastric peptide cholecystokinin (CCK) [34.44, 45]. TRPA1 channels are also expressed in vagal airway neurons and activation of TRPA1 channels evokes cough in guinea pigs [34.46] and healthy humans suggesting that TRPA1 channels are part of the lungs defense system [34.30].

TRPM8, the cold sensor, is mainly expressed in sensory neurons but also in the bladder, the prostate, hippocampus, skin, brown adipose tissue, vascular smooth muscle, macrophages and sperm [34.13, 47]. Together with TRPA1, TRPM8 represents the cold sensors in human. Noxious cold is mediated by TRPA1 [34.48, 49]. TRPM8 agonists induce a transient body temperature rise by stimulating brown adipose tissue thermogenesis. Therefore, this might be an interesting target to treat obesity [34.50]. In addition, TRPM8 regulates lacrimation and basal tear flow [34.51]. TRPM8 channels are expressed in trigeminal ganglia but only sparsely in whole lung tissue [34.30, 52]. However, TRPM8 channels are expressed in human bronchial epithelial cells [34.53, 54]. The role of TRPM8 in lung in health and disease is rather unexplored even if there is the hypothesis that cough induced by cold air might be initiated via the activation of TRPM8 channels [34.30].

34.2 Secondary Plant Compounds Activating TRP Channels

Different ingredients of flavors and spices are potent TRPA1, TRPV1, TRPV3 and TRPM8 channel activators [34.6, 13, 20, 55, 56]. However, several of these compounds are not selective for one TRP channel or they even also activate other molecular targets, as is the case for curcumin. For TRPC1, TRPC4, TRPC6, TRPM4, or TRPM5, until now no compound isolated from spices is known to activate these channels.

Several pungent and irritant constituents of spices are TRPA1 activators. They are electrophilic compounds, which induce a covalent modification of N-terminal cysteins or lysines of TRPA1 channels. TRPA1 is for example activated by allylisothiocyanate (EC_{50} 22 μM in TRPA1-expressing CHO cells or 2.5 μM in TRPA1-expressing HEK293 cells (HEK: human embryonic kidney cell)) [34.35, 57], allicin (EC_{50} 2 μM) [34.36], diallylsulfide (EC_{50} 125 μM) [34.36], cinnamaldehyde (EC_{50} 61 μM in TRPA1-expressing CHO cells or 6.8 μM in TRPA1-expressing HEK293 cells) [34.35, 58], hydroxyl-α-shangool (EC_{50} 69 μM) [34.58], shogaol (EC_{50} 11.2 μM) [34.58], 6-gingerol (EC_{50} 10.4 μM) [34.57], carvacrol (EC_{50} 690 μM) [34.59], curcumin (pronounced TRPA1 activation in TRPA1-expressing HEK293 cells at 30 μM). Allylisothiocyanate is responsible for the pungent taste of mustard, radish, horseradish and wasabi [34.55]. Allicin gives garlic its specific odor. Allicin is a conversion product when young garlic is chopped and the alliinase converts alliin into allicin which is again degraded to diallylsulfide. Cinnamaldehyde is the ingredient of cinnamon which is important for the flavor and odor of cinnamon. Carvacrol, the isomeric of thymol, is a main component of oil obtained from oregano, thyme, pepperwort or wild bergamotte.

TRPV1 channels are activated by capsaicin (EC_{50} 0.037 μM) [34.58], camphor (5 mM) [34.60, 61], carvacrol (not determined), thymol (EC_{50} 100 μM) [34.62], piperine (EC_{50} 38 μM) [34.63], allicin (51 μM), 6-gingerol (EC_{50} 3.3 μM) [34.57], hydroxyl-α-shangool (EC_{50} 1.1 μM) [34.58], shogaol (EC_{50} 0.2 μM) [34.58]) or eugenol (EC_{50} 10 μM) [34.62] (Fig. 34.2). The hotness of chili peppers is mediated by TRPV1 activation by capsaicin. Camphor has a strong aromatic odor and can be found in evergreen trees, the camphor laurel, kapur trees or dried rosemary leaves. Thymol is found in thyme and

many other different plants. Piperine is the constituent of pepper which induces its pungency. Eugenol is one of the active ingredients of cloves.

TRPV3 channels are activated by eugenol (10 μM) [34.62], carvacrol (0.5 μM) [34.64], camphor (EC_{50} 6 μM) [34.64], incensole (EC_{50} 10 μM) or thymol (EC_{50} 0.9 μM) [34.64].

TRPM8 is activated by menthol (EC_{50} 80 μM) [34.65], linalool, geraniol, or eucalyptol (EC_{50}7700 μM) [34.66].

34.3 Health Benefits of Spices

Most of these compounds have a pungent note, which might even be slightly painful. The question that arises, is why do humans consume hot and pungent spices and what could be the evolutionary advantage [34.67]? There are several hypotheses ranging from hedonic experiences to certain health benefits [34.67]. These potential health benefits were already known in ancient cultures and were passed down to present time. Antifungal, antibacterial effects, anticancer, anti-inflammatory, cytoprotective, cardioprotective as well as anti-pain effects are discussed [34.67]. Pungent spices were first used in countries with subtropical and tropical climate and the addition of antifungal and antibacterial spices to daily food makes perfect sense. However, these effects are not mediated via TRP channel activation. Regarding the other discussed health benefits, the important and still unanswered question remains if the constituents are able to reach pharmacologically active concentrations if they are taken up with the daily food. Most data supporting the protective health effects on cancer, inflammation or the cardiovascular system are mainly epidemiological studies. In the case of epidemiological studies, it is very hard to distinguish whether the intake of a certain spice or the overall life style might influence the prevalence of certain diseases. Further supporting data comes from preclinical studies using cell and animal models where often very high concentration of the respective compound is used. Whether or not the required concentration can be reached by daily consumption of spices in our food or by intake of highly enriched food supplement is controversially debated. Only for a few compounds, data regarding bioavailability is available. One of the best-investigated molecules is curcumin, which is the principal curcumimoid of turmeric [34.68]. Its bioavailability is low; several clinical trials investigate its anticancer properties in patients suffering from pancreatic cancer where curcumin was applied in a high concentration of 8 g/d, a plasma concentration of 85 ng/ml was reached [34.69]. However, the plasma concentration, which would need to be achieved to activate TRPA1 channels, is 100-fold higher. Even if we assume that a certain accumulation in different organs is possible, this concentration gap between the detected plasma concentration and the concentration needed to activate TRPA1 channels seems to be too pronounced. In daily food consumption, around 250 mg curcumin in an indian curry can be reached. This complex food matrix also contains fatty ingredients and surfactants such as lecithin which might improve curcumin absorption [34.13]. However, the pharmacological needed concentration will probably not be reached by daily food consumption. In addition, several TRP activating compounds also activate other targets, which might also contribute to beneficial effects in the mentioned diseases. Again, curcumin is a very good example. Over 30 targets are discussed such as the nuclear transcription factor NF-$\kappa\beta$, the matrix metalloproteinase, the cyclooxygenase-2, or the vascular endothelial growth factor just to name a few [34.70]. For most odorants and spices further data is required to estimate if they might have systemic effects after they are inhaled or orally consumed.

The best-characterized spice ingredient regarding its effects on gastrointestinal function and weight control is the selective TRPV1 activator capsaicin. Capsaicin inhibits gastric acid secretion in modest concentrations which can be reached by spicy food consumption and also shows protective effects in the gastric mucosa [34.71, 72]. However, in higher concentrations capsaicin is able to enhance gastric acid secretion and to damage the gastric mucosa confirming an old toxicologic/pharmacologic principle *the dose makes the poison*. Capsaicin also shows an antibacterial effect against Helicobacter pylori, the main trigger for gastric ulcer. However, its protective effect is not mediated by TRPV1 activation. Similar effects on gastric acid secretion and protective effects on mucosal integrity are also described for 6-gingerol, also a TRPV1 activator [34.73].

In addition, an increase in TRPV1 receptor expression was detected in the gut mucosa of patients with conditions associated with visceral hypersensitivity, including in the esophagus of patients suffering from the nonerosive reflux disease and in the colon of patients with irritable bowel syndrome (IBD) belonging to the class of functional gastrointestinal disorders (FGID) [34.74]. Patients with functional dysplasia

and IBD show a hypersensitivity to singular oral capsaicin application reflected in higher abdominal pain and burning symptom scores. These results and others from animal experiments suggest that the hypersensitivity of TRPV1 pathways in patients with FGID is likely a result of low-grade inflammation and may be an important pathogenesis of gut hypersensitivity, abdominal pain, and abdominal burning symptoms in FGID [34.74].

However, chronic ingestion of capsaicin is considered to have different effects due to TRPV1 desensitization. A small clinical trial in 30 patients suffering from functional dyspepsia receiving 2.5 g/d of red pepper powder or placebo showed that the overall symptom scores, epigastric pain, and nauseas improved compared to placebo [34.75, 76]. In a clinical trial including 50 patients with IBD treated with 600 mg red pepper/d, abdominal pain and bloating mean score values was reduced in week 6 of the treatment compared to placebo [34.77]. These results are supported by the epidemiological findings that the prevalence of heartburn is much lower than the prevalence of acid regurgitation in Asian countries with a high intake of spiced food compared to western countries [34.74]. However, further clinical trials are needed to confirm the hypothesis that chronic chili intake might facilitate pain and burning symptoms in FGID patients.

Several lines of evidence suggest that TRPV1 agonists, especially capsaicin, trigger cellular mechanisms against obesity. In a clinical trial, healthy lean individuals received once a hedonically acceptable red pepper dose of 1 g/d. Postprandial energy expenditure and core body temperature were increased, and skin temperature was lower after the intake of red pepper [34.78]. Interestingly, the desire to consume fatty, salty, and sweet foods were decreased more in the individuals who did not consume spicy food regularly before. This finding suggests a desensitization of individuals with long-term intake of spicy foods. Two recent meta-analyses recapitulate clinical trials investigating the effects of capsaicin and the nonpungent TRPV1 activator capsiate found in CH-19 sweet peppers [34.79, 80]. The authors conclude that the studies provide evidence for the role of capsaicin and capsiate on weight control [34.79, 80]. However, the magnitude of the thermogenic and appetitive effects is small and their long-term sustainability is uncertain. The authors calculate that a 10 kcal negative energy balance, which is predicted for hedonically acceptable capsaicin doses in an average weight, middle-aged man would produce an ultimate weight loss of 0.5 kg over 6.5 years, whereas a 50 kcal negative energy balance (predicted for encapsulated dihydrocapsiate) would yield a total weight reduction of 2.6 kg over 8.5 years [34.79]. Several mechanisms of action are discussed ranging from the enhanced expression of proteins involved in lipid catabolic pathways and thermogenesis in skeletal muscle, liver or white fat tissue. Capsaicin was also found to stimulate the brown adipose tissue which is a metabolic active tissue responsible for nonshivering thermogenesis [34.13].

TRPA1 channels are expressed in cranial visceral vagal neurons. These neurons convey chemical signals from the gut to the solitary nucleus participating in peripheral satiety signaling as mentioned above. The TRPA1 agonist cinnamaldehyde, one of the active ingredients of ginger, reduced cumulative body weight gain and improved glucose tolerance without affecting insulin secretion in obese mice in a daily dosage of 250 mg/kg BW reflecting a daily dose of 3 g [34.81]. This dosage can only be reached by food supplementation and not as a hedonically pleasant ingredient of our daily food. 1 g of a specific Ceylon cinnamon hydroalcoholic extract reduced postprandial glycemia in 18 healthy volunteers by 21% without affecting insulin secretion [34.82]. However, these initial results need to be supported by additional clinical trials in obese patients.

Interestingly, the food supplementation with TRPM5 inhibitor quinine (0.1%), which tastes bitter, showed in mice fed with a regular balanced diet a significantly lower increase in body weight and fat mass [34.83]. However, no clinical data is available which would support these preclinical results.

Another organ where pharmacologically active concentration of TRP channel activators can be reached is the lung. Menthol, camphor as well as eucalyptus oil are the active ingredients of Vicks Vaporub and are traditionally used as topical agents to relieve symptoms of chest congestion or other upper respiratory disorders [34.84]. Interestingly, many remedies contain menthol to counteract coughs. In conscious guinea pigs, the antitussive effect of menthol, 1,8-cineole and camphor was investigated. Menthol showed the best activity and reduced the cough frequency at concentrations of 3 and 10 µg/kg about 28 and 56%, respectively [34.85]. Its antitussive effects are considered to increase mucosal blood flow, relief in bronchoconstriction and direct or indirect effects on cough-initiating sensory neuronal pathways [34.66, 86, 87]. Importantly, in patients with chronic cough, pre-inhalation of menthol reduces cough sensitivity to inhaled capsaicin and influences inspiratory flows [34.88]. However, the outcomes of clinical trials using menthol against cough, which was initiated by a single application of capsaicin or citric acid, are controversial with positive effects [34.89, 90] as well as no effects [34.91] on cough suppression. These differences might be due to the administration route – menthol inhalation seems to be more effective than topical application of menthol. The underlying molecular

mechanism of cough suppression by menthol seems to be initiated by a reflex initiated from the nose via TRPM8 channels [34.92, 93].

The suppression of respiratory irritation by menthol is also used in cigarettes. Menthol is present in 90% of commercial cigarettes sold in the United States including brands which are not labeled to be mentholated [34.94]. Epidemiological studies suggest that mentholated cigarettes are preferred in young smokers and thereby might increase smoking initiation [34.95]. In addition, smoking menthol cigarettes seems to be associated with reinforcing nicotine dependence, impeding cessation, and promoting relapse compared with nonmenthol smoking [34.96]. How does menthol interfere with smoking? Cigarette smoke contains numerous irritants that stimulate chemosensory nerves. *Willis* and *Morris* [34.97] investigated the effects of 16 ppm menthol in mice, which is a concentration lower than found in the smoke of mentholated cigarettes (200 ppm $\approx$ 8 μM) on the respiratory sensory irritation response in mice elicited by the smoke irritants acrolein, acetic acid and cyclohexanone. Importantly, menthol was absorbed with high efficiency ranging from 65% or greater depending on the experimental group. Menthol as well as eucalyptol immediately suppressed the irritation response to acrolein, acetic acid and cyclohexanone via TRPM8 and thereby act as potent counterirritants against a broad spectrum of smoke constituents. These effects of menthol might interfere with smoke inhalation and thereby might promote nicotine addiction and smoking-related morbidities such as lung cancer.

Campher is an agonist of the heat sensing channel TRPV3 [34.60, 61], a partial agonist of TRPV1 [34.60], and an antagonist of the noxious cold sensor TRPA1 [34.60]. Interestingly, camphor shows a bimodal activity on TRPM8, it activates TRPM8 but also inhibits the response to menthol [34.98]. Camphor is also used traditionally to treat cough. Recently, *Patberg* et al. suggested that camphor might mediate its antitussive effects via TRPV1 channels [34.99]. They demonstrated that camphor initially stimulates but then strongly desensitizes TRPV1. However, camphor is not a selective TRPV1 desensitizer but also inhibits TRPA1 channels which are also involved in bronchoconstriction [34.60], and also activates TRPM8 channels which are also involved in antitussive action [34.98].

34.4 Negative Effects of TRP Channel Activators

Another organ where the pharmacologically active concentration of capsaicin is reached to activate TRPV1 channels is the lung. Everyone knows the tussigenic effect of inhaled red pepper powder. Importantly, capsaicin is slowly degraded in the lung and the metabolic inactivation is incomplete at the pulmonary level. In patients suffering from the cough variant of asthma as well as from the sensory airway hyper-reactivity syndrome, capsaicin provokes an exaggerated cough response [34.100, 101]. Therefore, these patients should be extremely cautious eating spicy food.

In addition, menthol should be used with caution in small children or patients suffering from asthma or chronic obstructive disorders because menthol might trigger an uncontrolled upper airway muscle reflex resulting in spasms and significant breathing difficulties [34.102]. It might also cause allergic reactions.

Umbellone, a TRPA1 activator, is known as the major volatile constituent of the leaves of the California bay laurel, which is also known as the *headache tree* because inhalation of its vapors can cause severe headache. Inhalation of umbellone caused a painful cold sensation in the nasal mucosa. *Nassini* et al. [34.103] demonstrated that umbellone induced a calcium-dependent release of calcitonin gene-related peptide (CGRP) from rodent trigeminal nerve terminals in the dura mater. Release of CGRP seems to play a prominent role in the activation of specific brain areas and the trigeminovascular system.

34.5 Conclusion

Several molecular constituents of spices and flavors are potent TRP channel activators. They are not only involved in the smell, taste or chemesthesis of our food but are also discussed to mediate health benefits in several diseases including gastrointestinal diseases, cancer, inflammation, pain just to name a few. Several preclinical experiments including cell and animal experiments and epidemiological data support this view. However, the bioavailability of the molecules often embedded in complex matrices is very low enabling to scrutinize their systemic effects. However, in the gastrointestinal system as well as the pulmonary system, the pharma-

cologically active concentrations are reached. In these organs, spices show pharmacological effects which are beneficial such as the moderate effect of TRPV1 agonists on weight control or the antitussive effect of menthol mediated by TRPM8 channels. Further research is needed to clarify the potential role of several odorants and spices in health and diseases. In addition, several odorants and spices as well as their metabolites are not yet investigated regarding their effects on TRP channels.

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35. Anti-Inflammatory Effects of Odor Compounds

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Food-derived odor compounds determine the flavor of foods. However, these volatile compounds are known to elicit biological activities beyond their flavor function. Plants rich in volatile compounds, for example mint, have been used in traditional medicines worldwide to improve wound healing and to treat inflammation-related illnesses. In this chapter, we focus on the anti-inflammatory activity of different plant essential oils and individual odor compounds. Here, it is reviewed that odor compounds possess anti-inflammatory activity in pre- and postabsortive model systems, in vitro, ex vivo, and in vivo. Monocytes, macrophages, and fibroblasts are cells that play an important role in the innate immune response. In vitro models of these cells are commonly used to identify the mechanisms of action of the anti-inflammatory activity of volatile compounds. An inflammatory stimulus initiates the toll-like receptor-mediated signaling pathway, resulting in the gene expression and further the release of cytokines. Thus, an anti-inflammatory effect of odor compounds is measured by a reduction of cytokine messenger ribonucleic acid (mRNA) expression or release from stimulated cells. For example, eucalyptol, borneol, and camphor have been identified as anti-inflammatory active compounds that may be used to prevent or treat inflammation-related diseases.

35.1 Relevance of Identifying Anti-Inflammatory Active Odor Compounds

Increased standard of living has caused the population to constantly grow older. This wealth has been accompanied by an increase in average body weight, cases of obesity, and obesity-associated diseases. Among those diseases are cancer, coronary heart diseases, and diabetes mellitus type II, which have also been linked to

chronic inflammation [35.1–3]. Increasing rates of patients with chronic inflammatory diseases show the urge of treatment and prevention. Thus, research focused on counteracting inflammation by treatment with anti-inflammatory compounds. The advances in research on an anti-inflammatory activity of aroma active compounds are determined by technological progress in the field of analytical food chemistry and sensory, the access to documentation of traditional medicines and the biochemical and molecular biological research investigating inflammatory signaling. Odorants are small volatile molecules that interact with chemical receptors in the olfactory cells. However, these small molecules are discussed to exhibit biological activities beyond their odor activity.

35.1.1 Analytical Advances in Chemistry of Odor Compounds

The flavor of foods and beverages is determined by the composition of odorants and gustatory compounds, responsible for aroma and taste, respectively. The aroma is characterized by the interaction of different compounds in the essential oil. However, the essential oil constituents that contribute to the flavor are called key aroma compounds. These key compounds, may not necessarily dominate in quantity, but have a low aroma threshold level [35.4, 5]. Sensory analyses determine flavor thresholds of volatile compounds, allowing the identification of flavor profiles and characteristics of foods [35.4]. Moreover, method development played a crucial role in the analytics of flavor compounds. Here, olfactory gas chromatography [35.6] allows the simultaneous detection of a compound by chromatography and the assertion of its smell. These advances in analytical chemistry over the past decades allowed the identification and quantification of hundreds of aroma compounds in different natural and processed foods [35.7, 8] and beverages [35.9–11].

35.1.2 Odor Compounds Used in Traditional Medicine to Treat Inflammation

One strategy to identify anti-inflammatory active compounds is to search for the literature reports about plants used in traditional medicines to treat pain, fever, and local inflammation and test their anti-inflammatory potential. The access to historic documents and the preservation of traditional uses of plant material allows the gain of knowledge in which plant and plant preparations have been used to treat symptoms of inflammation.

Little is known about traditional medicines of South America and Africa. In Europe, historic documents like the *Naturalis historia* by Pliny the Elder report about the use of plants to treat health problems, for example, mint to treat headaches, gastric problems, and nose malfunctions when drinking or inhaling mint juice. Today, mint aroma is not only popular in beverages, drops, or chewing gums, but is also used in hygienic products, toothpaste, or medical formulations for inhalation. Even though alternative medicine exists in many Western countries, the majority of people use pharmaceutical products to treat illnesses and diseases. In contrast, the practice of traditional medicine is still very common in China, but the documentation is sparse. The Western approach to use Chinese medicine in drugs was to identify individual compounds for testing their individual pharmaceutical impact on different diseases. Traditionally, herbs, herbal extracts and plant extracts are used in combination, which hinder the identification of active ingredients and also raise the question whether combinatory effects are required to gain curing success. However, to prove a potential anti-inflammatory activity, both extracts and individual compounds are to be considered.

35.1.3 Traditional Applications of Odor Compounds

Traditionally, odor compounds were not used as individual products but in complex mixtures, like infusions, crèmes, or pastes. Reports include the use of plants to treat inflammation, symptoms of inflammation like swelling or fever, or inflammatory-linked diseases like tooth aches or cardiovascular diseases. A review article summarized the medicinal plants used in Chile and their applications, for example, treatment of inflammatory diseases [35.12]. The described application forms range from chewing stem latex to treat tooth

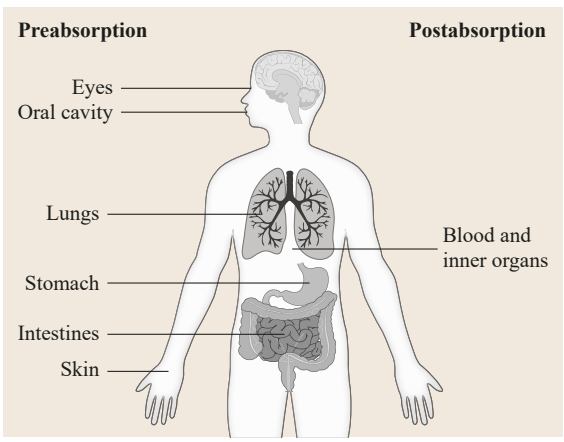

Fig. 35.1 Target sites of plant extracts containing odor compounds to treat inflammation

aches to topical applications and infusions of leaves and stems. Infusion of *Cestrum parqui* stem and leaves were used to treat inflammation, coughs, heart and bladder pain, stomach ache, and also for wound washing [35.12].

In African medicine, infusions and decoctions were the preferred forms of medical preparations for cattle. The plant infusions were either applied topically or as drinks. The preference toward fresh plant material indicates a role of odor compounds [35.13]. Italian traditional medicine used infusions of chamomile to treat eye inflammation and skin conditions like dermatitis [35.14]. Chamomile has been registered as a medicinal plant in Germany for treating mucous membrane and skin inflammation as well as bacterial skin disease.

In summary, plant extracts containing odor compounds are widely used to treat symptoms of inflammation systemically as well as directly. Figure 35.1 summarizes the pre- and postabsorptive inflammatory sites, where plant extracts containing odor compounds have been described as beneficial. Treatment of inflammation of skin, eyes, oral gum, or mucosal tissue of the gastrointestinal tract and the lung do not require absorption of the odor compound. In contrast, a treatment of symptoms like fever and pain may require the absorption of the odor compounds, including possible structural changes of the compounds upon metabolism.

35.2 Mechanisms Underlying the Anti-Inflammatory Activity

The mechanisms underlying ananti-inflammatory activity are closely connected to the mechanisms inducing inflammation. Inflammation, as part of the innate immune response, is a reaction of an organism to pathogens, for example, viruses or bacteria. If pathogens pass the mechanical barriers (Table 35.1), they enter the body, may damage cells and allow foreign substances (antigens) to enter the organism. Antigens can be proteins, toxins, or other foreign chemicals that are specific for a pathogen. Thus, bacterial endotoxins or the filament protein flagellin or viral ribonucleic acid (RNA) represent potential recognition targets. In the host, immune-active cells detect and react to these antigens. Toll-like receptors play a crucial role in the detection of antigens from viruses and bacteria and transmit the information by signaling cascades.

Table 35.1 Key factors of the mechanical, cellular, and humoral innate immune response

Defense type	Barrier	
Mechanical defense	Skin	*Sweat*
	Gastrointestinal tract	*Gastric acid*
	Nasopharynx	*Saliva*
	Lungs	*Mucus*
	Eyes	*Tears*
Cellular defense	Epithelial cells	
	Fibroblasts	
	Natural killer cells	
	T-Cells	
	Phagocytes	
Humoral defense	Chemokines	
	Interleukins	
	Enzymes	
	Adherence factors	
	Interferons	

35.2.1 Antigen Detection by Toll-Like Receptors

The mammalian toll-like receptor (TLR) family can be divided into at least 11 members and are part of the pattern recognition receptors. Humans possess functional TLRs 1 to 10, while TLR11 has only been found to be functional in mice. Toll-like receptors are trans-membrane proteins and are located in the outer membrane as well as in the endosomal membrane intra-cellular [35.15, 16]. TLR2 forms heterodimers with TLR1 or TLR6 and recognizes a variety of patterns, while other TLRs are active as monomers or homodimers with specific ligands. The ligand-binding domain is located in the extra-cellular region or in the endosome. The cytosolic Toll/IL-1 receptor (TIR) domain of TLRs is linked to an adaptor protein, myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF) [35.17].

The different types of TLRs (TLR1-9) recognize different specific patterns, so-called pathogen-associated molecular patterns (PAMPs) [35.18]. The patterns recognized by the TLRs are diacyl or triacyl lipopeptides, flagellin, lipopolysaccharide (LPS), single or double stranded RNA, or CpG desoxyribonucleic acid (DNA), all different types of microbial components. LPS is one of the most effective PAMPs and is known to activate TLR4 [35.19]. However, it has been shown that LPS from an oral bacterium, *Porphyromonas gingivalis*, is recognized by TLR2 and exploits different actions as *Escherichia coli* LPS [35.20]. Thus, the TLR type responsible for the recognition of LPS depends on the source and structure of the endotoxin. Ligand binding to a TLR results in the onset of signaling cascades

and gene expression (Fig. 35.2), depending on which TLR is activated [35.17].

35.2.2 Intracellular Signaling Pathways Involved in Inflammation

The most important signaling pathway involved in inflammation is the nuclear factor kappa B (NF-κB) pathway (Fig. 35.2). In immune active cells, cytosolic NF-κB is bound to inhibitory kappa B (IκB) and thereby inactivated. After ligand binding to TLRs, the adaptor molecule MyD88 recruits an intracellular interleukin-1 receptor-associated kinase (IRAK) in the cytosol, which activates TNF receptor-associated factor 6 (TRAF6). This signal molecule further activates the IκB kinase (IKK) complex, which phosphorylates IκB resulting in a cleavage of IκB and NF-κB [35.21]. The free NF-κB migrates into the nucleus and binds to NF-κB binding sequences in the DNA, leading to a gene expression of inflammatory cytokines in the early phase and interferon-β (IFN-β) in the late phase. TLRs that are linked to TRIF mediate a signal via kinases (e.g., IKK) resulting in the activation of the interferon regulatory factor 3 (IRF-3) and gene expression of IFN-β [35.22].

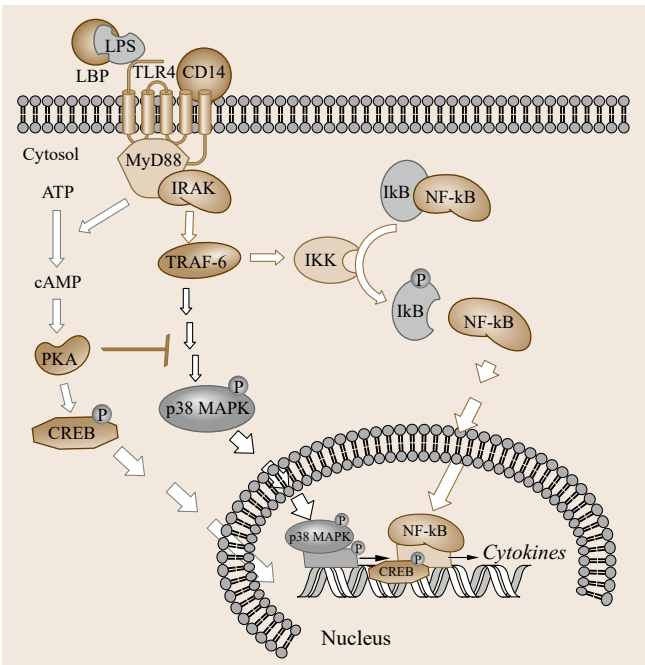

Fig. 35.2 Toll-like receptor 4 (TLR4)-mediated signaling. Lipopolysaccharides (LPS) and LPS-binding protein (LBP) activate the complex of TLR4 and CD14, leading to the recruitment of IL-1 receptor-associated kinase (IRAK) by the adaptor protein MyD88. TRAF6 further activates inhibitory kappa B (IκB) kinases (IKK), which phosphorylate IκB, inducing its cleavage from NF-κB. TRAF6 also induces elements of the p38 MAPK pathway, resulting in the phosphorylation of p38 MAPK. In the nucleus, both NF-κB and pp38 MAPK induce the gene expression of cytokines. Induction of the TLR also activates cAMP-mediated signals, inhibiting the MAPK cascade

Additionally, other signaling pathways, the mitogen-activated protein kinase (MAPK) pathway (Fig. 35.2), have been shown to be involved in inflammatory gene expression. TLR-stimulated TRAF6 activation also leads to the onset of the p38 MAPK phosphorylation cascade. In the nucleus, p38 MAPK phosphorylates and, thereby, activates transcription factors leading to the expression of genes encoding pro-inflammatory factors [35.23, 24].

Furthermore, TLR-independent signaling pathways, including cyclic AMP (cAMP) and calcium signaling, modulate the TLR signaling cascades (Fig. 35.2). Protein kinase A, activated by cAMP, inhibits elements of the MAPK pathway and also phosphorylates and activates cAMP responsive element binding protein (CREB). After entering the nucleus, pCREB competes with NF-κB for the binding site on CREB-binding protein and thereby inhibits the NF-κB-induced gene expression of pro-inflammatory cytokines [35.22].

35.2.3 Pro-Inflammatory Factors Induced by TLR Signaling

The stimulation of TLRs results in the mRNA expression of pro-inflammatory cytokines and enzymes, for example, inducible nitrite oxidase (iNOS) or cyclooxygenase-2 (COX-2). After mRNA expression and translation, iNOS produces NO, which is released by the immune cells as an anti-microbial compound. The COX-2 protein triggers the reaction of arachidonic acid to prostaglandins. Prostaglandin E2 (PGE2) has been known as a pro-inflammatory factor in the early stage of inflammation. However, in later stages of the inflammatory process, PGE2 activates the cAMP pathway and inhibits functions of NK cells or granulocytes [35.21, 22, 25].

Cytokines include chemokines, tumor necrosis factors (TNF), interferons, lymphokines, and interleukins (IL). The nomenclature does not follow a specific pattern. One strategy was naming after the location of production: monokines (monocytes), lymphokines (lymphocytes), and originally interleukins (leukocytes). However, interleukins can also be produced and released by other cell types, such as fibroblasts. Furthermore, it has been shown that IL-8 may also be considered a chemokine. Chemokines are named after their function (chemotraction), recruiting immune cells to the center of inflammation. All cytokines bind to specific receptors and mediate a target signal.

35.3 Assessing the Anti-Inflammatory Activity of Odor Compounds

Inflammation plays a crucial role in the defense against pathogens. During inflammation, partial tissue destruction is used to remove the pathogens from infected cells by cells of the immune system, macrophages or T-cells. Local tissue cells like fibroblasts are known to recruit leukocytes by releasing pro-inflammatory cytokines [35.26]. If the epithelial cells continue their immune activity beyond the fight against pathogens, chronic inflammation may lead to the development of an inflammatory disease, for example, inflammatory bowel disease [35.27] or asthma [35.28]. Nevertheless, inflammation is an important part of the innate immune response and is controlled by the presence of mechanical, cellular, and humoral immune defense (Table 35.1). The body reacts to an attack with the following symptoms: swelling, redness, loss of function, and heat. The latter is also responsible for the term *inflammation* which means *setting on fire* or *ignition*.

To assess an anti-inflammatory activity, immune competent model systems need to be chosen. The outcome markers of an immune response are mRNA expression and release of cytokines. Thus, model systems of the innate immune response, expressing, or releasing cytokines, are the most common cell models in investigations of anti-inflammatory effects. Endothelial cells, fibroblasts, monocytes, macrophages, and peripheral blood mononuclear cells (PBMCs) provide immune competence and are therefore preferred in vitro or ex vivo model systems. However, an effect seen from in vitro experiments should be confirmed in vivo. Here, working with odorants, originating from foods, ethically allows both animal studies and human intervention studies. Effects that require an induction of inflammation using LPS or other toxins may be done in animal models or using ex vivo experimental settings.

35.3.1 In Vitro Cell Models to Investigate Anti-Inflammatory Effects of Odorants

The choice of an in vitro cell model is determined by the research question and how the test compounds enter the organism. Odorants that can be inhaled may pass through the skin, or are consumed with foods. Thus, a variety of endothelial cells, lung, oral or esophageal, as well as connected fibroblasts serve as a well-established immune active model systems. Human gingival fibroblasts (HGF) are known to express TLRs and respond to stimulation with different types of LPS [35.29]. The cell line HGF-1 expresses mRNA and release cytokines, IL-6 and IL-8, upon stimulation with LPS from *P. gingivalis* for at least 6 h [35.30]. Thus, HGF-1 cells have been established as a cell culture model to investigate anti-inflammatory effects of food compounds or orally ingested extracts [35.31]. However, the endothelium is the first barrier of the oral cavity in response to pathogens. Thus, an oral endothelial cell-line is also used as a model system to study the anti-inflammatory properties of food constituents on periodontal tissue. These cells have shown to respond to 24 h stimulation with 10 $\mu g\,ml^{-1}$ LPS from *P. gingivalis* with an increased release of IL-6, IL-8, and CCL-5 [35.32].

Furthermore, pharmacokinetic studies showed that after consumption, volatile compounds or their metabolites can be detected in plasma. In rats, α-cedrene was detected in the plasma in concentrations of up to 2.4 μM after oral administration of 25 $mg\,kg^{-1}$ [35.33]. After intake of 30–40 oz lemonade, peak plasma concentrations of the main limonene metabolite perillic acid were reached within the first hour with $37 \pm 24\,\mu M$ in 12 healthy volunteers [35.34]. Thus, blood cells, involved in the innate immune response, may also serve as model systems. Especially, monocytes and macrophages are widely used as cell model system to investigate the effects of odor compounds and their respective plasma metabolites. Different animal, murine macrophages (RAW 264.7), and human, THP-1 or U937, cell lines have been established. Both, THP-1 and U937 cells are monocytic cells which can be differentiated into macrophages using phorbol esters, for example, phorbol 12-myristate 13-acetate. LPS stimulation leads to mRNA expression of *iNOS*, *COX-2*, *TNF*α, and different interleukins (*IL-1*β, *IL-6, IL-8*, and *IL10*) in THP-1 monocytes and macrophages. Therefore, these cell lines were established as model systems to identify anti-inflammatory effects of food compounds [35.35]. In differentiated U937 cells, IL-1α/β, IL-6, and TNFα mRNA expression have been detected, and an anti-inflammatory effect of the red wine constituent resveratrol and its monosulfated metabolite has been determined [35.36]. The advantage of the murine cell line RAW 264.7 is that no differentiation is needed to obtain macrophages, which also can be stimulated to express pro-inflammatory cytokines [35.37].

Experimentally, different strategies can also be chosen for the determination of preventive or therapeutic effects. For a preventive effect, a preincubation with the potential anti-inflammatory compound followed by stimulation of the immune response, should be the experimental set-up. In contrast, co-incubation of the

compound with a stimulant shows the therapeutically anti-inflammatory potential of the compound in the presence of a given pathogen or endotoxin.

35.3.2 In Vivo and Ex Vivo Experimental Settings to Investigate Anti-Inflammatory Effects of Odorants

In vivo studies primarily focused on the reduction of symptoms related to inflammatory diseases, testing for anti-asthmatic [35.38], anti-colitis [35.39], or wound-healing [35.40] effects of odor compounds in animal model systems. In contrast, human intervention studies on anti-inflammatory effects of food constituents are sparse. No conclusive data on anti-inflammatory effects of odor compounds in vivo have been collected. Broadening the search for information, including gustatory compounds, the bitter tasting flavonoids can be referred to as aroma-active compounds. A meta-analysis of the effects of flavonoids on the plasma concentrations of TNF-α and IL-6 showed no effect of the flavonoid on either of the two parameters in a random model. However, decreased levels of plasma TNF-α were detected with a fixed effect model after flavonoid intake [35.41].

Ex vivo studies generally use whole blood [35.42] or biopsied tissue [35.43], and stimulate an immune response outside the organism. Moreover, investigation directed toward anti-inflammatory diseases of the oral cavity may be done in primary gingival endothelial cells. These cells, isolated during dental surgery, can be treated with LPS ex vivo [35.44].

35.4 Identification of Anti-Inflammatory Odorants

Foods rich in essential oils are fruits, herbs, and spices. Essential oils of all of these foods have been studied for their anti-inflammatory potential. However, in the essential oil of a plant, a variety of different structured molecules are present. Aromatic food constituents can be separated into different groups, according to their chemical structure, for example, aldehydes, phenols, lactones, or terpenes. Previous research studies of anti-inflammatory effects of odorants either used the crude essential oil or further identified individual compounds of the essential oil that are responsible for the anti-inflammatory effect. Furthermore, depending on the target site (Fig. 35.1), absorption and structural changes to the compounds due to metabolism have to be considered. Preabsorption target sites, the oral cavity or the lung mucosa, are in direct contact with the essential oil, while in the blood metabolites may be present in higher concentrations than the parent odor compounds.

35.4.1 Anti-Inflammatory Effect of Odor Compounds Preabsorption

In previous studies, our research group has shown anti-inflammatory effects for odor compounds on cells of the oral cavity. Orange juice essential oil was shown to reduce the intra-cellular IL-6 concentration in buccal cells [35.45]. The orange juice volatile, α-terpineol, has been identified as the anti-inflammatory active compound, reducing intra-cellular IL-6 concentration in buccal cells, stimulated with PMA/ionomycin (PMA: phorbol-12-myristate-13-acetate) [35.45]. In HGF-1 cells, sage infusion inhibited the PMA/ionomycin-stimulated release of IL-6 and IL-8. The volatile fraction was analyzed using gas chromatography with mass spectrometry (GC-MS) and five compounds were identified, 1,8-cineole, borneol, camphor, and α- and β-thujone. These individual compounds exploited anti-inflammatory properties in sage infusion representative concentrations. Furthermore, using a combination of the individual compounds, reconstituting the essential oil, inhibited the PMA/ionomycin-stimulated IL-6 and IL-8 release, proving that the volatile compounds contribute to the anti-inflammatory potential of sage infusion [35.46].

While limited information is available on the anti-inflammatory effect of thujone, borneol, and camphor as individual compounds, the anti-inflammatory potential of eucalyptol (1,8-cineole) has been investigated in vitro and in vivo. Thereby, one research group focused on the anti-inflammatory activity of 1,8-cineole with regard to a treatment of airway infections. In animal models, the monoterpene decreased biomarkers of colitis [35.47] and attenuated LPS-induced lung inflammation by suppression of TLR4 and NF-κB expression [35.48]. Moreover, in a double-blind placebo-controlled human intervention study, 200 mg cineole, administered three times per day over a period of 6 months improved the symptoms of asthma, respiratory rate, and subjective quality of life [35.49]. A similar study was performed by *Juergens* et al. [35.50], using the same dose of cineole and also investigating a beneficial effect of the compound against asthma. After 12 weeks of intervention, the patients who received cineole tolerated lower doses of steroids and were stable on lower steroid doses for a longer period of time than the patients of the placebo group.

35.4.2 Postabsorptive Anti-Inflammatory Activity of Odor Compounds

Various essential oils and individual odor compounds have been tested for an anti-inflammatory potential in postabsorption test systems, monocytes, and macrophages. The essential oil of striped African pepper, traditionally used to flavor soups, was demonstrated to possess anti-inflammatory properties in vitro. In concentrations $\geq 6\,\mu g\,ml^{-1}$, this essential oil inhibited the $1\,\mu g\,ml^{-1}$ LPS-stimulated NO production in RAW 264.7 macrophages after a 24 h co-incubation [35.51]. In the same test system, essential oil of cinnamon (*Cinnamomum osmophloeum*) inhibited the LPS-induced NO production by 69% and the PGE_2 release by 65% in concentrations of 25 and $10\,\mu g\,ml^{-1}$ cinnamon twig essential oil, respectively [35.52]. α-Thujone (48.3%), β-thujone (12.7%), and camphor (6.7%) have been identified as the main constituents of the essential oil of *Artemisia fukudo* Makido, a plant used in South Korea and Japan to flavor cosmetics. The essential oil of this plant was shown to inhibit NO and PGE_2 production, *iNOS* and *COX-2* mRNA expression as well as cytokine mRNA expression and release via the MAPK and NF-κB pathway in LPS-stimulated RAW 264.7 macrophages [35.53].

Furthermore, essential oils of common herbs used to flavor food exploit anti-inflammatory properties systemically [35.54]. Rosmary (*Rosmarinus officinalis* L.) oil inhibited the leukocyte migration in different animal model systems for inflammation [35.55, 56]. In a murine macrophage model, *Thymus vulgaris* L. essential oil inhibited the mRNA expression of *iNOS* and NO release. In THP-1 cells, $10\,\mu g\,ml^{-1}$ sage (*Salvia officinalis* L.) and oregano (*Origanum vulgare*) reduced the oxidized LDL-stimulated release of TNF-α, IL-6, and IL-1β and increased the release of the anti-inflammatory cytokine IL-10 (LDL: low density lipoprotein) [35.57]. The volatile compounds 1,8-cineole, borneol, camphor, and thujone are found in many herbal essential oils. In THP-1 monocytes, 1,8-cineole inhibited the translocation of the MAPK activated transcription factor early growth response protein 1 (egr-1) to the nucleus, suggesting an anti-inflammatory potential [35.58]. In a study in 2004, the authors showed that $1\,\mu M$ 1,8-cineole inhibits the release of IL-1β, IL-6, IL-8, and TNF-α in human monocytes after 20 h concomitantly stimulated with PMA/ionomycine. Additionally, the release of IL-1β, IL-4, IL-5 and TNF-α by stimulated lymphocytes was decreased by $10\,\mu M$ eucalyptol [35.59].

In most of the here cited studies, the essential oil composition was identified, individual constituents quantified, and tested for their anti-inflammatory potential. Of the different types of volatiles, terpenes have been determined to exploit anti-inflammatory properties. Among the terpenes, especially the monoterpenes possess anti-inflammatory potential [35.60].

35.5 Risk Assessment

Eucalyptol (1,8-cineole) is available in Germany as licensed medicinal product. In a human intervention study, patients receiving 200 mg three times per day reported adverse effects, for example, severe headache, heartburn, or gastritis, all considered as side effects of the intervention [35.50]. This raises the question whether essential oils and volatiles are safe to consume, even in large amounts. To evaluate the safety of a volatile compound, the following points need to be considered:

1. In vitro toxicology data
2. Administration and exposition
3. Distribution
4. Metabolism
5. Excretion.

Food and food additive safety is controlled by the Food and Drug Administration (FDA) in the United States and the European Food Safety Authority (EFSA) in Europe. Volatiles are known to be added to foods and cosmetics as flavoring compounds. Hence, volatiles are not only taken up by oral ingestion and inhalation but also through the skin in cosmetics. It has been shown that camphor and menthol were absorbed dermally after 8 h application and detected in the plasma [35.61]. Here, two examples of volatile compounds and their toxic potential and risk assessment are presented: (1) thujone, the absinthe constituent and (2) eugenol, found in clove oil, nutmeg, or bay leaves.

35.5.1 Toxicity of Thujone

Absinthe has already been known in Roman times as a psychoactive drug and was popular over centuries among artists like van Gogh, Degas and Picasso, who consumed and painted the *green fairy*. Later, absinthe was banned in Europe until the 1990s. A characteristic sensory constituent of this beverage is thujone. How-

ever, the maximum thujone concentration in absinthe is now limited to 35 mg kg^{-1}. The abuse and chronic ingestion of absinthe is associated with stimulation, hallucinations, and depression [35.62]. The toxic potential of thujone was reviewed in 2012 by a Finnish research group [35.63]. In vitro data on the mechanism of neurotoxicity showed that α-thujone modulated the γ-amino butyric acid $(GABA)_A$-gated chloride channel. The intake sources of thujone are sage-flavoured sausages and other meats, herb vinegar, liqueurs/bitters, and sweets, resulting in an estimated mean total daily intake of 1.175 mg per person per day.

In different animal studies, single dose toxicity was determined. LD_{50}-values of mixtures from α- and β-thujone were measured with 192 mg kg^{-1} in rats, 230 mg kg^{-1} in mice and 396 mg kg^{-1} in guinea pigs when orally administered. To determine a no observed adverse effect level (NOAEL), repeated dose toxicity experiments were needed. In 1963, a study was conducted in rats with gavages in doses of 0, 5, 10, and 20 mg kg^{-1} and six times per week for 14 weeks. In addition, studies with doses up to 100 mg kg^{-1} per day were performed in mice and rats. In summary, a NOAEL of 30 mg kg^{-1} per day was released. Based on human intervention data and the NOAEL, an acceptable daily intake (ADI) of 0.11 mg kg^{-1} bodyweight was calculated [35.63]. For a normal adult (70 kg) this would mean a daily intake of 7.7 mg thujone or, considering the maximum concentration of 35 mg kg^{-1} thujone in absinthe, 220 g (approx. 235 ml) absinthe per day.

35.5.2 Toxicity of Eugenol and Related Compounds

The EFSA reviewed the toxic potential of eugenol and related compounds in 2006 and adopted this review in 2008. The EFSA procedure is based on the opinion of the Scientific Committee on Food (SCF), derived from the joint FAO/WHO expert committee on food additives (JECFA). The JECFA estimated the intake based on the maximized survey-derived daily intake (MSDI). However, the more realistic approach considered the modified theoretical added maximum daily intake (mTAMDI), which is based on the normal levels used by industry. For eugenol, estimated intakes based on the MSDI model of 0.950 mg per capita per day in Europe and of 3.364 mg per capita per day in the United States were calculated. Based on the mTAMDI approach, an intake of 2.3 mg eugenyl isovalerate per capita per day was determined. First, the toxicology and genotoxic data were evaluated with generally no genotoxic potential for in vitro and in vivo toxicology of eugenol even at doses of up to 800 mg kg^{-1} bodyweight (bw). A NOAEL for eugenol has been determined with 300 mg kg^{-1} bw.

In addition to eugenol, typical volatile food compounds were evaluated in the same report, for example, thymol and carvacrol. Thymol, found in the essential oil of thyme, did not possess any genotoxic potential in the Ames test. The compounds evaluated by the expert panel of the EFSA remained to be safe for a regular and normal intake with foods.

35.6 Summary and Outlook

In summary, food odorants have been shown to possess anti-inflammatory potential in various model systems. Both, the complex mixture of volatiles in essential oils and individual compounds, for example, 1,8-cineole, borneol or camphor, exploit anti-inflammatory effects in cell model systems of inflammation-related diseases and systemic inflammation. These anti-inflammatory effects resulted in the inhibition of the NF-κB signaling pathway, the mediated mRNA expression, and release of cytokines. The main outcome measures were mRNA expression of *iNOS*, *COX-2*, *IL-1β*, *IL-6*, *IL-8*, and *TNF-α* as well as the release of the encoded interleukins, NO and PGE_2, although further studies are needed to provide more comprehensive mechanistic data. Moreover, toxicological information is needed for safety evaluation and risk assessment of food and cosmetic flavor compounds and essential oils. However, volatile food constituents comprise beneficial anti-inflammatory properties and, therefore, are a promising class of compounds in the treatment and prevention of inflammatory-related diseases.

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36. Skin Sensitization of Odorant Materials

Andreas Natsch, Graham Ellis

Many natural and synthetic odorant materials contain structural features such as aldehyde functionalities or conjugated double bonds which lead to a certain chemical reactivity. Such molecules have the intrinsic ability to modify skin proteins, and if they are applied to skin at too high doses this may trigger an immune reaction leading in sensitive individuals to an allergic reaction. Here we review the underlying molecular mechanisms, the key structural classes of sensitizing odorant molecules, the predictive tests to identify fragrance allergens, the epidemiology of fragrance allergy, and the measures taken to avoid such reactions based on a risk assessment.

A skin sensitizer may be defined as a chemical which has the intrinsic ability (hazard) that, given sufficient skin exposure, can lead to the modification of the immunological state referred to as induction of skin sensitization or, clinically, as contact allergy. The induction of sensitization (contact allergy) refers to a nonsymptomatic condition which an individual has when they are sensitized to a specific chemical. Once sensitized, a subsequent and sufficient skin exposure to the same material in an individual who has contact allergy may lead to a skin reaction (eczema) also referred to as allergic contact dermatitis (ACD; Fig. 36.1 for an allergic reaction to a deodorant). The primary aim of safety assessment and risk management must therefore be to avoid the induction of contact allergy by skin sensitizers.

Materials with odorant properties may be naturally or synthetically sourced and cover a wide and varied possibility of chemical structures which provide for a large diversity in toxicological as well as olfactive properties [36.1]. Some of these odorant materials may have the ability to induce a skin sensitization response which may be predicted through animal, human, or in vitro and in silico models.

This chapter describes the mechanisms behind skin sensitization and how this relates to different classes of odorant materials with potentially sensitizing properties. Currently available methods to determine the intrinsic skin sensitising property (hazard) of a material and tools used to determine skin sensitization potency and therefore allow appropriate risk management are also presented. Finally, an overview of what is currently known about the epidemiology of allergy to odorant materials in the clinical and general population is discussed along with the tools used to manage safe use of such materials.

36.1 Molecular Mechanism Behind Skin Sensitization

Skin sensitization is a complex chain of reactions, involving a number of chemical and biological steps. It is generally divided into two key phases: (a) the sensitization phase, when the organism for the first time comes in contact with a sufficient amount of a chemical to trigger an immunological reaction (i. e., expansion of specific T-cell clones) and (b) the elicitation phase, when the expanded T-cell clones recognize their corresponding antigen and trigger an inflammatory reaction in the skin leading to the disease state known as allergic contact dermatitis. Some of the molecular events described in the following are common to both the sensitization and the elicitation phase, whereas others are specific to one phase. The key steps were recently described as an *adverse outcome pathway* (AOP) [36.2].

In a first step, a topically applied molecule must reach the viable epidermis, and skin penetration or more specifically *bioavailability in the skin* therefore is thought to be a prerequisite for the skin sensitization reaction. However, bioavailability appears not to be a key rate determining factor as chemicals of very different physicochemical properties can trigger sensitization [36.3]. Indeed, odorant molecules are sufficiently small and lipophilic that they can gain access to the epidermis if topically applied.

Chemicals with low molecular weight (MW < 500 Da) are too small to be immunogenic and cannot directly trigger an immunological response. However, if these molecules are able to change the molecular conformation of larger molecules, especially proteins, they can render endogenous proteins in the body immunogenic. Therefore reactive chemicals, which are able to covalently modify proteins under physiological conditions, do have the ability to generate novel antigens in the skin. Such protein-modifying chemicals are also referred to as *haptens* [36.4]. Once the hapten binds to the skin protein/peptide, a novel epitope is formed, a novel structure not previously known to the immune system. This new structure may then be recognized as *foreign* by the immune system. Chemically speaking, sensitizers therefore are mostly electrophilic chemicals able to react with nucleophilic residues (predominantly cysteine and lysine) in proteins. The key step for the initiation of skin sensitization is therefore the formation of a covalent adduct between the skin sensitizer and endogenous proteins and/or peptides in the skin. This event is also referred to as the molecular initiating event (MiE) [36.2]. Certain molecules are considered *pro-haptens*, indicating that they themselves are non-sensitizers and need to be metabolically converted by skin enzymes to form reactive metabolites which then react with the proteins [36.4].

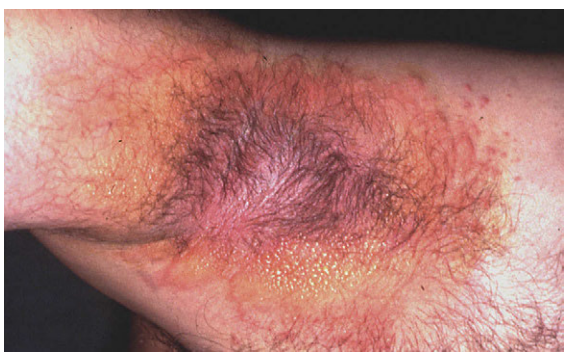

Fig. 36.1 Allergic contact dermatitis to a deodorant (photo by An Gossens)

In the next step, the modified peptides must be processed and presented by the Langerhans cells (the dendritic cells in the skin) on their major histocompatibility (MHC) proteins in order to be recognized by T-cells. The Langerhans cells must also migrate to the lymph nodes, as only there they come in contact with the T-cells to present the novel epitopes. This migration must be actively initiated, and it is thought that the mere binding of modified peptides on the dendritic cells is not sufficient to initiate the activation and migration of dendritic cells, but that a second signal often referred to as *danger signal* is needed at this stage [36.5]. The danger signal appears to be a part of the innate immune reaction and it is formed in the absence of specificity conveyed by T-cells. Recent work shows that activation of Toll-like receptors (TLRs) [36.6] and the inflammasome [36.7, 8] to form interleukins (IL-1β and IL-18) play a central role in the danger signal. These pathways are also involved in the innate reactions against pathogens. At the molecular level, the sensitizer-triggered release of ATP [36.9], the formation of fragments of hyaluronic acid [36.10], and the formation of reactive oxygen species (ROS) [36.11, 12] appear important to trigger chemical-induced TLR-signaling and inflammasome activation.

Stimulated by the danger signals, the dendritic cells maturate and emigrate from the skin into local lymph node where they finally present the hapten-modified peptides to the T-cells and stimulate the proliferation and differentiation of specific T-cell clones. Cytotoxic CD8+ T-cells are then the key effector cells. This complete process depicted on the left hand of Fig. 36.2 is referred to as the *sensitization phase* of the skin sensitization reaction. In the *elicitation phase*, the specific expanded cytotoxic T-cell clones then trigger skin inflammation on secondary contact with the causative

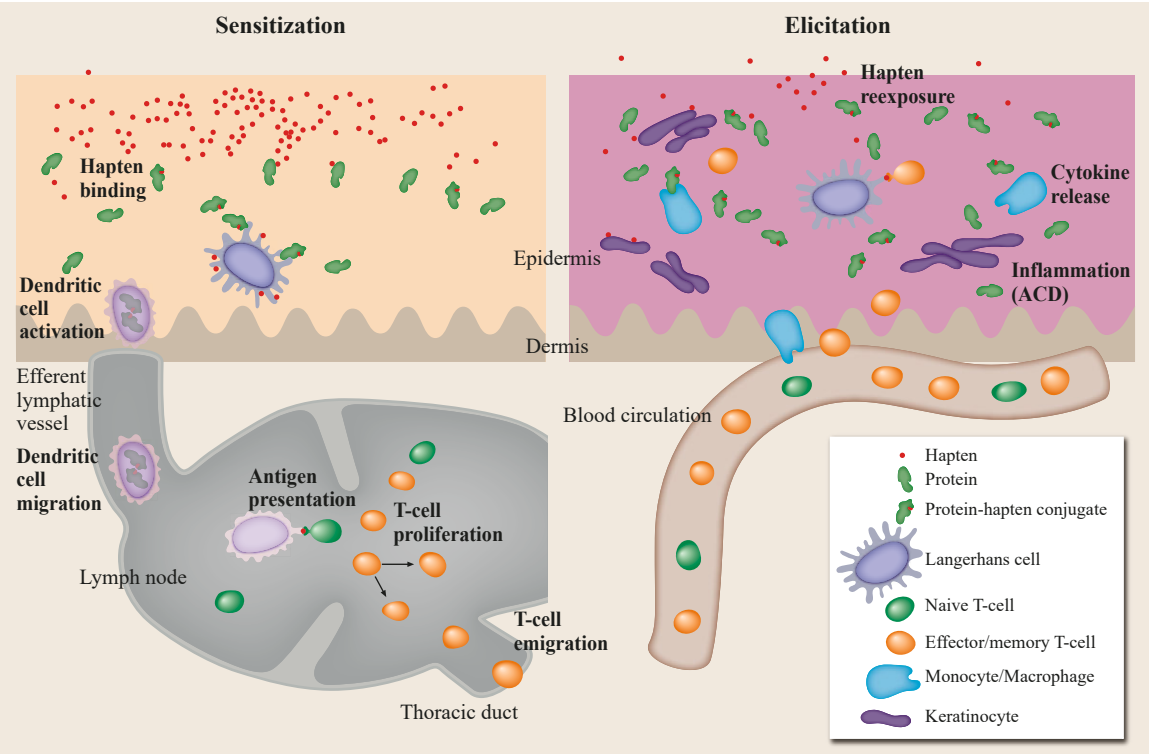

Fig. 36.2 The mechanistic steps leading to allergic contact dermatitis (after [36.4])

agents when they recognize the hapten-modified peptides presented by the keratinocytes.

In summary, according to the current model, skin sensitizing molecules must be able to both (i) form novel epitopes due to their reactivity and to (ii) trigger the danger signal. Since both requirements are needed, skin sensitization in most individuals only occurs above a given threshold. For each sensitizer, a given dose is therefore tolerated [36.13], and it appears that only above a certain threshold-dose sensitizers trigger sufficient danger signal and form sufficient epitopes to initiate the sensitization phase.

36.2 Structural Classes of Sensitizing Molecules with Special Emphasis on Odorants

Based on the molecular understanding of the MiE, skin sensitizing chemicals can be classified according to their chemical reactivity and whether they are directly reactive (haptens) or whether they need metabolic or oxidative activation to become haptens (pro-haptens). Classification can be performed either by structural alerts (reactive functional groups) [36.14] or by predicted chemical reaction pathways [36.15]. Here we give no systematic overview on all classes of sensitizing molecules, but highlight the key functional groups present in odorants and the key reaction pathways which have been associated with their skin sensitization potential. A more general overview can be found elsewhere [36.16]. It is noteworthy that key structural features determining the odor of important natural scents are often also determining their reactivity – thus, aldehydes, α,β-unsaturated aldehydes, α,β-unsaturated ketones, substituted phenols, and aromatic aldehydes: These are the main structural features highlighted as follows but these structural features are also present in key ingredients in many essential oils. Our olfactive

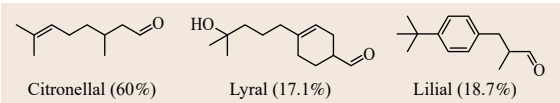

Fig. 36.3 Aliphatic aldehydes and their skin sensitization potential according to the LLNA

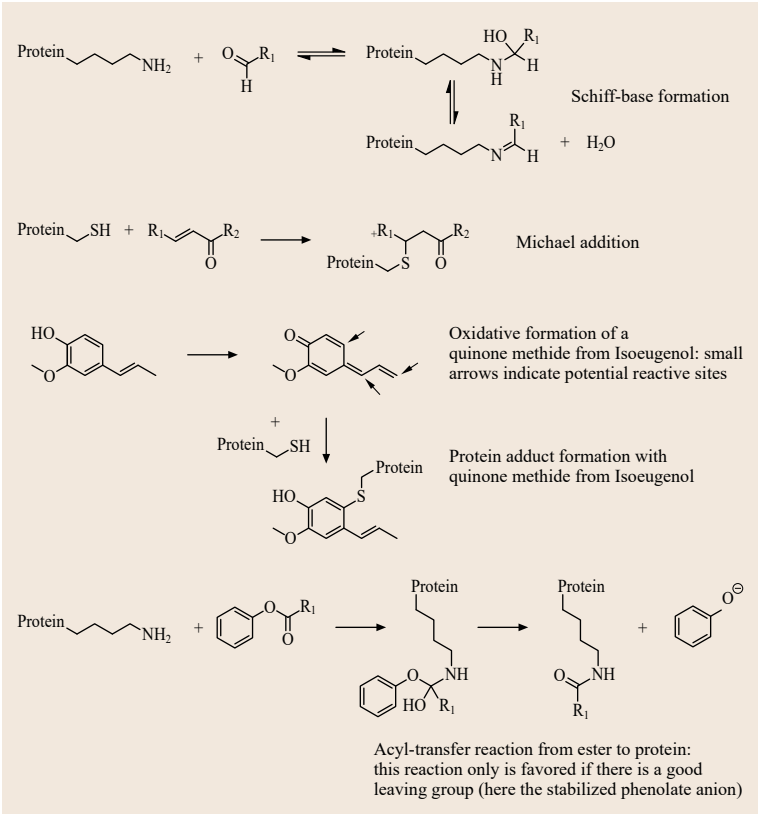

Fig. 36.4 Theoretical reaction mechanisms between the discussed classes of skin sensitizers and skin proteins (mainly cysteine and lysine side chains). Note: these mechanisms are largely based on chemical knowledge and in vitro experiments – the actual proteins modified and the epitopes formed in vivo are unknown

system appears to be tuned to these structural features in natural scents, which makes formulation of appealing scents without any structural alerts for skin sensitization challenging, if not impossible. In the following, the key classes are discussed and exemplary structures are given in the figures, associated with the EC3 values from the local lymph node assay (LLNA) to indicate their potency in animal test (concentration in percentage to induce three-fold lymph node cell proliferation; see as follows).

36.2.1 Aliphatic Aldehydes

Branched and unbranched aliphatic aldehydes (some containing ring systems in the chain) are widely used as odorants and include natural compounds such as citronellal or synthetic chemicals, such as the lily-of-the-valley aldehydes exemplified here by Lilial and Lyral (Fig. 36.3). As aldehydes, they can in principle form Schiff-bases with skin proteins (Fig. 36.4 for overview of putative reaction mechanisms). However, in the aqueous milieu of biological tissues this reaction is not favored due to rapid reverse reaction. This is most likely the reason why this group is mostly rated as weak sensitizers in animal tests and frequency of positive reactions in humans is quite low, despite widespread use. An exception here is Lyral which has given rise to frequent positive reactions in humans [36.17] – it is unclear currently whether this is due to high use or an unknown intrinsic feature of this molecule.

36.2.2 Aromatic Aldehydes

A number of aromatic aldehydes are very important for their unique scent. Most widely used is not just vanillin, but also benzaldehyde and heliotropin are the key balsamic odorants (Fig. 36.5). With the exception of benzaldehyde, these are very weak sensitizers – an observation which could be linked to a very low reactivity of these molecules [36.18]. This structural group also contains two aldehydes found as minor components in oakmoss extract: atranol and chloratranol. These two molecules are potent skin sensitizers [36.19–21] and were found to be the culprit for the high frequency of sensitization to oakmoss. Their sensitization potential appears to be due to their ability to form Schiff-bases which are stabilized by tautomerism in aqueous conditions [36.18]. Commercial oakmoss qualities now must follow stringent limita-

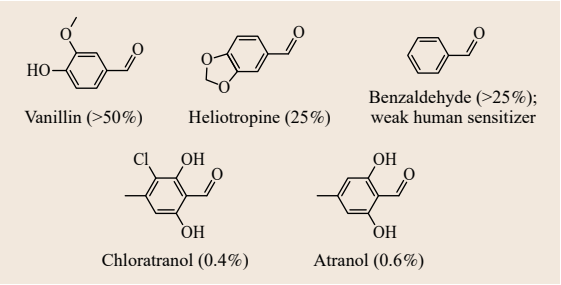

Fig. 36.5 Aromatic aldehydes and their skin sensitization potential according to the LLNA

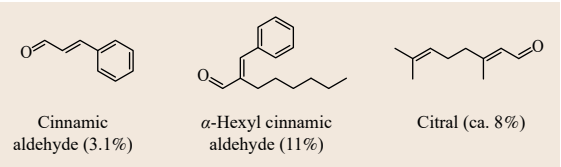

Fig. 36.6 α,β-unsaturated aldehydes and their skin sensitization potential according to the LLNA

tions for these two chemicals to avoid the sensitization risk.

36.2.3 α,β-Unsaturated Aldehydes

These aldehydes are more reactive as compared to the aliphatic aldehydes as they can react (next to potential Schiff base formation) with a Michael addition at the conjugated double bond (Fig. 36.4). The key representative in this group is cinnamic aldehyde, the main component of cinnamon bark essential oil. Aldehydes that are branched at the α-atom, such as α-hexylcinnamic aldehyde or at the β-atom such as citral have much lower reactivity, accompanied with a reduced sensitization potential (Fig. 36.6).

36.2.4 α,β-Unsaturated Alcohols

The α,β-unsaturated alcohols are pro-haptens and are thought to be predominantly oxidized by skin enzymes to the corresponding aldehydes (Fig. 36.7). This is reflected by frequent cross-sensitization: Thus, patients who are sensitive to cinnamic alcohol often do also react to cinnamic aldehyde [36.22], and this observation is most elegantly explained by oxidation of cinnamic alcohol in the skin and subsequent reaction of sensitized individuals to the formed cinnamic aldehyde. However, cross-sensitization is not the case for all individuals and, therefore, alternative metabolic routes such as formation of an epoxide at the double bond of cinnamic alcohol and subsequent addition of the nucleophile to the epoxide bond were proposed [36.23]. A similar pair

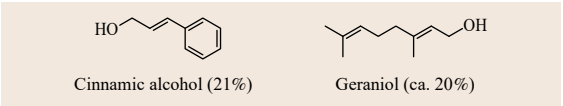

Fig. 36.7 Unsaturated alcohols and their skin sensitization potential according to the LLNA

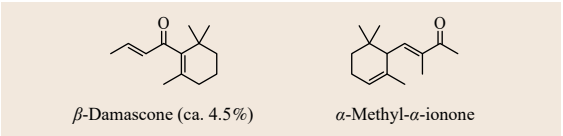

Fig. 36.8 α,β-unsaturated ketones and their skin sensitization potential according to the LLNA

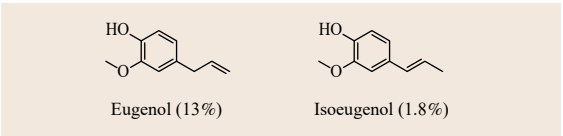

Fig. 36.9 Phenols and their skin sensitization potential according to the LLNA

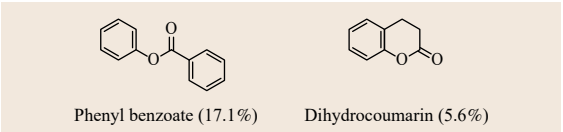

Fig. 36.10 Reactive acyl transfer esters and their skin sensitization potential according to the LLNA

is geraniol and citral. Also in this pair, cross-reactions are frequent and the true sensitizing hapten may often be identical.

36.2.5 α,β-Unsaturated Ketones

As for the α,β-unsaturated aldehydes, α,β-unsaturated ketones (Fig. 36.8) contain an activated double bond which is prone to Michael addition, especially by free sulfhydryl groups in proteins. This group comprises the damascones, key contributors to rose odors. As for the aldehydes, the substitution pattern strongly affects reactivity and sensitization risk. Thus, to note a striking example, while damascones are highly peptide-reactive, the substituted α-methyl-ionone has no detectable reactivity and was found nonsensitizing even if applied at very high doses in human tests.

36.2.6 Substituted Phenols

This chemical group comprises key allergens in other product types (such as hair dyes and biocides), but only few odorants belong to this class. The most frequently

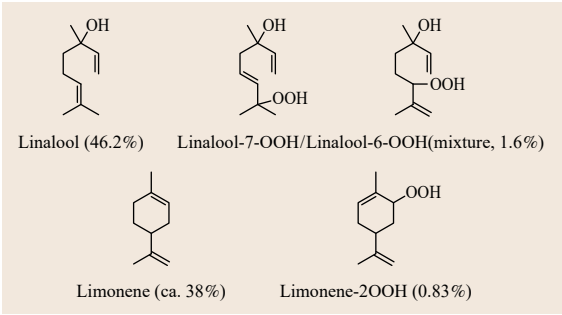

Fig. 36.11 Unsaturated terpenes and their primary oxidation products: skin sensitization potential according to the LLNA

cited odorant sensitizers in this group are isoeugenol (a key component for carnation scents) and eugenol (key component in clove buds and cinnamon leaf oil, which is essential in spicy scents) (Fig. 36.9). Eugenol appears to be a pro-hapten, requiring metabolic activation to become reactive, but isoeugenol appears to become reactive already upon abiotic oxidation (Fig. 36.4). This difference is also reflected in a clearly higher sensitization potency of isoeugenol. However, the enzymes in the skin which may be involved in activation of for example eugenol are unknown. It is proposed that both enzymatic and abiotic oxidation can lead to quinone methides as highly reactive intermediates which then can modify skin proteins (Fig. 36.4 for putative mechanism).

36.2.7 Reactive Esters

Esters with a good leaving group such as the phenolate anion are prone to acyl transfer reactions (Fig. 36.4). Such chemicals, thus, have the ability to modify proteins by the transfer of the acyl group onto the skin proteins also rendering them immunogenic. This appears to be the reaction mechanism explaining the sensitization potential of 3,4-dihydrocoumarin or phenyl-benzoate (Fig. 36.10). Although esters are very widely used in perfumery, the use of such phenyl esters has largely been phased out.

36.2.8 Terpenes

Unsaturated terpenes are unreactive as such and when tested in predictive tests are rated in most cases as very weak or nonsensitizing (Fig. 36.11). However, it was shown that essential oils containing such molecules or pure preparations of certain chemicals are prone to autoxidation, and that the oxidation products do have sensitization potential [36.24]. This risk is being mitigated by careful quality assurance of raw materials, but nevertheless frequent reactions to high concentrations of such oxidation products were recently reported [36.25]. Currently, it is not known whether these reactions are highly specific (whether patients only react to the selected oxidation products and whether sensitivity was indeed induced by exposure to these oxidation products from fragrances).

36.3 Testing for Skin Sensitization

Many methods are available and have been described for determining the skin sensitization potential of chemicals. Early developments relied on human studies [36.26] before the development of animal studies, such as the Guinea Pig Maximization Test (GPMT) [36.27], which have been the cornerstone of determining skin sensitization hazard. Nowadays, the LLNA performed in mice is the animal model of choice as it provides an indication of the potency of a material to cause sensitization which allows classification into stronger versus weaker groups of sensitizers and risk assessment [36.28]. Significant progress has been made in the last decade on the use of in vitro methods to predict skin sensitization hazard to the point where suitable and authority approved nonanimal alternatives are now available (OECD test guidelines 442c, 442d and 442e). The challenge over the next few years will be to adapt the understanding of the information from the in vitro methods to allow a reliable prediction of potency as is currently available from the LLNA. In silico tools complement the picture and remain most effective when used and interpreted with expert judgement, with the better tools providing not only predictive information, but also a detailed and well-referenced rationale behind each prediction. Finally, the use of human studies such as the human repeat insult patch test (HRIPT) to confirm the no effect levels observed from animal studies remain a useful tool when performed to high ethical standards. Clinical data is also instructive to provide information on materials that are able to cause sensitization in humans.

Here we provide an overview of the more commonly used methods with specific observations for the prediction of sensitization to odorant materials.

36.3.1 Animal Tests

Animal studies have long been the methods of choice for investigating the sensitization properties of chemicals. Several methods have OECD approved guidelines,

the GPMT and the Buehler Test (OECD 406) as well as the LLNA (OECD 429).

In the LLNA, dilutions of the test chemical are applied to the ears of mice three times on consecutive days. After a two days rest period, the mice are injected with radiolabeled thymidine into their tail vene (alternatively nonradioactive labels are also used), 5 h later the animals are sacrificed and the local lymph nodes draining the ears are excised. Pools of cells of the lymph nodes are then evaluated for the inclusion of labeled thymidine (and hence active DNA synthesis). This measure correlates to active proliferation of cells in the local lymph nodes, and thus provides for a quantitative measure of the sensitization phase (Fig. 36.12).

The LLNA has been the most commonly used assay on odorant chemicals in recent years and large datasets of information have been published which include many odorant chemicals [36.29, 30]. The LLNA has proved to be most useful for a quantitative evaluation of the sensitization potential and classification of materials into extreme, strong, moderate, weak, and very weak or nonsensitizers. This is based on the reported EC3 value: the concentration required to induce a threshold positive response [36.31]. The EC3 value has been demonstrated to generally correlate well with the no observed effect levels (NOEL) from human sensitization tests designed to confirm lack of induction [36.31–36], and it is therefore used for risk assessment. For odorant materials, some limitations to the use of the LLNA have been observed. For example, certain classes of ingredients show a strong trend of being over-predicted for their sensitization potency when compared with other animal studies and human data. Salicylate esters are an important class of odorant materials which are routinely over-predicted as moderate sensitizers in the LLNA whereas human experience and other animal data indicate they are not or only very weakly sensitizing, although the reason for this discrepancy is not well understood. Conversely, some materials, in particular those with high volatility, may have their sensitization potency under-predicted by the LLNA. Two examples are benzaldehyde and 2-hexenal which show a sensitization potential under occluded conditions in humans which is not seen following open application in the LLNA. For odorant materials, the avoidance of false positive results in the LLNA due to irritation is also an important factor and measurements of ear weight and thickness are important indicators in addition to the visual observation of irritation when conducting a study. For odorant mixtures, such as essential oils, attempts have been made to determine sensitization potential in the LLNA [36.37]. Whilst a good correlation is seen between the EC3 value for a simple mixture with one main component versus the EC3 value of that main component, no such correlations are seen for more complex mixtures. It is worthwhile to note that during validation, the LLNA was not tested on complex mixtures such as essential oils (which were not included in the chemical validation set) and therefore the value of conducting a study on such materials remains questionable.

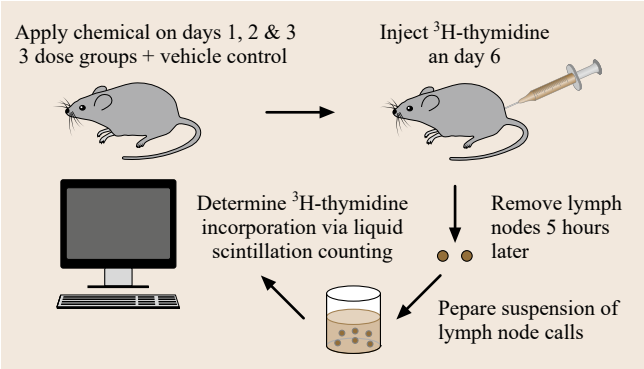

Fig. 36.12 A schematic representation of the experimental steps in the LLNA

Other methods such as the GPMT are nowadays less commonly used but much historical data exists which provide important information. While the GPMT has been used primarily for the hazard prediction of sensitization, some potency information may be obtained to be used in risk assessment. Other adjuvant tests in animals such as the Freund's complete adjuvant test (FCAT), mouse ear swelling test (MEST) and nonadjuvant tests such as the Buehler Test, open epicutaneous test (OET) and closed epicutaneaous test (CET) may be used to contribute information to a potency assessment [36.38].

36.3.2 Human Tests – Predictive and Diagnostic Tests

The predictive test most typically conducted is the human repeat insult patch test (HRIPT) [36.39]. The HRIPT is the most reliable test method by which confirmatory human data can be made available for use in risk assessment following pre clinical studies. The test exaggerates exposure from normal use of consumer products and can be used to confirm a safe level determined from animal studies such as the LLNA. Details of the standard HRIPT protocol used for odorant materials are described by *Politano* and *Api* [36.40]. A standard HRIPT protocol consists of a 3 week induction phase (weeks 1−3), a rest period of 14−17 days (weeks 4 and 5), and a 1 week challenge (elicitation) phase (Week 6). In the induction phase, patches are applied for 24 h, three times a week for a total of nine patches. This is then followed by a rest period which allows time for

any immunological response to develop and also allows any previous reactions developing at the induction sites to subside prior to the re-application of the test substances at challenge. Challenge patches are applied to the original application site and to a naive alternate site for 24 h. The sites are visually graded for skin responses 48 and 96 h after patch application, although intermediate or longer readings may be performed. This phase is used for the assessment of delayed skin responses, which may be indicative of contact sensitization. A re-challenge can be performed between 4 and 12 weeks after the initial challenge phase to provide more information about doubtful reactions. Each test typically includes just over 100 panellists.

All studies must follow good clinical practice (GCP) which includes requirements for the pre study safety assessment, informed consent, and Institutional Review Board (IRB) or Ethics Committee review. The HRIPT receives criticism, particularly in Europe, from an ethical perspective but remains a useful tool which is ethically acceptable when done under GCP and is routinely performed in the United States and other major geographies. A review of the ethical situation [36.41] concludes that where there is a specific rationale for testing, for example, to substantiate a no-effect level for a sensitizing chemical or to ensure that matrix effects are not making an unexpected contribution to sensitizing potency, then rigorous independent review may confirm that an HRIPT is ethical and scientifically justifiable. However, the possibility that sensitization may be induced in volunteers dictates that HRIPTs should be conducted rarely and in cases where the benefits overwhelmingly outweigh the risk. Today, for odorant materials, the HRIPT is conducted only as confirmatory assay at a dose where no induction in humans is expected based on pre clinical data.

The use of patch testing to diagnose allergic contact dermatitis was first developed by Josef Jadassohn in 1895 [36.42]. In diagnostic patch tests, patients with symptoms of allergic contact dermatitis are tested with multiple materials (grouped into the so-called baseline series). Materials are applied as occluded patches to their backs for 2 days, and development of symptoms are visually scored after 3–7 days. Patch testing indicates whether a patient has contact allergy to a particular substance or group of substances. Although patch testing remains the gold standard for the diagnosis of ACD, the actual procedure involves several steps and is usually carried out at specialized clinics. Selection of an appropriate series of allergens requires the physician to have sufficient knowledge of numerous allergens and to have them on hand in his or her clinic. Obtaining a history of the patient's exposures both at home and work is vital in determining the appropriate materials to patch test. The preparation of the patch test panels can be time consuming, but can be done ahead of time and standard series on fragrance materials are commercially available. Patients need appointments both for the application as well as first and second readings. When reading a patch test, correctly identifying a positive allergic versus irritant reaction requires experience and skill. When patch testing is complete, identifying clinical significance of the positive allergen in the patient's environment requires detective work and patient education. All of these factors may contribute to a dermatologist's decision to not offer patch testing in his or her office [36.43]. However, patch testing remains a very useful tool. Future development of in vitro and in situ diagnostic tools to complement the patch test and to allow a rapid and more readily available determination of contact allergy and the causative agent has been recognized as a need and development of such methods may lead to complementary diagnostic tools in the future [36.44].

Information from clinical studies such as the diagnostic patch test performed in specialized dermatological clinics may inform the risk management of substances, in particular where causative information linking the allergy to a specific exposure (product or occupational) is available. A scheme for the identification of the cause of allergy to fragranced products has been proposed [36.45]. However, causative information is often not available and closer cooperation between dermatologists and the industry is required to maximize the value of diagnostic patch test information for use in appropriate risk management decisions.

Finally, use tests such as the provocative use test (PUT) or repeated open application test (ROAT) are available and used to better understand the clinical significance of patch test results. The repeat open application test (ROAT) procedure is a useful tool to determine whether the positive patch test result may be relevant and a cutaneous reaction is seen from normal use of a product [36.46]. The ROAT protocol may vary according to product type tested but typically involves repeated once or twice daily applications of a product (either neat or in diluted form as used) to the volar forearm over several weeks with inspection for irritant or allergic contact dermatitis reactions between each application. ROATs are a valuable tool to provide information in the risk management of common allergens and studies have been conducted on fragrance ingredients. For example, *Schnuch* et al. [36.47] demonstrated that 90% (50%) of people allergic to the odorant material hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) would be protected by restricting presence in products to 0.009–0.027% (0.18–0.34%) depending on product type. A recent ROAT study simulating day-

to-day use showed that the weak sensitizer Eugenol would not elicit a reaction in patients with a positive patch test to Eugenol when used at levels as allowed within the current International Fragrance Association (IFRA) Standard [36.48].

36.3.3 In Vitro/In Silico Assessment

For cosmetic ingredients, a ban on animal testing has been put into force in 2013 in the European Union. Thus, chemicals used only for cosmetic purposes shall no longer be tested in the predictive animal tests. This legislation is a major driving force behind significant recent research initiatives to find alternative testing strategies. Basically different steps of the mechanism as formalized in the AOP and summarized in Sect. 36.1 can be tested in isolated in vitro tests. Thus, a number of different peptide reactivity assays were proposed to assess reactivity with model peptides [36.49–51]. The extent of the reaction, especially the kinetics of the reaction, does correlate to sensitization potential and can at least be used to make predictions within some structural classes [36.52–54]. Such reactivity assays are also called *in chemico* assays. A standardized protocol was developed and nicknamed DPRA (for direct peptide reactivity assay [36.55]) and has undergone validation at the European Center for the Validation of Alternative methods to animal testing (ECVAM). Measuring reactivity, which is the key MiE and most likely also the rate limiting step in the sensitization reaction, is clearly the most straightforward approach.

A different approach focused on the activation of dendritic cells. Contact allergens were found to trigger phenotypic changes and intracellular signaling pathways in dendritic cells cultivated in vitro. The key phenotypic markers identified were: up-regulation of the surface markers CD86 and CD54 on dendritic cells, release of Interleukin-8, and activation of the mitogen activated kinase p38 [36.56]. Based on these findings, a predictive test for dendritic cell activation (h-Clat, human cell line activation test; [36.57]) was developed and also underwent ECVAM validation. Next to phenotypic changes, also changes at the transcription levels triggered by sensitizers were investigated in detail. A common pathway triggered by sensitizers found in several studies is the electrophile-sensing pathway involving the sensor protein Keap1, the transcription factor Nrf2 and its cognate binding site EpRE/ARE (elecrophile/antioxidant response element) present in the promotor region of many cytoprotective genes. This pathway responds to most electrophilic skin sensitizers. An assay based on this pathway (KeratinoSens) has also been validated by ECVAM [36.58].

These three assays measuring chemical reactivity and cellular responses do not directly measure immunogenicity; nevertheless a combination of these assays may be sufficient to recognize the majority of skin sensitizing chemicals [36.59, 60]. A more relevant assay would be to directly measure *ex vivo* with a mixed lymphocyte reaction the clonal expansion of specific hapten-specific T-cell clones. This is now feasible for very strong sensitizers [36.61, 62] but so far not sufficiently developed for weaker sensitizers.

Several in silico tools exist for the prediction of skin sensitization with the most useful of these commercial tools providing mechanistic explanation of the prediction such a DEREK and TIMES-SS [36.63, 64]. These tools remain best used in knowledgeable hands and when combined with expert judgement and information from other methods (in vitro and in vivo) in providing understanding and a weight of evidence prediction of sensitization hazard and potency of substances.

The key remaining hurdle is to quantify the sensitization potency: as discussed earlier it is not always possible to create odorants without any chemical alert for sensitization, and therefore a careful risk assessment is needed to stay well below the threshold for sensitization in product applications. As point of departure for the risk assessment, quantitative data are a key requirement – up to now these were mostly generated in animal tests.

36.4 Epidemiology of Allergy to Odorants

36.4.1 Fragrance Allergy in Dermatology Patients

Dermatological patients represent a subset of the general population suffering from allergic contact dermatitis who present themselves to specialized dermatological clinics for evaluation and diagnosis. Extensive data on contact allergy to odorant materials is available from different regions around the world and standard patch test series are available to aid the diagnostic process. Routinely Fragrance Mix I (FM I), a mixture of commonly found fragrance substances, is used where allergy to fragrance may be suspected [36.65] and Fragrance Mix II (FM II) and Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) [36.66] have also been introduced into the European baseline se-

ries. Globally, it has been reported that 6–14% of patients presenting with allergic contact dermatitis react to FMI, reactions to FMII are lower (0.6–9.3% of patients) and 0.4–2.4% positive reactions were reported for HICC [36.67]. Geographical variation to fragrance allergy in patients and in particular that to HICC in patients is seen with a general, although variable, trend to lower rates in the USA and southern Europe versus northern and central Europe. Significantly lower rates of reaction to HICC are seen in the United States as compared to Europe and a recent report shows declining rates in EU possibly following tighter industry restrictions on the use of this substance [36.68]. Data on clinical relevance, that is, the confirmation that the patch test response in the patient is relevant to the current illness (allergic contact dermatitis), is mixed. Clinical or *current* relevance has been explored by *Frosch* et al. [36.69, 70] who estimated between 2% and 55% of reactions seen to FMI were relevant depending upon whether relevance was classified as *certain*, *probable*, or *possible*. Data on other fragrance ingredients and subsequent studies by the same group showed similar ranges.

Significant amounts of information are available in this area and for an extensive information source the reader is invited to review the relevant chapters in the recent European Scientific Committee on Consumer Safety report on fragrance allergens [36.67].

36.4.2 Fragrance Allergy in the General Population

The earlier given data refer specifically to patient populations. The most comprehensive study to date in the general population was conducted recently by the European Dermatological Epidemiology Network (EDEN) sponsored by The Research Institute for Fragrance Materials, Inc. [36.71]. This study recruited over 12 000 people from 6 centres in Europe and patch tested a random sample of 3000 subjects [36.72]. Overall the crude prevalence of patch test positive responses to FMI, FMII, and single substances was between 1.0% and 2.4%. When a conservative estimate of clinical relevance was considered, it was 0.4–1.1%. Reactions to individual materials within FMI and FMII, with the exception of HICC, were all at or below 0.5% again accounting for lifetime clinical relevance. This data is similar to that of the SCCS which reported 1–3% of the general population as having contact allergy (a positive patch test response) to fragrances. Data on actual current cases of allergic contact dermatitis due to odorant materials in the general population are missing and therefore extrapolation has to be made from the above; however, data collected from adverse effects reported by consumers may be useful in this regard.

36.4.3 Risk Assessment and Risk Management to Control Skin Sensitization

Significant progress has been made in recent years in the risk assessment for skin sensitization due to the availability of better exposure data and the use of the LLNA and HRIPT which has allowed determination of safe levels of use for skin sensitizers using a quantitative approach versus. previous more qualitative assessments. The quantitative risk assessment (QRA) methodology [36.73] is now widely used when assessing and setting risk management measures for fragrance materials. Since the introduction of the QRA in 2008, the International Fragrance Association (IFRA) has gradually introduced Standards setting limits for the use of fragrance ingredients, with now over 100 substances restricted in their use. The QRA approach follows general toxicological risk assessment principles where a no effect level for sensitization (so-called no sensitization induction level – NESIL) is defined and a series of safety factors accounting for inter individual, vehicle/product matrix effects, and product use considerations are applied to derive safe use levels in consumer products. Using the QRA, safe use levels may be defined for individual product types provided adequate exposure, use, and product formulation information are available. In implementing safe use levels, IFRA has adopted 11 categories within which over 50 common consumer products are defined. For fragrance ingredients with sensitization potential, each category is assigned to a specific maximum use level determined to ensure induction of sensitization to the substance does not occur [36.74]. More information and guidance may be found on the IFRA Website [36.75]. The QRA as applied by the fragrance industry is currently limited in scope to consumer products. Occupational exposures, pharmaceutical products, aromatherapy and massage oils, natural exposures, and other unregulated areas are not covered by the fragrance industry approach and IFRA standards and this may still represent an important gap for risk management of consumer safety and prevention of allergy. As with all toxicological risk assessment approaches, further investigation and refinement will undoubtedly occur in the future to ensure continuous improvement of approaches.

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37. Aroma Therapy in Neonatology

Michael Thiel

Aroma therapy has become a well-established part of complementary and alternative medicine in pediatrics mainly because it is accepted and desired by many parents. However, the scientific base of several of these methods is often contradictory. This chapter provides an overview of data on aroma therapy in neonatology and tries to explain its physiological background.

The prognosis of preterms and seriously ill newborns has substantially improved during the last 25 years. To reduce or avoid the adverse effects of conventional therapies like antibiotics or mechanical ventilation, several attempts have been made to practice gentle medicine, including complementary and alternative methods.

A number of tools from this field, such as kangaroo care [37.1, 2], glucose [37.3], breast milk [37.4, 5] or massage [37.6, 7], have played a crucial part in this improvement. Often the aim is to reduce pain, but also to stabilize vital parameters like oxygen saturation, heart rate and blood pressure, or to foster bonding. A highly important aspect in this context is developmental care, being an inherent and almost natural part of the holistic approach of neonatology [37.8].

However, the exact pathway of these methods is not always clearly defined. For example, it is a common assumption that the pain score is lower in infants that received glucose. But recent findings have suggested that it is actually not the pain itself that is influenced by glucose, but rather the changing facial expression which coincides with measuring pain. So the validity of the evidence ultimately depends on the question of what has actually been measured [37.9].

Furthermore, the use of complementary and alternative methods is not always based on evidence. For instance, although there is some significant data on music therapy, it is not as widely spread as other methods [37.10]. The same is true for some therapies like homeopathy, which are being used without or with only little evaluation or evidence [37.11]. There are theoretical approaches to apply acupuncture in neonates, but again, very little data is available [37.12, 13].

Figure 37.1 illustrates the discrepancy of use and evidence in some complementary and alternative medicine (CAM) methods in neonatology.

Even though a systematic review on phytotherapy in neonatology showed some encouraging aspects of aroma therapy [37.14], it is still not very common in the care of preterms. The following section provides an overview of the current literature on this topic and suggests possible explanations of the observed effects.

37.1 Data on Aroma Therapy in Neonatology

37.1.1 Aroma Therapy and Pain in Neonatology

Pain has an enormous impact on the development of a preterm. Many complementary and alternative attempts have been made to improve analgesic management in neonatology, such as glucose, music therapy, massage, mother milk, or kangaroo care.

Probably the most common painful procedure is taking blood from the child. Therefore, numerous nonpharmacologic pain-relieving interventions are practiced and have been evaluated, including swaddling, holding, skin-to-skin care, pacifiers, sweet-tasting solutions and breastfeeding [37.15].

There are several studies on aroma therapy whose results suggest its use at least as an additional means to reduce pain and stress, thereby improving the prognosis of the preterm.

Vanillin

In a study, 31 healthy preterms (32 weeks of gestational age, mean birth weight 1730 g, and aged 6 days) were divided into three groups. The infants in the first group were exposed to vanillin flavor prior to venipuncture on the hand or a capillary puncture of the heel, the second group smelled vanillin for the first time, and the third group had no odor exposure during the procedure. An independent video score before, during and after venipuncture assessed the preterms' reaction. Exposure means that a swab with 10 drops of vanillin solution was placed in the incubator for 10 min. If the infant was lying in an open bed it was placed close to its head. The neonatal pain score was significantly reduced if vanillin was familiar to the child before venipuncture. An interesting incidental finding of this study – beside its main outcome – was that heel sticks were more painful than venipuncture [37.16].

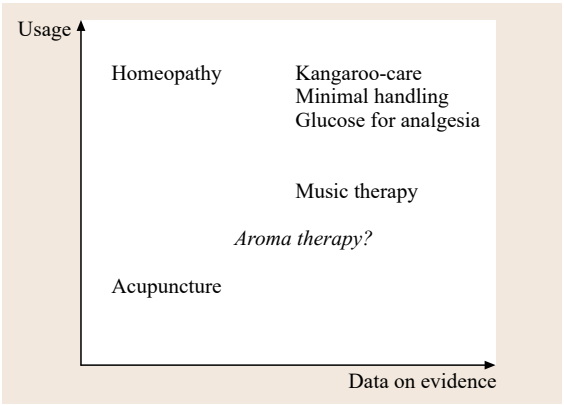

Fig. 37.1 Discrepancy of usage and evidence of some complementary and alternative methods in neonatology

Test and results of another study with 44 healthy newborns were quite similar: Odor of the infants' mother's milk, vanilla smell, unfamiliar odor and no odor were compared. The pain score before, during and after a routine heel stick was significantly lower in the group of children with familiar odors, i. e., vanillin and mother's milk. All children were breastfed [37.17].

A very similar study revealed a reduction of the pain score in the group of vanillin and mother's milk in 44 healthy newborns. Again, mother's milk, vanillin familiar, vanillin not familiar and no odor were compared before, during and after venipuncture [37.18].

To discriminate the effect of vanillin from other influences, a further study of 44 healthy newborns (gestational age 39 weeks and mean birth weight 3440 g) was performed. Vanillin being familiar, venipuncture in bed, vanillin unfamiliar, venipuncture in bed, vanillin not familiar, venipuncture on mother's arm and venipuncture without no vanillin were compared, again by measuring the pain score before, during and after venipuncture. In this study, the pain score was significantly lower in the group with exposure to vanillin before venipuncture [37.19].

In yet another study, 135 neonates were divided into 3 groups. An arterial puncture was performed on the infants. The first group was exposed to vanillin overnight and then exposed to vanillin during the painful procedure. The second group had no exposure before, but during arterial puncture, while the third group was not exposed to vanillin at any time. Less crying and less fluctuation of oxygen saturation – probably indicating less pain – were observed in infants exposed to vanillin before and during the procedure. These findings were interpreted as a pain-relieving effect of vanillin, especially when the infant is familiar with this odor. The focus was set on the assessment of a nonmaternal, but familiar scent. Familiarization was achieved by placing a scented gauze pad in the incubator next to the infants' head for an average duration of 8.65 h. Another difference to the other studies results from the fact that arterial puncture is known to be more painful than venipuncture or a heel stick. So with regard to the neonates' reaction to pain, the findings of this study indicate a benefit of vanillin even with stronger pain triggers [37.20].

All of the studies suggest that vanillin can be helpful in decreasing pain in neonates, especially when the

Table 37.1 Comparison of studies on the effects of vanillin on pain in neonates

	Patients	Interventions	Results
Goubet et al. [37.16]	31 healthy preterms	• Exposure to vanillin before and during venipuncture • No exposure to vanillin before and during venipuncture • No odor before and during venipuncture	Significant reduction of pain score when vanillin was familiar before venipuncture
Rattaz et al. [37.17]	44 healthy newborns	• Odor of mother's milk during heel stick • No exposure to vanillin before but during heel stick • Unfamiliar odor during heel stick • No odor during heel stick	Significant reduction of pain score before, during and after heel stick when odors were familiar, i. e., vanillin and mother's milk
Williams et al. [37.18]	44 healthy newborns	• Odor of mother's milk during heel stick • Exposure to vanillin before and during heel stick • No exposure to vanillin before but during heel stick • No odor before and during heel stick	Reduction of pain score in the group of vanillin and mother's milk
Goubet et al. [37.19]	44 healthy newborns	• Exposure to vanillin before and during venipuncture • No exposure to vanillin before and during venipuncture • Venipuncture without odor	Significant reduction of pain score when exposed to vanillin before the procedure
Sadathosseini et al. [37.20]	135 preterms and newborns	• Exposure to vanillin before and during arterial puncture • No exposure to vanillin before but during arterial puncture • No odor before or during arterial puncture	Less crying and less fluctuation of oxygen saturation when exposed to vanillin before and during the procedure

Table 37.2 Comparison of studies on the effects of vanillin on apneas in preterms

	Patients	Intervention	Results
Marlier et al. [37.21]	14 extreme preterms, 24–28 gestational weeks, with severe apnea under intensive therapy	Day 1: standard therapy Day 2: plus vanillin Day 3: standard therapy	Addition of vanillin reduces apnea
Edraki et al. [37.22]	36 preterms, 32–33 gestational weeks, without present apnea	Control (standard therapy) versus vanillin for 5 days	Vanillin significantly reduces apnea, bradycardia and fluctuation of oxygen saturation

child is exposed to the odor before undergoing a painful procedure.

The results are summarized in Table 37.1.

37.1.2 Aroma Therapy for Stress in Neonatology

Stress is a more general parameter than pain, but the tools used for measuring stress and pain are quite similar. Animal studies have shown that genetic differences in temperament may determine how lavender oil influences anxiety [37.23].

Lavender

One published study has tried to assess the influence of lavender on stress parameters of the child, but also of its mother.

In this study, 30 healthy mother-newborn dyads were exposed to lavender, compared to no odor and lavender plus the information given to the mother that lavender has a positive influence on her and her baby. The results indicate a reduction of stress parameters on behavior, sleep duration and cortisol level in saliva in the lavender group. A *pseudo-placebo effect* by providing additional information about the positive effects of lavender could not be confirmed. However, the results of this study were not significant [37.24].

37.1.3 Aroma Therapy and Apnea-Bradycardia-Syndrome of the Neonate

Apnea and bradycardia due to immaturity of the brain stem are severe problems in the care of neonates. At a heart rate below 80 beats/min, a reduction of the cerebral blood flow can be observed. Yet the influence on the long-term neurological development is not clear.

Vanillin

There is one study that assessed the influence of a specific odor on high-risk preterms. 14 infants, gestational age 24–28 weeks, suffering from severe apnea despite analeptic therapy with caffeine and/or Doxapram, were part of this study. On day 1 of the study, apneas were counted as baseline, on day 2, the infants were exposed to vanillin, and on day 3, vanillin exposure was stopped. The infants showed significantly less apnea on day 2 [37.21].

Focusing on the same problem, another study with 36 preterms was conducted. In contrast to the study

mentioned before, the infants did not belong to a high-risk group. These infants had a mean birth weight of 1936 g in the intervention group and 1848 g in the control group. Their gestational age was 33 and 32 weeks. The study started when the infants were 2 days old. Every 12 h, 2 ml of a 2% vanillin solution was dropped on a cotton swab placed in the incubator while vital parameters were measured. After 5 days, the intervention group had significantly less apneas than the control group. There was also a significant difference regarding oxygen saturation and heart rate between the two groups [37.22].

It is remarkable that both high-risk preterms and late preterms seem to benefit from vanillin exposure regarding apneas and bradycardia.

A comparison is shown in Table 37.2.

37.1.4 Aroma Therapy and Energy Expenditure in Neonates

Based on the assumption that there is an influence of odors on the metabolic system by vegetative and endocrine pathways, attempts have been made to evaluate this correlation. It has been observed that several factors influence the reactions of preterms and newborns when exposed to milk, for instance whether they are hungry or not [37.25].

Vanillin

In the only published study in neonates, 20 healthy preterms were exposed to vanillin and energy expenditure was measured by indirect calorimetry. An influence on energy expenditure could not be observed [37.26].

37.1.5 Weaknesses of the Studies

Unfortunately, pain scores are not sufficiently precise. Thus it is difficult to discriminate pain from stress or just discomfort. Objective measurement tools would be helpful [37.27].

In addition, cortisol is not an obligate stress parameter. Endonasal suctioning is known to be painful for a preterm. However, it was shown that cortisol levels are not significantly changing during the procedure [37.28].

37.2 Possible Explanations

37.2.1 Considerations of Physiology

The rhinencephalon and the limbic system are phylogenetically and ontogenetically old parts of the human brain. Olfactory sensations are being transported by the olfactory bulb and then reach structures of the thalamus and hypothalamus, which explains vegetative reactions as well as effects on endocrine function. The amygdala and limbic system connect these sensations to the orbito-basal, fronto-basal and temporal cortex which mediate olfactory reactions to our consciousness.

It has been shown by near-infrared spectroscopy studies that this theoretical background is present in the physiology of newborns as well. They revealed that olfactory stimulation is followed by reactions in the olfactory cortex of newborns [37.29, 30], to the extent that even a relatively precise discrimination of odors is possible [37.31]. Furthermore, it is known that the fetus is able to smell and that the food of the mother has an influence on the fetus as well [37.32–35].

Phylogenetically, smelling is a mechanism of noticing danger. This behavioral pattern can be transferred into our long-term memory by several mechanisms. So it is still reflected in our reactions to particular olfactory stimuli: An uncomfortable odor is often followed by holding breath, escape or vomiting. By remembering odors that have led to an uncomfortable situation, the odor itself can cause such reactions. For example, a particular smell can evoke a feeling of sickness even years after having consumed soured milk or rotten fish. In many people, the odor of vomitus is enough to induce sickness. But on the other hand, an odor can also be associated with a pleasant situation and act as a trigger to recall this situation unconsciously [37.36, 37].

37.2.2 Parallels to Adults

The findings in neonates outlined above are underpinned by various results of studies on adults.

Development and Involution

It is known that the early olfactory development starts in the first trimester. As we grow older, more and more junctions to cerebral functions, memories, moods and cognitive abilities develop [37.38]. But this development can be reversed when we reach higher ages. One of the most extreme forms of this reversal is dementia. This raises the question whether aroma therapy could also be a useful tool in the treatment of dementia as a pathological condition. In fact it has shown benefits for disorders associated with dementia [37.39].

These positive results can be explained by pathways similar or even identical to the ones described above

for neonates. However, the results on aroma therapy are still ambiguous [37.40].

Pain

Another clinical condition where aroma therapy is being used in adults is pain. In patients undergoing hemodialysis with arteriovenous fistulas, the authors of a study showed reduction of pain when lavender essence was presented during needle insertion [37.41].

Vital Signs

Similar to the findings of studies on infants, a recent study tested the effect of lavender oil inhalation not only on patients' vital signs, but also on the perceived quality of sleep in an intermediate care unit. Like in neonates, it was an underlying aim of this study to save drugs for sedation. Effects were observed in modulation of vital signs. Also quite similar to the study on infants mentioned above (Sect. 37.1.2, *Lavender*) [37.24], the quality of sleep improved under lavender aroma therapy. This difference was not significant [37.42].

37.2.3 Essence

For aroma therapy in neonates, the database is small. Yet, the published data is encouraging and new hypotheses could be developed. For example, the positive effect of kangaroo care could partially be explained by the odor of the mother. No adverse effects were reported. Even though effects of early exposure to certain odors are well known [37.43, 44], the long-term effects of aroma therapy, i. e., the application of odors for therapeutical use, cannot be estimated due to the lack of data [37.43, 44].

So it can be concluded that the theory appears to be plausible, but is not sufficiently precise yet. Further studies are needed before a general recommendation is possible.

37.3 Neonatology-Related Research and Aroma Therapy

In modern neonatology of the 1980s, intubation was the most common treatment of the immature lung. Then, in the 1990s, intubation was complemented by the application of surfactant, which strongly improved the prognosis of a preterm. Since the early 2000s, the application of surfactant was not necessarily followed by endotracheal intubation, but by continuous positive airway pressure (CPAP), a method for noninvasive ventilation.

The historical development of neonatal management also included an increasing tolerance towards pH, pCO2, pO2, blood pressure and oxygen saturation. All of this was accompanied by the general insight that children are not little adults, and moreover, that preterms are not simply little children.

The historical change started with a practice of *maximal handling*, few analgesia, invasive ventilation and progressed to minimal handling, adequate analgesia as well as rooming-in, kangaroo care, general reduction of stress, breastfeeding, the use of glucose for analgesia and a general awareness for developmental care. Data on music therapy as well as aroma therapy might lead to even smoother neonatal medicine where complementary methods like aroma therapy have their evidence-based place. The further we go, the more simple principles become relevant: touching, i. e., body contact by kangaroo care or massage, glucose (at least partially) as taste, music therapy as auditory stimulation as well as aroma therapy as olfactory stimulus – all of these methods are based on phylogenetically and ontogenetically old mechanisms, but at the same time, they are simple, basic tools with increasing evidence. Besides, they are cheap and easy to perform.

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Part E

Psycho-P

Part E Psycho-Physical and Cognitive Aspects of Odor Perception

The human body has learned to detect and respond to odorants by physiological responses and cascades, as detailed in the previous section, as did the human brain and mind. Humans are not only consciously aware of smells, provided the threshold levels of odorant mixtures and concentrations are met, but they are also capable of generating meaning of these smell impressions, to a lesser or greater extent.

Of course an important aspect of generating meaning is (lifelong) learning, and learning again is strongly dependent on motivation and experience. This dedication might arise from a person's interest in learning about smells, for example, in a professional context to become a perfumer, flavorist, enologist, or odorant scientist. However, specific situations and circumstances may also enhance learning attention in humans, especially situations that are linked to strong emotional experiences or those that are clearly linked with survival. Humans do not only learn to link specific smells to objects, situations and environments, but they also learn to rate them, to judge if smells are good or bad (or rather, their related sources), if they should respond with aversion, attraction, or not at all. As experience is a major dimension in these processes, odor memory, interpretation, and reaction may be strongly divergent in different phases of human life. Adults may even acquire preferences for smells or aromas that are rejected by young children, as adults learned to enjoy these smells, for example, in a social context. On the other hand, experiences with smells in relation to threatening situations or toxic objects might translate from early childhood into a lifelong aversion.

Unconscious processing of smells is also of major importance. Sometimes we even do not really know or cannot explain why we react in a certain way to specific smells. Moreover, smells are rarely perceived and interpreted in a mono-sensory dimension, but commonly in a complex interplay with input from other sensory modalities such as taste, appearance, and/or sound (i. e., via verbal communication) alongside the respective smell. If these diverse inputs do not fit the expectation or individually acquired learned concepts of the receiver, even pleasant smells can be rejected, or conversely, unpleasant smells might be accepted because they fit into a specific concept. In any case, it becomes clear that psycho-physical and cognitive aspects of olfaction are highly individual, as are human responses to smells.

38. Cortical Olfactory Processing

Jessica Freiherr

The act of smelling is a fundamental perceptual process mediated by the evolutionary very old olfactory system. Smells influence human behavior strongly related to survival, such as food consumption, hazard avoidance, sexuality, and reproduction. Hence, olfactory stimuli are of high ecological importance and are processed in phylogenetically old brain areas. This anatomical deviation leads to changes in the cortical organization of networks responsible for olfactory processing in comparison to other sensory systems that can be perfectly examined with the help of neuroimaging methods.

Within this chapter insights about the anatomy of the peripheral and central olfactory structures will be provided and physiological processes that are the basis for olfactory perception will be explained. The way of the olfactory information processing – starting with the molecules that are sniffed and bind to the receptors in the olfactory epithelium, to information transmission to the olfactory bulb and onward to olfactory cortical areas – will be traced. Alongside this, the reader will be informed about the clinical implications of the sense of smell.

38.1 Sniffing of Odors

To sniff or to draw air into the nose is the physiological precondition of olfactory perception. A sniff is defined as a reflex elicited by chemicals – either irritants or odors. This olfactomotor activity causes a certain level of turbulence in the nostrils responsible for the transport of odors to the olfactory epithelium located in the upper part of the nasal cavity. A sniff is traditionally viewed as a simple transport mechanism carrying the odors to the receptors; however, it can also be considered the earliest stage of olfaction as compared to eye movement in vision [38.1]. Sniffing does not only influence olfactory intensity but also odor identity or quality perception, and sniffing patterns can be quite divergent between individuals, and depending on task [38.2, 3]. Odorant molecules depending on their shape have a distinct sorption rate on the mucosa and high sorption rate odorants are better perceived at high velocities and vice versa [38.4–6]. This is of special interest since the human nose underlies a nasal cycle during which the mucosa alternates between congestion and decongestion from one nostril to the other leading to low and high velocities of the sniffed air [38.7]. In other words, at a given time we find low velocity and a better uptake of low sorption rate odorants in the congested nostril and vice versa in the decongested nostril. Consequently, a different olfactory percept is conveyed through each nostril at a given time [38.5].

Natural sniffing provides optimal chemosensory perception [38.8] and sniff magnitude is largely related to olfactory pleasantness – humans sniff less intense in response to an unpleasant as compared to a pleasant odorant [38.8, 9]. Additionally, breathing patterns during olfactory perception and olfactory imagery are similar [38.10] and even imagery of pleasant odors evoked larger sniffs in comparison to unpleasant odors [38.11–13]. Sniff magnitude is not only of interest with regard to common odors but also due to chemosensory emotional cues. Evidence stares that sniff magnitude increases when smelling fear sweat and decreases after smelling disgust sweat. This phe-

nomenon is interpreted as sensory acquisition and rejection behavior [38.14].

As stated earlier, sniffing is needed for the conscious perception of odorants; however, the adjustment of sniff response also appears during unconscious odor perception. Therefore, the measurement of sniff response offers a great opportunity to examine processing of consciously undetected stimuli [38.15] and renders possible an implicit measure, for example, during sleep [38.16].

Another interesting aspect of sniffing behavior is that humans unconsciously mimic olfactory acquisition behavior of their conspecifics: *Arzi* et al. used the movie *Perfume* to show that the observers sniffed as soon as the characters in the movie started sniffing. This mirror sniffing effect was strongest when subjects heard the sniff but did not see the object emanating a smell. Mirror sniffing is interpreted as a form of mimicry, contagious behavior, such as laughing or yawning or a form of orienting response [38.17].

Regarding the activation of brain areas, sniffing of odorless air alone leads to an activation in the olfactory bulb [38.18] as well as in piriform and orbitofrontal cortices [38.19, 20]. Probably, this activation of typical olfactory brain areas is related to the airflow in the nostrils and provides important hints for the computation of olfactory information. In other words, the sniff prepares the olfactory cortical network for incoming chemosensory information. Further, the hippocampus and the cerebellum are considered the neural control centers of olfactomotor response. The hippocampus receives olfactory information from the entorhinal cortex and projects to respiratory centers [38.1, 21]. Activation of the cerebellum during olfactory perception is interpreted as a feedback mechanism adjusting breathing volume according to odorant concentration [38.22].

Since sniffing is necessary for odor perception, neurological or psychiatric disorders that are accompanied with smell dysfunction might be related to impairments of sniffing [38.1]. Sniffing deficiencies that are at least partly related to olfactory impairment have been proven for patients suffering from Parkinson's disease [38.23]. Additionally, children with autism spectrum disorder showed an altered sniff response in comparison to typically developing children and did not adjust sniff magnitude with regards to odor pleasantness [38.24]. The measurement of sniff response can thus be considered as novel diagnostic tool and biomarker for several neurological and psychiatric diseases. To go one step further, sniffing is also a helpful tool in emergency medicine: sniffing enables communication and environmental control in severely disabled people. In an elegant study, *Plotkin* and colleagues [38.25] established that sniffing provides a control interface to write text and drive an electric wheelchair in patients suffering from locked-in syndrome.

38.2 Olfactory Epithelium

The molecules that are sniffed, reach the olfactory epithelium situated in the upper part of the nasal cavity. The olfactory epithelium lines the mucosa right below the cribriform plate and the mucosa of the superior turbinate and houses the olfactory receptors, G-protein coupled receptors [38.26]. Humans possess approximately 340–400 different functional olfactory receptor genes coding for olfactory receptors [38.27–29]. Odor molecules retain different functional groups activating different receptors. In other words, the olfactory receptors are units that detect molecular features of the odorants [38.30]. One study provides a first hint for a topographical organization of the olfactory receptors in the epithelium based on pleasantness of the odors that activate the receptor [38.31].

Olfactory receptor cells are bipolar cells; their apical end is situated in the nasal mucosa where the odorants bind to the receptors. This causes an action potential that travels along the long axon of the receptor cell (fila olfactoria, that are grouped into the olfactory nerve – cranial nerve I) through the lamina cribrosa directly into the olfactory bulb [38.26]. Please see Chap. 27 for more details on odorant sensing.

By means of a combinatorial code, it is possible that thousands of different odor molecules are recognized with a relatively low number of receptors [38.32]. In scientific literature of the last decades it is assumed that humans can perceive about 10 000 different odors. Recently, psychophysical testing was used for an estimation of the actual number of discriminable smells. In contrast to the existing literature, the authors approximate that humans can discriminate more than one trillion different substances by their smell [38.33] and hence provide evidence that the olfactory system outperforms other sensory systems with regards to the number of physically different perceivable stimuli.

Although humans can distinguish such a great number of smells, they perform surprisingly bad at naming familiar olfactory impressions [38.34]. The interactions between language and olfactory perception are complex (see also Chap. 53).On the one hand, it is more difficult to assign words to an olfactory experience than to any

other sensory experience. It is supposed that this shortcoming is based on the early development of the olfactory system in relation to the relatively late development of the language system during evolution [38.35]. On the other hand, olfactory stimuli can be processed without any linguistic involvement – smelling an odor without identifying it can lead to episodic memory recall and a strong emotional response. Controversially, in case a word and an odor are presented concomitantly and thereby linked to each other, words and verbal context are more powerful modulators of olfactory information than stimuli of any other sensory modality [38.36]. More recent studies established that the odor-language integration network consists of the orbitofrontal cortex and the anterior temporal lobe/temporal pole for odor object coding and semantic integration and the inferior frontal gyrus for olfactory naming and verbalization [38.37–39]. Another area that might be of importance for olfactory identification processes is the olfactory bulb. In a voxel-based morphometry study, olfactory bulb volume of the subjects predicted behavioral performance in an olfactory identification test – the larger the olfactory bulb the better are humans in identifying the odor [38.40]. Within the network of the brain areas described earlier, smells are linked to their name and odor identification takes place.

38.3 Olfactory Bulb

The olfactory bulb is considered the first relay station in the olfactory system. In the glomeruli of the olfactory bulb, olfactory information is transmitted from the primary neuron, the olfactory receptor cell to a secondary neuron, the mitral cell. Glomeruli respond to a range of different odorants that share a particular pattern of molecular characteristics. Further, glomeruli that receive input related to a similar molecular range group together and form molecular-feature clusters in the bulb [38.30]. Thus, odorant-specific activation of receptors is translated into an odorant-typical spatial activation pattern in the olfactory bulb [38.41, 42]. At this stage, we find a high level of convergence from thousands of neurons onto a few glomeruli.

The olfactory bulb also contains periglomerular neurons and interneurons transmitting inhibitory signals to neighboring glomeruli, as well as to the contralateral olfactory bulb. Those cells also receive retrograde inhibitory signals from higher order brain areas. The functional importance of those cells is to enhance the contrast between olfactory stimuli and background noise resulting in an increase of discriminability and sensitivity of the system.

Until recently, the olfactory bulb was largely underestimated regarding its function and importance. Nowadays, it is recognized that the olfactory bulb fulfills tasks comparable to that of other primary sensory cortices. In the olfactory bulb, the signal is condensed and amplified and a basal cognitive processing already takes place. Although traditionally the piriform cortex (pirC) has been termed as primary olfactory cortex, due to the discovery of the sophisticated functions of the pirC (see in the following) this concept does not hold true. Thus, the olfactory bulb serves as primary olfactory cortex encoding the chemical features of odorants and organizing them in a spatial pattern [38.43–46].

One of the anatomical deviations of the olfactory system is that olfactory information can be transmitted to cortical areas without a thalamic relay [38.47]. Thereby smells have the capability to bypass attentional gating processes. The olfactory bulb can hitherto also be considered the olfactory thalamus [38.48]. This is reasoned since the olfactory bulb has a bottleneck and relevance filter function regarding information processing before the cortex, comparable to the function of the thalamus in processing of other sensory stimuli.

38.4 Central Olfactory Pathways and Networks

From the olfactory bulb, the information about the chemical structure of an odorant is transmitted via the lateral olfactory tract to a set of small structures: the pirC, the entorhinal cortex, the amygdala and periamygdaloid cortex, the olfactory tubercle, and the anterior olfactory nucleus. An overview about the olfactory pathways and network can be found in Fig. 38.1.

The pirC is one of the key nodes in the cortical olfactory system. In a recent meta-analysis of olfactory brain imaging data the pirC revealed the highest activation consistency across all included functional imaging studies (Fig. 38.2) [38.50]. The pirC is anatomically divided into two parts that are responsible for different tasks. The anterior or frontal part of the pirC is

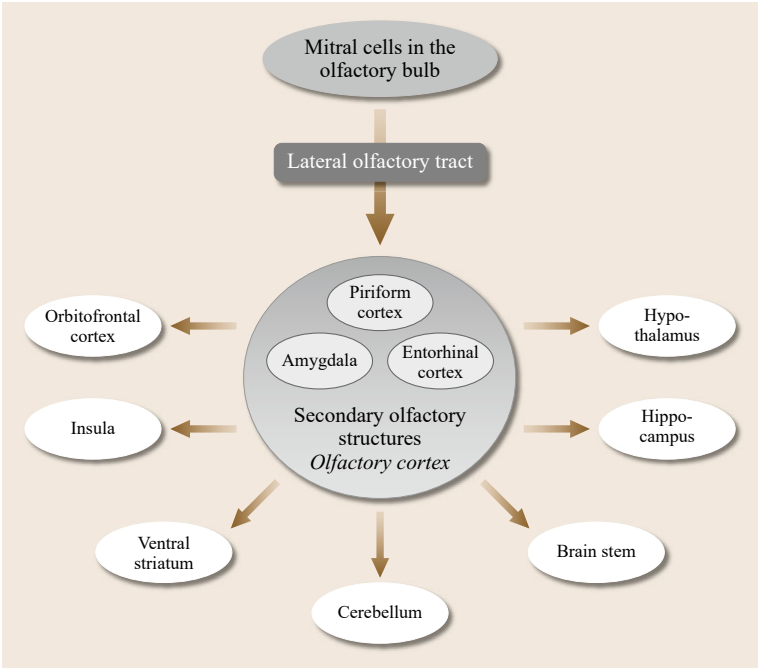

Fig. 38.1 Brain areas involved in olfactory processing (after [38.49])

related to an initial neural representation of an odorant and encoding of its molecular features [38.51], whereas the posterior or temporal part of the pirC is responsible for a holistic odor object formation, hedonic quality coding, and categorization [38.52, 53]. In the pirC the perceptual quality of an odor is shaped by attention [38.54], expectancy [38.55–57], learning [38.58], recognition and memory [38.59], as well as valence-dependent responses [38.60]. The pirC can consequently be considered association cortex connecting olfactory perception with behavioral, cognitive and contextual information [38.43, 46].

The olfactory bulb and pirC send projections to the entorhinal cortex that renders the gateway to the hippocampus responsible for memory processes [38.61]. From rodent studies, it is suggested that the entorhinal cortex projects back to the pirC and the olfactory bulb and thereby serves as a *powerful top-down modulator of olfactory cortical function and odor perception* [38.62]. The entorhinal cortex, especially the transentorhinal region is one of the first brain areas showing a neuropathology in patients suffering from Alzheimer's disease [38.63, 64] and is thus responsible for early olfactory deficits seen in Alzheimer's disease patients. For further insights about disrupted olfactory perception please refer to Chap. 31.

The amygdala and periamygdaloid cortex receive input from the olfactory bulb and the pirC and are responsible for a cognitive evaluation of olfactory input. In a recent meta-analysis about predictors of amygdala activation it was established that olfactory and gustatory stimulation had the highest amygdala activation probability in comparison to other sensory modalities [38.65]. Traditionally, the amygdala was considered the neural substrate for odor pleasantness assignment [38.66, 67]. More recently, evidence arose

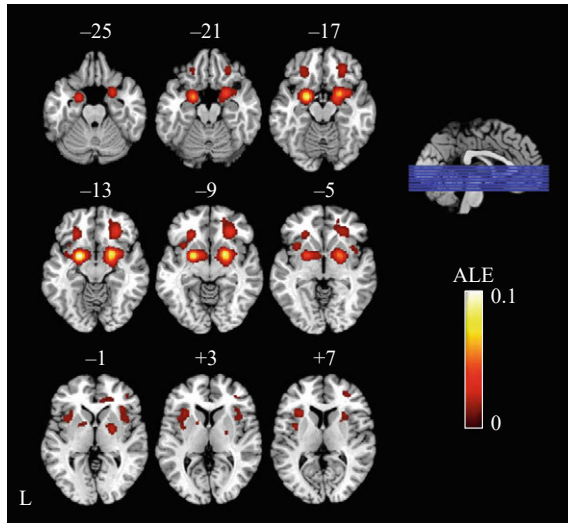

Fig. 38.2 Typical brain activation during olfactory stimulation. Depicted are the results of a meta-analysis of olfactory brain activation in 45 functional imaging studies. Bilateral activation of the pirC, the OFC, as well as anterior insula can be found (courtesy of Elesvier, after [38.57])

that the amygdala is rather responsible for olfactory intensity coding but only if the odor is considered pleasant or unpleasant. In other words, emotional or behavioral salience encoding takes place in the amygdala [38.68], whereas odor pleasantness or valence is coded in the orbitofrontal cortex [38.69]. Please see Chap. 39 for a detailed review on olfactory valence.During studies using chemosensory stimulation, amygdala response was correlated with response in pirC, orbitofrontal cortex, and insula hinting toward a network of those areas responsible for processing of chemosensation [38.70].

An attempt to describe the temporal pattern of odor perception is the cascade model of olfactory perception by Jonas Olofsson. This model states that odor detection is the first and fastest step followed by a direct odor valence determination. An indirect path links odor detection with odor valence by way of identification of the odor object. The slowest perceptual process is the rating of edibility of the odorant [38.71]. Hence, olfactory perception is based on a hierarchical and partial parallel organization of the olfactory system [38.72].

From this set of smaller brain areas, olfactory information is passed on to brain areas that are not specific for olfactory processing but rather convey cognitive processes related to the perception of odors: the orbitofrontal cortex (OFC), the insula, hippocampus, thalamus, hypothalamus, ventral striatum, cingulate cortex, and the cerebellum.

Here the OFC is considered a key node in the olfactory system and a crucial center for cognitive odor processing. In the OFC higher order processes shape the odor signal coming from the pirC and create the final conscious smell percept. While olfactory bulb volume predicted olfactory identification of healthy subjects, OFC volume rather predicted threshold scores and olfactory discrimination ability [38.40]. Thus, the OFC mediates processes related to olfactory sensitivity and discrimination tasks. Further, experience-dependent modulation of the signal as well as affective coding (as stated earlier) are computed in the OFC. It is also known, that the OFC is the brain area in which olfactory information is integrated with information from other sensory modalities [38.73] and the language system [38.39]. With regards to food odor perception, the OFC involves mechanisms of reward assignment [38.74]. Hence, the OFC does not exclusively process odor information but rather engages in processes important for human perceptual decision-making and resolving sensory uncertainty.

The insula is traditionally considered primary gustatory cortex [38.75]; however, displays frequent activation as response to odors. It receives input from not only pirC and amygdala, but also from OFC [38.76]. Evidence exists that the anterior insula plays a role in integrating chemosensory information especially with regards to food [38.45] leading to an integrated flavor perception [38.77]. Furthermore, the anterior insula is crucial for the elicitation of avoidance behavior and is thus correlated not only with the perception of negatively valenced olfactory or trigeminal stimuli [38.78] but also negative multisensory stimulus combinations [38.79]. The insula moreover is involved in regulation of autonomic interoception. In the context of a strong connectivity of the insula with pirC, amygdala, and OFC it can be suggested that chemosensation influences interoception and interoceptive processes are regulated within this network [38.70].

The olfactory system is unique in the sense that sensory information passes only two synapses and reaches brain areas involved in emotion and memory processing. The hippocampus conveys memory processing associated with odorants. Smells indeed can elicit very vivid and emotional memories. On a timeline, smells in comparison to pictures or language have the capability to evoke memories of early life – smells remind us of earlier events in comparison to pictures or language [38.80]. For a more detailed description of olfactory memory processes please refer to Chap. 42. The hippocampus further seems to be involved in integration of olfactory with other sensory stimuli [38.73].

As already mentioned, the main direct pathway of olfactory information processing is from the olfactory bulb and pirC to the neocortex; however, an indirect pathway through the mediodorsal thalamus to the neocortex also exists. The mediodorsal thalamic nucleus (MDT) is considered a part of the thalamus responsible for odor processing as it receives afferents from pirC, amygdala, enthorhinal cortex, and projects to the OFC [38.81, 82]. In an elegant study, *Plailly* and colleagues [38.83] demonstrated that attention toward an odor strengthened the connectivity between the MDT and the neocortex (OFC). As described earlier, in olfaction the OB and pirC inherit functions of a primary sensory thalamic control including sensory coding, gain control and state-dependent modulation. Hence, the MDT might rather be seen as a higher order olfactory thalamus [38.82].

The cingulate cortex, especially the anterior cingulate is traditionally involved in odor–taste integration and is therefore considered a part of the flavor network [38.84]. Cingulate cortex belongs to the limbic system and its activation is also frequently found following olfactory stimulation and might therefore be

involved in attention processes in relation to odors. Especially the anterior cingulate is responsible for detection of conflicts of attention [38.85].

As stated earlier, the involvement of the cerebellum in olfactory processing was established as a kind of regulation center that adjusts sniff magnitude to concentration of the smell [38.22]. The sniffs of patients with cerebellar lesions were invariant with regard to odor concentration and thus olfactory identification was impaired. The authors suggest an olfactocerebellar pathway that is important for identification of odorants [38.86].

38.5 Conclusion

Taken together, the olfactory system involves three anatomical deviations. First, the main central gate for olfactory input is the olfactory bulb; here we find a topical map of odor representation. Olfactory information does not pass a thalamic relay on its way from receptors to neocortex, which might be the reason for a multitude of unconscious processes involved in olfactory perception. Second, olfactory information is first processed in the paleocortex (olfactory bulb, pirC) and then passed on to the neocortex while sensory information in other modalities is mainly processed in neocortex. Third, the olfactory system is strongly connected to the limbic system resulting in strong emotionally toned responses to odors and robust relation to memory processing. Those characteristics of the olfactory cortical system form the basis for smell perception which renders unique among other sensory perceptions.

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39. Behavioral and Neural Determinants of Odor Valence Perception

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This chapter serves as an introduction to our current understanding of the stimulus-driven and experience-driven mechanisms, which give rise to affective evaluation of odorants. We will start by focusing on the potential evolutionary benefits of rapid elicitation of affective responses to odors and provide an overview of paradigms and approaches that can be used to quantify these experiences in an experimental setting. We will then outline evidence in favor of stimulus-driven and experience- or learning-driven accounts of odor valence perception, representing two prevalent theories. Finally, we provide an overview of the cortical networks that support the assignment of valence to odors.

The pleasantness or unpleasantness of an odor, which is commonly referred to as its valence, is usually experienced as its most pervasive perceptual property. This is eloquently described in Marcel Prousts' book *In Search of Lost Time* (Vol. I, Swann's Way, 1913) in the *episode of the madeleine* (a famous description of an involuntary memory episode which the protagonist experiences while enjoying a piece of French small cake). These emotional reactions to odors, whether they occur in the context of a food aroma or the perfume of a loved one, not only color the emotional evaluation of a situation, but also form the basis of many traditions that constitute an integral part of human culture: for example, the shared consumption of familiar foods, or the usage of scents, such as incense or patchouli, during spiritual gatherings [39.1, 2]. Unlike visual information, where conscious identification of the object generally precedes the elicitation of approach or avoidance reactions, affective responses to odors can occur even in the absence of any knowledge about the identity or source of the odorant. It is this immediacy that gives smells the unique ability to involuntarily and profoundly change affective experiences of our sensory surroundings, supporting the prevailing notion that valence, rather than quality, may constitute the principal dimension of olfactory perception [39.3–5]. Emotional evaluations of odors range from strongly appetitive, as might be the case for the smell of freshly cut grass, to strongly aversive, as when we smell a pair of dirty socks. While this valence attribution appears to arise naturally and apparently without conscious effort, the mechanisms behind the generation of positive or negative responses to odors are far from trivial: while smells like *freshly cut grass* or *dirty socks* are perceived as holistic objects, they are formed by complex mixtures, and may even overlap in a number of their odorous compounds. Sometimes, a fraction of the molecular components of an odor can make a fundamental difference to its evaluation, for example, when distinguishing between *grilled fish* and *rotten fish*. The matter is further complicated by the observation that emotional responses are quite unstable over time and

susceptible to changes based on additional knowledge about the emitting object: finding out that the disgusting smell of *dirty socks* is actually released by a fancy French cheese quickly results in a reappraisal of the odor, which is now perceived as appetizing and edible, and no longer elicits disgust and withdrawal tendencies [39.6]. Despite this fundamental importance of valence to odor perception, our understanding of how it is formed, modulated, and processed is still poorly understood.

39.1 Odor Valence and Behavior

39.1.1 Relevance of Odor Valence Perception for Adaptive Behavior

Emotional experiences are commonly classified along two principal axes: valence and arousal [39.7]. Valence is commonly considered the principal dimension underlying more complex odor-elicited feelings [39.3] (see Chap. 23 for an in-depth discussion on these).Forming a core element of motivation [39.8], valence describes the intrinsic attractiveness or aversiveness of an experience [39.9], while arousal, discussed in more detail below, quantifies the amount of physiological or psychological reactivity.

Valence qualities also form the basis of many evolutionarily adaptive behavioral responses, which occur as a direct consequence of olfactory perceptual experiences. These responses fall into two broad categories: those which subserve a behavioral warning mechanism through elicitation of a withdrawal impulse (avoidance responses, [39.10, 11]), and those which assist the elicitation of appetitive responses as a contribution to the maintenance of health. These include for example the elicitation of a desire to consume food, or affiliative behaviors toward potential mates and social groups (approach responses).

In a recent overview, *Stevenson* [39.12] further separates the evolutionary functions of odor valence perception into three broad category domains in parallel to the approach/avoidance dimension: ingestion, hazard avoidance, and social communication. *Ingestion* hereby refers to responses to odors relevant for food intake, and includes the elicitation of an appetitive response toward familiar edible items, but also to the regulation of appetite through state-dependent elicitation of approach or avoidance behaviors toward them: although a chocolate chip cookie generally constitutes an edible object, we may not feel a strong preference for its aroma after just having finished a brownie. On the other hand, *hazard avoidance* describes aversive reactions contributing to an olfactory warning system. This olfactory-based warning response aids detection of environmental dangers such as smoke and fumes (through elicitation of fear, as during detection of the smell of a gas leak), as well as the prevention of spoilt food items (through elicitation of disgust, as during detection of the smell of rotting meat). Finally, *social communication* includes appetitive aspects relating to the perception of body odors [39.13], which may contribute to the communication of emotions and fitness detection [39.14, 15], but also avoidance aspects, as evident in the negative responses to body odors signaling high genetic overlap to avoid inbreeding [39.16], and aversive reactions to the smell of illness [39.17].

Given that odor valence evaluations can bypass object identity identification, which typically precedes valence attributions in other sensory modalities, their role as a warning signal can be considered of foremost importance. For example, quick and reflex-like negative emotional responses are crucial when it comes to spitting out a spoilt food object or withdrawing from toxic fumes.

Correspondingly, valence attributions to unidentifiable odors, without *knowing* what is smelled, tend to be subject to a negativity bias, as they are usually less positive than attributions to familiar odors [39.18]. For example, people frequently report the odor of 1-octen-3-one to be unpleasant when presented by its chemical name; the same odor, however, elicits positive valence ratings when presented as *mushroom odor*. This response pattern meets the criteria for a behavioral warning system as put forward by error management theory [39.19]: any system designed for self-protection should, in doubt, be biased toward elicitation of a warning response, given that an erroneous failure to respond could potentially lead to fatal consequences [39.11]. The health impact resulting from impairment of the sense of smell further demonstrates the importance of these immediate avoidance reactions to odors for survival: decrease or complete loss of olfactory function, for example, during old age, results in failure to elicit withdrawal reactions and thus an increase in hazardous events, for example, a fire or gas leak [39.20], and a lowered rejection threshold for rotten food [39.21]. A recent retrospective cohort study demonstrated that the chances of experiencing such a hazardous event increases from 18% in individuals with an intact sense of smell to 39.2% in anosmic patients [39.22].

Immediate rejection responses, however, are of course not the only pathway for odor valence assignment; many odors that we encounter in our day-to-day lives are a source of pleasant affective experiences. These positive emotional responses are thought to be more strongly driven by the perceived *identity* of the smelled objects. Typically, such processes are labeled as *top-down processing* to distinguish them from the stimulus-driven, or *bottom-up*, negative evaluations explained above. These more cognitive, top-down controlled attributions are associated with a so-called *high-road* of olfactory perception, meaning that *object representations activate emotional responses downstream* [39.23, p. 1209] before a conscious valence attribution to the olfactory percept arises.

Absence of the ability to perceive the positive affective properties of an odor can equally result in severe consequences: In the elderly, the loss of olfactory function is thought to be a key factor contributing to appetite loss, and thereby to the incidence of undernutrition observed in this population [39.24, 25].

39.1.2 Assessing Odor Valence Perception in an Experimental Setting

Qualitative and quantitative measures of odor valence evaluation are predominantly assessed by studying the participant's perceptual experience, as observed through stimulus ratings or response time toward certain stimuli. The other dimension of emotional experience, its arousal value [39.26], tends to be less reliably captured by behavioral measures, however; it is thus more commonly characterized by measurements of central and peripheral nervous system responses to the stimulus material.

Behavioral Measures

Self-reported stimulus ratings and response times are frequently used methods that provide experimenters with a holistic picture of the participants' subjective odor perception and evaluation (Fig. 39.1).

In a typical experimental setup for self-report of olfactory properties, participants are directly asked to evaluate the sensory attributes of odorants, which are presented to them in a controlled manner. This presentation can take place manually, via glass jars that are opened and closed by the experimenter, or by means of an air-delivery device, such as an olfactometer [39.28] (see Chap. 24 for a detailed description).

Most studies assess stimulus valence by means of a rating procedure, in which the pleasantness of each odorant is evaluated on either a continuous scale between *highly pleasant* and *highly unpleasant* (digital analog scale, [39.29]), or by a two-alternative forced choice task, which yields a *pleasant* or *unpleasant* rating outcome [39.23]. Depending on the research question, the identities of the presented odors are either openly presented to the participants [39.6], or concealed from them [39.30]. Familiarity of the odor may bias toward top-down or bottom-up evaluations of valence, and thus constitutes an additional variable of interest in this context. Familiarity may change over the course of an experiment and thus can also be used as an experimental manipulation to study recognition effects of previously unfamiliar odors on valence perception [39.31].

Participants' response times, another measure of differential effects of positive and negative odor valence, have been shown to be influenced by the direction of valence [39.2]. In addition, the ecological relevance of the presented stimulus, particularly as related to food ingestion [39.32], affects the speed at which an affective evaluation is reached. As an example, a study by *Boesveldt* and *colleagues* [39.10] showed that the detection of unpleasant compared to pleasant food odors was faster and more accurate, strengthening the idea of the olfactory systems' preferential processing of odors that constitute ecologically relevant warning signals such as rotten food smells. Other studies have used the dimension of *edibility* as a measure capturing the motivational aspects (approach versus avoidance) of odors [39.23]. On the other end of the valence spectrum, the concept of *wanting* captures an odor's incentive value, salience, and propensity to elicit consumption in addition to mere *liking* [39.33]. *Wanting* tends to change in relation to a person's satiety state, while *liking* remains less affected.

Neurophysiological Measures

Physiological indicators of arousal, such as sweaty palms or a fast heartbeat, are prominent features of the experience of an emotional event. As such, they offer a unique window into the intensity of the experience of odorants' affective properties [39.34], which com-

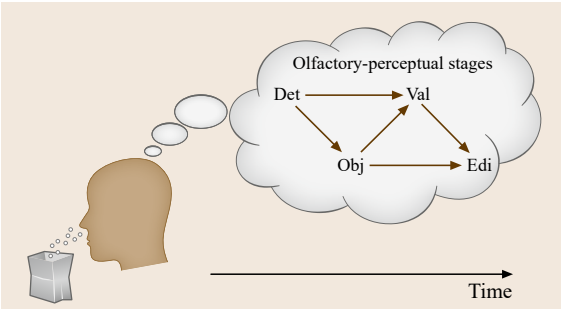

Fig. 39.1 A cascade model of olfactory perception (with permission from [39.27])

plements valence assessments. Physiological indicators further provide valuable insights into the biological basis of psychological constructs underlying affective odor perception and the evolutionary relevance of the associated emotional responses [39.35]. Changes in both autonomous and central nervous system activity, are recorded in the periphery of the nervous system and generally occur independently of the participant's awareness.

Alaoui-Ismaïli and *colleagues* [39.30] were among the first to include physiological markers to an odor hedonic rating task. These markers included skin potential and resistance, skin blood flow and temperature, as well as instantaneous respiratory frequency and heart rate. The authors showed that the presentation of odors engaged several of these autonomic markers, demonstrating that odors carried an emotional load comparable to the presentation of emotional auditory or visual signals. It was further shown that skin conductance and heart rate measurements significantly correlated with the intensity of participants' emotional experience, which indicated a direct relevance of these autonomous measures for subjective emotional experience (However, note that there is evidence that frontotemporal lesions can lead to impairments in autonomic responses but not of subjective ratings, limiting the interdependence of these two [39.36]). Furthermore, skin potential data showed a difference based on whether pleasant or unpleasant odors were presented, demonstrating that this activity was additionally modulated by the direction of valence.

Neurophysiological brain imaging techniques, such as electroencephalography (EEG) measurements, offer an indirect way to capture central nervous system activity through scalp recordings of postsynaptic potential change in large assemblies of neurons. Desynchronization of alpha-band activity in the EEG-signal, an indicator of cortical activity or arousal [39.37], has long been shown to be sensitive to odor stimulation [39.38]. A study by *Brauchli* and *colleagues* [39.39] investigated the valence-dependence of this autonomous response to odors, and found a significant decrease in the upper alpha-band activity of the EEG (9.75–12.5 Hz) within 8 s after stimulus presentation of an unpleasant odor. On the other hand, a pleasant odor condition did not result in a change of alpha-band activity, therefore suggesting that observed changes in cortical arousal captured by alpha desynchronization could be specific to odors with a negative valence. Similar to the above studies, the authors observed increased skin conductance amplitudes to negative odors, which were not, however, mediated by the amount of decrease in upper alpha-band activity. This indicates that central and peripheral physiological responses may capture independent processes. Another EEG study by *Schupp* and *colleagues* [39.40] determined the time course of the EEG signal following olfactory stimulus presentation, and found a difference in the amplitude of the late positive event-related potential (LPP) to odorants as a function of valence. The LPP is a positive potential arising approximately 400–500 ms after stimulus onset, which has been generally implicated in coding affective content and motivational relevance. An association of LPP amplitude change with different odor valences therefore suggested an important role of the late even-related potential in signaling the perceived pleasantness of odors [39.41, 42].

The studies above manipulated odor valence perception by presenting different odors, and thus cannot dissociate whether the observed effects are a direct result of perceived quality, or of specific influences of the odorant's different chemical composition. A more recent study by *Lundström* and *colleagues* [39.43] addressed this concern through the usage of androstenone, an odor for which a common genetic polymorphism results in fundamentally different perceptual experiences. Depending on a person's genetic variation of the olfactory receptor *OR7D4* [39.44], the odor is either perceived as urinous, hence negative, or flowery, hence pleasant [39.43]. Another portion of the population, who is completely anosmic to the odor, was excluded from the study. The main difference in cerebral processing of the androstenone odor between subjects with positive and negative valence perception was specific to the peak of the LPP amplitude; thus demonstrating its clear link to odor valence processing [39.43] and individual variation in genetic composition [39.44].

A final aspect of the experimental assessment of odor valence that we will discuss here relates to its relevance for social communication of affective information. The human face contains over 100 facial muscles, which, in certain combinations, express the elementary emotions of joy, anger, disgust, fear, and sadness, but can also communicate more subtle and culture-dependent mood states and feelings. Facial muscle activity is measured by electromyography (EMG) [39.45, 46]: small surface electrodes are hereby attached to the facial skin above the specific muscle and measure its electromyographic activity in relation to the stimulus presentation. Electromyographic activity is quantified by an amplitude change between baseline and response level. One particular study by *Jäncke* and *colleagues* [39.47] assessed facial EMG responses toward pleasant and unpleasant odors and demonstrated higher EMG activity toward disgust-eliciting stimuli at the highest odor concentration; however, no direct correlations with odor valence ratings were observed. An additional experimental manipulation investigated the

effects of the *social context* in which the odors were presented: individuals smelled the different odors either in a private setting or in the presence of another person (the experimenter). As expected, the display of facial emotions to unpleasant odors, as measured by the EMG response, was significantly stronger when the experimenter was present than when the participants were tested in solitude, indicating that facial responses are driven by social factors rather than directly by olfactory–facial reflexes (see [39.48] for a discussion). Facial muscle activity accompanying odor valence perception is thus thought to be embedded in a context-dependent higher order modulation, and dissociable from peripheral nervous responses that occur as a result of autonomous nervous system activity, such as skin conductance changes. Pleasant odors and positively rated odors, in contrast, were *not* associated with a facial response (participants smiling). This suggests that communication of odor-evoked emotion may primarily serve the evolutionary function to communicate danger or contamination to our conspecifics, rather than joy and content.

39.2 Determinants of Odor Valence

While the evidence outlined above clearly demonstrates that odor valence shapes both our behavior and physiological responses, the perceptual processes determining whether an odor is perceived as pleasant or unpleasant have long remained elusive. As we elaborate below, two separate lines of research can be identified, focusing on the one hand on the relationship between the molecular properties of odors and their perceived valence, and on the other hand on the cognitive mechanisms, which give rise to an affective valence percept on the cortical level. Both these factors need to be taken into account to grasp the construction of an odor percept with distinct valence properties.

39.2.1 Effects of Molecular Features on Odor Valence Perception

Similar to the colors, edges, or spatial frequencies that make up visual scenes, monomolecular volatile chemicals, such as alcohols and aldehydes, form the smallest perceptual units of all odors. These structures can be viewed as basic features that construct the olfactory objects we perceive, and as such, their influence on perceived qualities of odors are of significant interest for understanding the emergence of the pleasantness or unpleasantness of the final odor percept. Research has focused on the identification of possible innate approach or avoidance responses to individual chemical compounds, which may subserve the evolutionarily adaptive functions of odor valence perception outlined above. Indeed, work on rodents has convincingly demonstrated that the detection of specific volatile chemical compounds can elicit hard-wired emotional reactions, which likely constitute evolutionary advantages in the context of threat detection. Laboratory rats, for example, while never having been exposed to cats in their lifetime, still demonstrate strong avoidance reactions when exposed to a cat odor: relative to their peers who are exposed to a control odor, they spend significantly more time in a *hide box* and assume a distinctive defense-related body posture [39.49]. Immunoreactivity patterns further indicate that these effects do not originate from accessory olfactory bulb activity, which is frequently implicated in reflex-like adaptive behavior in rodents, but rather from the main olfactory bulb. This strengthens the possibility that analogous mechanisms may exist across species. More specifically, a later study by *Kobayakawa* and *colleagues* [39.50] identified the locus of innate odor-elicited fear responses in the dorsal olfactory bulb, indicating that a distinct subset of receptors may be responsible for these responses. While the precise coding mechanisms for aversive responses to specific chemical compounds remain unclear, recent work by *Dias* and *Ressler* [39.51] indicates that, at least in part, aversive responses to odors in mice may be produced by epigenetic inheritance mechanisms. Traumatic odor exposure in a parental generation was found to carry over to the following, unexposed, two generations who showed neuroanatomical and genetic modifications contributing to behavioral manifestations of increased sensitivity to the conditioned odor.

In humans, however, conclusive evidence for hard-wired behavioral responses to odors remains scarce. Earlier work has argued in favor of some innate preferences for specific odorants in humans [39.52] by demonstrating differences in facial expressions to odors in neonates. However, recent data has demonstrated relationships between early olfactory preferences and feeding modes [39.53], as well as prenatal exposure to food flavor by way of the newborns' mothers' diets during pregnancy [39.54]. These findings challenge the idea that postnatal behavior provides conclusive evidence for a hardwired biological basis of early odor preferences, and raises the possibility that even early olfactory preferences may be the result of learning mechanisms occurring during gestation and early life.

An alternative approach for the assessment of innate odor preferences, which has more recently gained popularity, explores relationships between the chemical composition of odors and olfactory perceptual space in humans with the help of big population-based datasets: these allow identification of robust correlations between human psychophysical assessments and structural molecular properties [39.55–58]. Stable relationships between perceptual ratings and molecular structures across participants are hereby interpreted as evidence for the existence of systematic and potentially innate relationships between affective evaluation, molecular structure, and interindividual variability in receptor expression.

In one of the most comprehensive studies of this type to date, *Khan* and *colleagues* [39.59] pursued a two-step procedure. First, they applied principal component analyses to an existent database of perceptual odor characteristics to reduce the dimensionality of perceptual descriptors. The component with the highest factor load emerging from this analysis, which the authors labeled *pleasantness*, accounted for 30% of the explained variance in perceptual feature space, and thus provided support for the idea of valence as the primary dimension of olfactory perception, much as described above. Second, they applied an identical approach to the molecular and physical descriptors of the same odorants; here, a primary axis emerged that was anchored by the attributes of *molecular weight* and *molecular extent*. Comparisons of the ranks of each odorant on the respective principal components in perceptual and molecular space indicated a high degree of overlap between these factors. This suggests that certain chemical properties are, in the absence of any contextual information, simply perceived as more pleasant than others. It is important to note, however, that the study was conducted on isointense concentrations of all odors only. Given that intensity perception is known to interact with pleasantness perception, future studies will need to incorporate intensity modulations to reflect this complexity.

While this work provides support to the idea of hardwired relationships between chemical properties and affective value independent of individual learning experiences, population-based models, which do not account for effects of interpersonal variability in perceptual experience, leave a substantial portion of variance unaccounted for. In a search for explanations for this remaining variability on the level of sensory encoding, studies have highlighted effects of interpersonal differences in olfactory-receptor expression on the phenomenological experience of odors. As explained above on the example of the odor androstenone, genetic dimorphisms in odor-receptor expression are linked to variances in glomerular activity to different odorants and, as a result, to differences in perceived odor quality and the resulting affective valence attribution [39.44, 58, 60]. Popular examples of such differences include dislikes for certain food items, which have been related to dimorphisms in receptors encoding distinct olfactory components of their aroma, causing some people to perceive a *soapy* note in cilantro that may be absent for other people, and a sweaty, urinous note in celery that others perceive as sweet and floral [39.61, 62].

In an effort to grasp this variability on a larger scale, *Mainland* and *colleagues* [39.58] identified agonists to 18 different odor receptors and demonstrated that polymorphisms in 63% of these were linked to differences in their in-vitro response patterns. To assess the functional consequences of this genetic variability, these patterns were related to receptor expression in a DNA population database, showing that on average, individuals differed in around 30% of these functional olfactory polymorphisms, and that geographical factors predicted the amount of observed genetic overlap between individuals (Fig. 39.2a). Finally, to demonstrate the behavioral relevance of this interpersonal variability, the authors singled out one receptor (Olfactory Receptor 10G4; *OR10G4*, which has four allele variants occurring with high frequency in the general population), for comprehensive behavioral testing (Fig. 39.2b). They found that possession of allele 3 or 4 accounted for a substantial increase in perceived valence of its agonist guaiacol. The perceived valence of two other agonists, as well as of 63 other odors not known to bind to *OR10G4* (Fig. 39.2c), remained unaffected.

These experiments show that receptor-binding patterns are not only likely to play an important role in the prediction of the perceived valence of an odorant, but also that variants within the population in the expression of these receptors significantly contribute to the variability in odor preferences between individuals.

39.2.2 Contextual Influences on Odor Valence Perception

As explained in the beginning of this chapter, any theory of odor valence perception needs to take into account the changes in valence evaluation that usually occur when a particular smell is linked to an anticipated positive or negative outcome. Highly variable between individuals and contexts, such top-down controlled evaluations are thought to account for a large portion of differences observed in odor valence assignments between individuals. For an impression of cognitively modulated influences on pleasantness perception, one only needs to revisit the above-mentioned example of a cheese odor that is presented as freshly grated parmesan cheese: it will likely stimulate appetite and

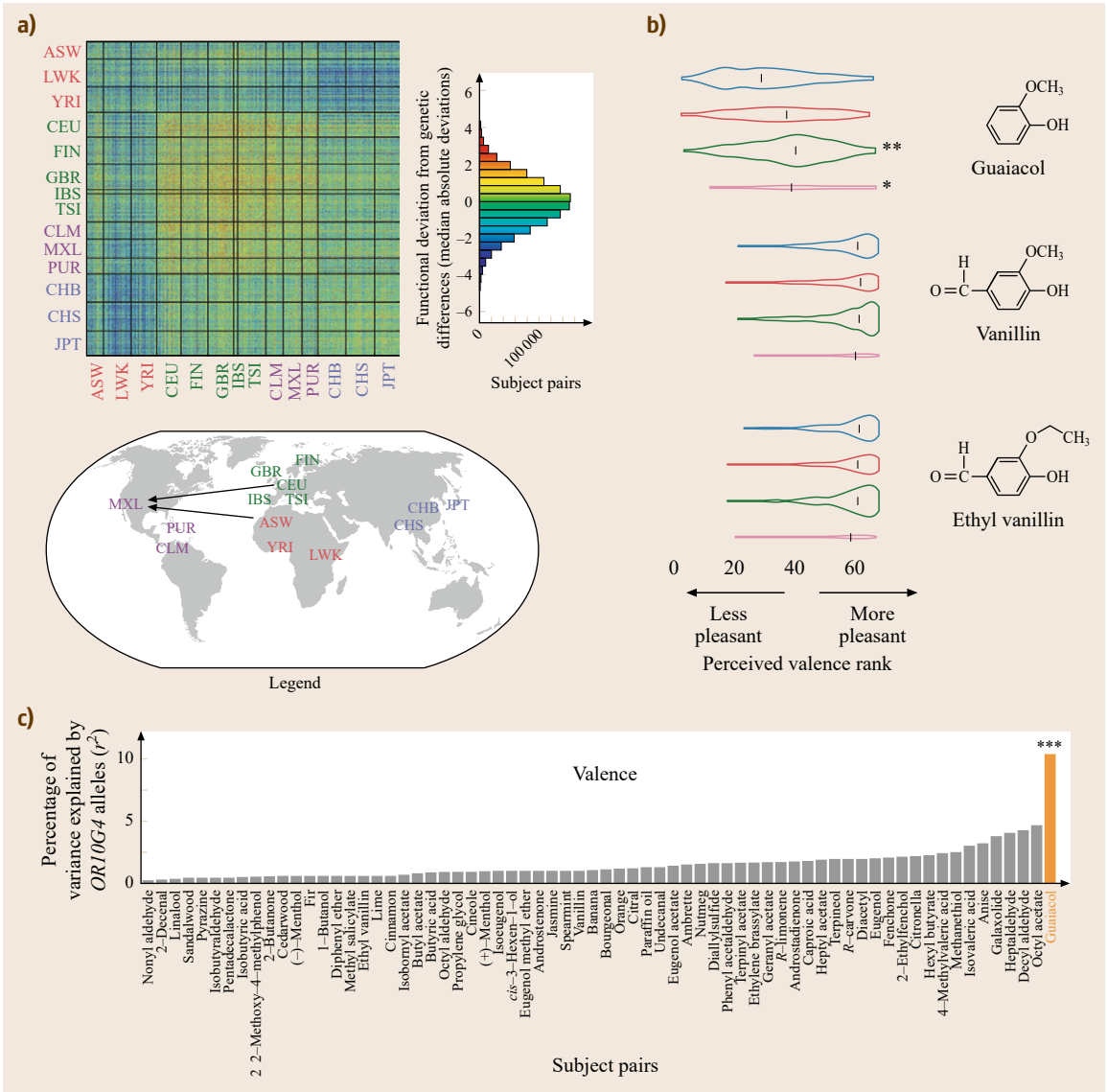

Fig. 39.2a–c Effects of functional variability in the human odorant receptor repertoire on the perception of odor valence (modified from [39.58], with permission from Nature Publishing Group). **(a)** *z*-Scored functional differences minus *z*-scored nucleotide differences among 27 odorant receptors of 1092 participants from the 1000 Genomes Project. Participant populations are labeled on the axes and separated by black grid lines. **(b)** Perceived intensity and valence rank for three in vitro *OR10G4* agonists by receptor variants of *OR10G4*. **(c)** Percentage of perceptual variance (r^2) in valence ranking explained by *OR10G4* allele types

will be perceived as pleasant, and even more so before dinner time than after finishing one's meal. The same odor, however, will likely elicit disgust and rejection when labeled as vomit or used sweaty socks. Similar effects have been demonstrated in experimental settings, and can be extended to a number of odors [39.6, 63, 64].

Difficulties to assign object labels to odors in the absence of additional cues are well documented; this instability of odor object representations makes us vulnerable to cognitively mediated shifts in valence assignments. Without the prior knowledge present in most real-life contexts, or the presentation of response alternatives, as in standardized tests of odor quality identification (Sniffin Sticks, see Chap. 31, University of Pennsylvania Smell Identification Test, UPSIT), humans perform extremely poorly when asked to name an

odorant [39.5, 65, 66] yet are overconfident in the veracity of their odor identifications [39.67]. Indeed, an equivalent of the surprisingly common confusion between parmesan and sweaty socks is difficult to imagine in any other sensory modality. The high dimensionality of odor mixtures likely plays an important role in this identification deficit. The large number of different chemical molecules with specific receptor-binding patterns means that humans can distinguish a near endless number of different odors [39.55]. In contrast to sounds and images, where any possible stimulus can be described on the basis of two dimensions (frequency and amplitude of its physical waveform), accurate memory retrieval of every possible odor encountered over one's life would require storage of a near infinite number of unique different receptor-activation patterns. In addition, the large extent of molecular overlap between odors from different sources means that matching a current perceptual impression to the memory of a previously encountered odor could yield several, equally plausible, response alternatives for an object source, all of which can vary vastly in their affective attributes.

Several authors have proposed that cortical processing of odor recognition solves these problems by matching odors to object-based templates, ultimately allowing the system to use similar mechanisms of pattern recognition, -completion, and -separation as observed in other sensory modalities [39.68–70]. Such a mechanism would reduce the high dimensionality of olfactory stimuli by matching noisy perceptual input to discrete memory templates, each of which are associated with specific object properties and affective connotations. Within this model, multisensory or memory cues would serve to facilitate access to particular object memory templates [39.69, 71] and, in turn, would lead to a particular identity assignment and elicitation of the associated affective response. In other words, cognitively mediated valence assignments would arise not directly from the sensory properties of the odorant per se, but rather from the learned affective properties of an olfactory memory trace.

In line with this idea, psychophysical evidence demonstrates that cognitively controlled identification processes might precede the conscious emergence of odor valence assignments. A behavioral study by *Olofsson* and *colleagues* [39.23] used mediation analyses on response times during identity and valence assessments to demonstrate that odor valence may only partly arise as the result of a direct feed-forward relationship between molecular properties and affective responses. Response times to identity judgments preceded those for pleasantness evaluations, favoring an indirect pathway to odor pleasantness evaluation based on the object properties of the presumed underlying source.

Work from other authors suggests that such cognitively mediated valence assignments may be particularly relevant in the determination of positive valence attributes; odors, rated as *pleasant* tend to be processed more slowly than unpleasant ones [39.72], indicating a higher cognitive processing load. In line with this idea, naming an odorant, and thus assigning an identity to it, was shown to influence hedonic judgments of neutral odors, making them likely to be perceived as more pleasant [39.73]. A negative object label, on the other hand, resulted in stimuli being rated as more intense and less pleasant compared to a neutral or positive name [39.6].

Li et al. [39.74] investigated learned odor valence responses through changes in odor perception following classical fear conditioning. After coupling an odor as a conditioned stimulus (CS+) with an electric shock (unconditioned stimulus, US), subjects were able to discriminate between that odor and a previously indistinguishable odor that had been presented as a CS− (i. e., without being coupled to the US). Odor preference can also be influenced by positive associative learning [39.75]. Depending on the valence of affective pictures presented to subjects, neutral odors were rated differently [39.76]: they were identified as more intense and unpleasant following negative picture presentation, while neutral odors following positive images induced greater odor pleasantness. Studies on populations from different cultural backgrounds have used existing differences in food object knowledge between ethnic groups to demonstrate systematic effects of learned associations on odor valence perception. *Distel* and *colleagues* [39.77] presented Japanese and German participants with odor samples that were either specific to the Japanese or German culture (such as fermented soybeans, dried fish, green tea, versus cheese, salami, incense) or familiar to both groups (such as chocolate, peanuts, beer). Unfamiliarity with an odor, and thus an inability to apply an object label with positive associations, resulted in the odor being rated as more unpleasant [39.18, 77]. These findings are in line with the above discussed idea that lacking identity information favors negative valence evaluation.

Top-down control of odor perception not only helps to understand contextual and interpersonal differences in valence assignment, but may also contribute to its fluctuations over time; most commonly, such effects are described in the context of metabolic changes, where hunger can heighten positive evaluations of food odors while satiety can dampen them [39.78, 79].

These state-dependent valence assignments in olfactory perception have been linked to similar phenomena in thermal perception, where the perceived pleasantness of temperature is evaluated relative to the self (warmth is perceived as pleasant when one feels cold,

while cold is perceived as pleasant during heat). Labeled *alliesthesia* [39.78], the appetitive value of an object is determined by the *milieu interieur*, internal signals, such as body temperature, or, in the case of chemosensation, by satiety status. Importantly, alliesthesia describes a phenomenon that generalizes to all stimuli related to the specific internal state in question: in the case of olfactory perception, valence ratings to all food-related odors, which are increased during a hungry state, and decreased during a satiated state. A related, but clearly distinct concept named *sensory-specific satiety* describes a phenomenon where state-dependent valence affects one distinct sensory stimulus: a particular food aroma is no longer perceived as appetizing after repeated exposure, however, without necessarily causing a generalization of valence changes in other food odors. Commonly, this phenomenon can be experienced during buffet-style meals, and is implicated as a potential cause for overeating [39.80]: because of the diversity of available sensory experiences, the motivational value of food flavors (a combination of their smell and taste) is maintained over much longer periods of food intake, compared to the rapid decline in affective responses elicited by a single dish with a comparatively monotonous flavor profile. Additional factors known to modify the occurrence of sensory-specific satiety include nutrient composition, texture, and visual impressions of food [39.79].

In sum, the perceptual phenomena described above demonstrate that the relationship between chemosensory properties and olfactory object identities, as well as the relationship between these object identities and their affective and motivational properties, are highly plastic and undergo constant changes depending on their perceived personal relevance. As a result, the role of olfactory valence evaluation during cortical encoding of odor perception should be viewed not just as a step in a linear processing cascade, but rather as a dynamic process that encompasses various feedback loops between molecular properties, receptor repertoire, learning experiences (such as associations with threat), and internal states (such as hunger). The next subsections of this chapter will address our current knowledge of how these processes are integrated during cortical and peripheral neural encoding of odors to give rise to an olfactory experience.

39.3 Valence Coding in the Human Brain

As outlined above, odor valence perception is not a unified concept, but rather constructed through the integration of bottom-up processing of relevant molecular characteristics combined with top-down learned assignments of object identities, which in turn evoke specific assignments of affective properties. These affective evaluations can be captured by various cognitive measures such as edibility judgments or incentive salience, and can be related to characteristic modifications of peripheral nervous activity. Given the multidimensionality of factors driving odor valence perception, the neural activations contributing to the emergence of affective perceptual properties need to be equally considered from this multidimensional perspective.

In the following, we will first discuss the role of direct integration of olfactory processing and emotional brain networks as a unique anatomical property of the olfactory system, which may provide a structural basis for the high automaticity of the emergence of affective properties in the absence of cognitive evaluation. We will then provide an overview of the extended network of subcortical and cortical structures known to contribute to the conscious assignment of affective properties through the identification of a distinct odor object identity. Finally, we will discuss data that demonstrates how the network activity regulating odor valence perception may change as a function of contextual characteristics and relevant experiences.

39.3.1 Subcortical and Limbic Processing of Odors – A Pathway for Automatic Threat Detection?

In primate olfactory perception, odor molecules bind to their primary sensing cells in the olfactory epithelium, which is located at the roof of the nasal cavity. All axons of neurons expressing the same receptor then converge onto the same two spatial locations in the olfactory bulb, yielding organization patterns, which are laid out in a chemotopic fashion [39.81–83]. Similarity between the bulbar patterns evoked by odorants has been linked to the experience of perceptual similarity [39.84, 85], and thus represents a crucial early stage for the representation of separable sensory experiences with distinct affective qualities. Importantly, behaviorally relevant stimulus knowledge formed during reward learning or aversive conditioning can lead to an enhanced separability between previously similar activation patterns of odors already at this early processing stage, as a result of lateral inhibition within the bulb [39.86–88], as well as a modification of receptor firing patterns. Early chemotopic odor representations are thus never, strictly

speaking, camera-like depictions of our chemosensory environment. Rather, they reach cortical and subcortical stages of processing as a selectively sparsened or enhanced code, which reflects the evolutionary significance of the stimulus at hand and thus contains information relevant for subsequent valence assignments.

Monosynaptic projections transmit olfactory signals directly from the olfactory bulb to the amygdala and hippocampus [39.89, 90], a unique anatomical feature of the olfactory system that is considered of key relevance for the speed and automaticity of odor valence assignments. This *low-road* pathway, which lacks the obligatory thalamic relay that gates information from other modalities before entering subcortical nodes implicated in affective processing [39.91], allows olfactory information to directly interface with memory and emotional processes in the absence of cognitive modulation, after it has passed the initial processing steps in the periphery.

Several studies have probed amygdala involvement in the affective experience of odors by separating activity changes to independent manipulations of odor valence and intensity. *Anderson* et al. [39.92] were the first to compare blood-oxygen-level-dependent (BOLD) signal changes measured with functional magnetic resonance imaging (fMRI) to a pleasant and an unpleasant olfactory stimulus, and found that both elicited increased activation in the amygdala, while separable patterns by stimulus valence were found in the orbitofrontal cortex (OFC). Importantly, an extension of this work by *Winston* and *colleagues* [39.93] demonstrated that the amygdala is not unresponsive to the emotional value of odor stimuli, but rather takes it into account in a more complex interactive manner; namely, by including an additional neutrally valenced odor, Winston et al. could demonstrate that the strength of the amygdala response increased with intensity for odors of affective relevance, as demonstrated by Anderson et al., but in contrast, remained unresponsive to intensity changes when the odor was emotionally neutral. The amygdala activation to olfactory stimuli, therefore, likely represents a merged representation of intensity – and valence information as an overall index for the emotional value of the percept. This pattern of amygdala reactivity ties in with current theories of its role in emotion perception postulated on the basis of research using visual stimulus material, which has equally demonstrated that experiences of both positively and negatively valenced stimuli can activate this region [39.94]. While earlier models have postulated a specific role of the amygdala in fear perception, it is now thought that it may respond more generally to valence-unspecific detection of saliency in terms of arousal and biological significance [39.95]. In the absence of cognitive control processes, automatic amygdala activation to odors may actually bias toward negative affective responses to presented stimuli, and thus play a crucial role in the above postulated odor-based threat-detection mechanism [39.12]. On the other hand, when an object has been identified as emotionally arousing, but cognitively evaluated as nonthreatening [39.96], such automatic amygdala responses are thought to be counteracted by cognitive reappraisal processes. These processes downregulate the elicitation of what, in this case, would constitute an unnecessary and resource-consuming avoidance response. In line with this idea, anxiety induction, which predisposes individuals to vigilance toward potential threats, has been demonstrated to induce shifts in olfactory network connectivity, creating a stronger bias toward amygdalar influences on cortical odor processing [39.97].

The elicitation of aversive reactions to odors is often accompanied by visceral and autonomic nervous responses, which go beyond a mere detection of threat and likely regulate the sensation of disgust or revulsion and an impulse for behavioral withdrawal [39.98]. A number of studies have demonstrated that such sensations toward odors arise from ventral anterior insular cortex, in addition to amygdala activation [39.99–101]. As a supramodal area forming a core part of all chemosensory pathways including smell, taste, and chemical irritation, this region has also been connected to the recognition of disgusted facial expressions [39.102, 103]. Studies incorporating both visual impressions of disgust and negative olfactory stimulus material [39.2, 104] have provided evidence that disgusting stimulus material shares a supramodal common neural substrate in the insula and have as such strengthened the idea of the insula as a key node in a *behavioral immune system* [39.105], which allows individuals to automatically engage in disease prevention behaviors, and, as such, should be considered to form part of an extended network for valence representation of olfactory stimuli.

39.3.2 Role of Cortical Object Processing for Odor Valence Perception

The subcortical pathways for odor valence perception are thought to permit a quick and highly automatized, almost reflex-like response to odor valence and chemical threats. Cortical encoding of odor attributes, on the other hand, underlies the emergence of affective qualities in a *high road* of affective evaluation, which permits the representation of complex emotions linked to the identity of the stimulus. Giving rise to learned representations of odor objects, prefrontal cortex is thought to provide the neural basis for the pleasant or unpleasant

feelings elicited by odors that we consciously experience as their *valence*.

As described in Chap. 38, the cortical mechanisms that transform the complex chemotopic bulbar codes into holistic identity and experience-based odor object representations with a distinct valence, have been the subject of intense research efforts over the last decade. A particular focus has hereby been placed on the role of the piriform cortex, as well as its tight reciprocal connections with the olfactory portion of the orbitofrontal cortex (OFC).

Receiving by far the largest portion of neural projections from the olfactory bulb, the piriform cortex is frequently labeled as primary olfactory cortex and is separated into an anterior portion, located in the frontal lobe, and a posterior portion, which forms part of the temporal lobe. The main secondary processing area of the olfactory system, human olfactory OFC, has reliably been placed rostrally to an analogous area in nonhuman primates, and is located at the intersection of the medial and transverse orbital sulci and bordered by visual, gustatory, and somatosensory areas [39.1, 106].

Evidence from the animal literature indicates that piriform cortex transforms the chemotopy of the peripheral olfactory signal into dispersed activation patterns [39.107, 108], which help to create a representation of an odor's behavioral relevance [39.109]. The presence of an analogous mechanism in humans is supported by functional neuroimaging studies: pattern analyses of MRI signal to odors in piriform cortex show that odors are grouped into similar activity patterns depending on their perceived quality or behavioral relevance in the external world [39.110]. This grouping pattern occurs even for odors that are substantially different in chemical structure, indicating additional processing steps beyond the chemotopic coding patterns of the olfactory bulb, which shift perceptual processing toward a representation of a perceived object identity.

Both the anterior portion of piriform cortex and the olfactory projection area of OFC have consistently been shown to be modulated by odor-valence characteristics [39.73, 111, 112]. Strongly connected with each other through reciprocal connections [39.111, 113], these areas are thought to form a core network for behavioral relevance assessment of olfactory stimulus properties. Most robustly, patterns of differential activation for pleasant and unpleasant stimuli (with higher activity levels tending to correspond to higher levels of aversiveness) [39.111, 114–116] have been reported in olfactory OFC, an area which has been implicated by lesion studies [39.74] and fMRI studies [39.117] to be essential for the emergence of odor representations into consciousness. These findings suggest, despite evidence for early sensory modulations by perceived valence in the periphery, that this region may play a necessary role in the emergence of affective properties into conscious perception.

Within OFC, some reports favor an anatomical separation between subsections that respond to positive and negative valence, with mediofrontal sections coding for stimulus reward, and dorsolateral portions preferentially responding to aversiveness [39.114]. To investigate the relevance of these spatially separable subsections for the formation of the final odor percept, *Grabenhorst* and *colleagues* [39.118] presented subjects with odors that were purely pleasant and purely unpleasant, as well as with a mixture of the unpleasant and pleasant odorant. By correlating BOLD signals with subjective ratings of pleasantness and unpleasantness, they were able to not only separate subsections of the OFC preferentially responding to pleasantness and unpleasantness, but also to show that the mixed odorant, while perceptually rated as pleasant, activated both subsections. The finding that pleasant and unpleasant valence associations can be *simultaneously* represented in higher order cortical areas potentially has important implications for top-down controlled changes in valence attribution: the latter might bias the conscious percept toward a preferential weighting of one or the other depending on the context in which the stimulus is perceived.

Grabenhorst and *Rolls* [39.119] further suggested a separation between continuous representations of stimulus value in the OFC and binary approach/avoidance decisions in the dorsolateral prefrontal cortex. This work ties in with the economic decision-making literature's view on prefrontal functions, where it is thought to serve an important role in the translation between reward encoding on the one hand and approach-avoidance decisions on the other [39.120]. Reflecting these influences, models of orbitofrontal involvement in olfactory perception have recently been extended beyond the notion of a linear representation of valence as a fixed property: rather, the emergence of a sensation of like or dislike is increasingly viewed as the result of evaluations of *actual* against *predicted* outcomes that are subsequently combined to signal reward value (a better outcome than predicted, rendering a positive valence assignment) or punishment (a worse outcome than predicted, rendering a negative valence assignment).

While studies investigating these questions in the context of flexible adjustments of valence perception are still lacking, recent work by *Zelano* and *colleagues* [39.121] has looked at the encoding of actual versus expected odor outcomes in the OFC, and thus provides a neural basis for potential encoding mechanisms of subjective value in olfactory cortical

networks. The authors tested the representation of real and predicted outcomes in the olfactory system by comparing neural responses to two odorants while paying attention to either one or the other (while the perceptual experience was identical, odor A would sometimes be represented as *YES, odor A*, and at other times as *NO, not odor B*). This modulation resulted in significant differences in activation between the two conditions within OFC and its projection area in the anterior piriform cortex. The neural response in both areas was strongly influenced by whether or not the sensory event matched the predicted event, representing the expected rather than the actual stimulus, and thus providing support for the idea of odor evaluation in the OFC as an inherently comparative task. In addition, recent work by *Rudebeck* and *colleagues* [39.122] has been able to demonstrate OFC involvement in olfactory stimulus–outcome expectancies more directly in a monkey model by means of single neuron recording, yielding first evidence that flexible OFC responses may contribute to reevaluations of odor stimuli dependent on current motivational states.

While further research is needed to form a unifying model of OFC function in olfactory valence perception, these findings demonstrate that valence representation in olfactory cortex is likely a multidimensional concept, rather than a clearly localized, linear representation of an abstract reward value. The OFC, due to its position at the intersection of various sensory systems, cognitive, and affective networks, appears to form an important relay function in this emergence of a unified perceptual experience. Along with a representation of valence in the form of a motivational value of a real or expected outcome, OFC maintains the flexibility to adjust this representation to varying requirements imposed by changes in internal and external states. Likely, these states include learned contingencies with positive and negative outcomes as well as multisensory influences, such as associations with positive primary rewards as is the case of sugar and salt taste; as such, they are likely contributors to some of the biggest public health challenges of the 21st century and deserve increased research efforts.

A reformulation of the role of OFC and anterior piriform cortex in valence perception as regions integrating the value of *real* in relation to *expected* outcomes also presents an opportunity for a reevaluation of the role of posterior piriform cortex in valence coding, which, despite its crucial involvement in the formation of identity-based perceptual quality, odor learning, and memory [39.110, 123], has traditionally been assumed irrelevant for valence encoding. The aforementioned study by *Zelano* and *colleagues* [39.121] directly compared response patterns between the two piriform subsections in addition to OFC activity and demonstrated that posterior piriform cortex maintained a state-independent representation of the stimulus while anterior piriform cortex activity was modulated by expected outcomes. In light of this functional separation, a model of cortical valence representation completely disregarding the posterior piriform cortex' role in pleasantness perception may fall short of providing a complete picture: a *high road of valence perception* relies on the integration of expected value on the one hand, and identification of perceptual evidence on the other. It could, therefore, be speculated that posterior piriform cortex, at the intersection of emotional and memory networks, might constitute an important relay for linking perceptual input to object-specific odor memories, which carry information on behavioral relevance [39.113]. In other words, posterior piriform cortex may play a key role in the retrieval of olfactory memories and the evaluation of these memories against expectations generated by present mental states constitute a crucial subprocess for affective valence perception. Future work will show whether such a model provides a reasonable approximation of the cortical processes that give rise to odor valence representations. In addition, the role of additional cortical support structures that might exert influences on olfactory valence encoding through multisensory influences, including, among others, the olfactory tubercle [39.124], and the entorhinal cortex [39.125, 126], needs to be defined.

39.4 Conclusion

As reviewed above, valence perception, or the ability to perceive olfactory input as pleasant or unpleasant, is not only one of the most basic and intuitive features of the perceptual experience of odors; this emotional experience is also fundamental to the most common interactions with our chemosensory environment, including the rapid rejection of contaminated materials or the elicitation of appetite by familiar foods. As such, valence perception of odors is a mechanism that contributes greatly to the maintenance of human health.

Given this immediacy and apparent one-dimensionality of odor valence perception, the complexity of neural processes contributing to its emergence and our continued search for answers to some of even the most basic questions, such as what makes an odor pleasant, remain

puzzling. Yet, as we review here, immense progress has been made over the last years, tackling the manifestation of emotional responses to odors from all possible angles, ranging from behavioral studies in humans and animals, through physiological and cortical responses, down to the level of early receptor coding patterns. Recent attempts to integrate these different levels of analysis begin to show a complex system, which, at high speed, captures not only the chemical composition of a stimulus at hand, but at the same time also attempts to identify possible sources, evaluates their potential behavioral relevance, and elicits the appropriate visceral response that, in the case of a negative evaluation, is often crucial to prevent a contaminated food item from entering our digestive system.

Taken together, the research reviewed above shows that odor valence coding in the human brain is a multilevel process involving early peripheral modulation as well as subcortical mechanisms for rapid behavioral responses and conscious cognitive evaluations. Given the growing public health issues that arise from dysfunctional approach and avoidance behavior to chemosensory stimuli, in particular during food consumption, understanding the molecular and neural mechanisms that cause some odors to spontaneously make our mouth water, while others make our stomach turn in disgust, will likely prove to be crucial for the development of strategies to counteract such dysfunctional valence attributions, and, as such, deserve our continued research efforts.

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40. Odor and Emotion

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This chapter advocates for adopting a theoretical and experimental approach that goes beyond the use of valence as the most interesting dimension in emotional reaction to odors.

Although valence is a dominant dimension of odor perception, limiting the description of emotional response to positive versus negative (valence) and activating versus calming (arousal) feelings is perhaps oversimplified and not well suited for a comprehensive view of odor-related effects. Just as inappropriate are basic emotions, usually defined as six states (fear, anger, sadness, surprise, joy or happiness, and disgust) putatively characterized by specific neural, physiological, expressive, and feeling components. Here, we present an appraisal approach of emotions as a plausible alternative. This kind of approach reconciles a priori incompatible characteristics observed in odor perception like the immutability and the flexibility of chemosensory preferences. After having exemplified this aspect with several studies from the recent literature, we will particularly emphasize feelings. We provide an empirical demonstration that feelings are broader than valence and both stable and variable across cultures. We argue that this approach provides an ecological model of the emotion process where olfactory emotions are understood considering their functional role, which is to adjust or to solve olfactory-linked survival-relevant problems.

Across cultures, odors have always been considered powerful elicitors of emotions [40.1, 2]. In the last few decades, a growing scientific literature has started to provide evidence for this claim [40.3–5], and a series of findings even speak in favor of a privileged link between odors and emotion as compared to other senses. For instance, it has been reliably shown that experiencing an odor as pleasant or unpleasant (its hedonic tone) is a key aspect of olfactory experience [40.6]. Accordingly, smells can influence mood – pleasant odors tend to induce positive moods while unpleasant odors tend to induce negative moods [40.7–9]. Olfactory-induced changes in physiological parameters, such as heart rate or skin conductance, correspond to typical emotional reactions elicited by emotional stimuli [40.10–19]. Results from numerous experiments suggest that odors can impact cognition and behavior in a similar fashion than do emotional stimuli in other perceptual modalities [40.20–26]. Such an effect is particularly studied on memory: odors can evoke autobiographical memories that are emotionally intense and thought to be forgotten [40.27]. These effects are usually interpreted as a interdependence of olfaction and emotion in overlapping neural systems [40.28]. Consistently, recent neuroimaging data has demonstrated that both the brain network processing olfactory stimuli and the circuitry processing subjective hedonic evaluations are modulated in a similar fashion after an anxiety induction [40.29].

In this chapter, after having proposed a definition of emotion, we aim at presenting an overview of the two dominant psychological approaches to emotion – namely basic emotion theories and dimensional theories – as they have been used as the major research frameworks when investigating the links between olfaction and emotion. We will underline the strengths and limitations of these two approaches and, then, we

will introduce an appraisal model of emotions that could be used as an alternative model to investigate odor-elicited emotions. These theories reflect a third approach in emotion research that has only been scarcely used as a framework in olfaction research. However, this approach deserves much more consideration, as we believe that it has the potential to lead to significant improvements in olfaction research, for reasons we will describe.

40.1 Emotion as a Multicomponential Phenomenon

Across almost all theoretical approaches of emotion, there is a clear consensus: emotion constitutes a multicomponential phenomenon.

The term emotion is usually defined as a short period of time during which several functionally defined components are coupled or synchronized to produce an adaptive reaction to an event that is considered central to the individual's well-being [40.30–32]. For instance, according to the *component process model* [40.33], these components are:

1. The cognitive system that is responsible for the evaluation of the situation and the determination of emotion.
2. The autonomic system that is in charge of the organism' regulation and physiological support.
3. The motor system that is responsible for the expressive aspects, for the communication of reactions and behavioral intentions.
4. The motivational system that is responsible for the preparation and direction of actions (approach/avoidance).
5. The monitoring system, in charge of the subjective feeling.

Similar to the emotion literature in general, experiments in the subfield of emotion and olfaction have focused on one or two of these components (subjective feeling and/or physiological response), mainly for practical reasons. Thus, it is important to keep in mind that the differentiation of emotions on the basis of only one of these components (hedonic ratings) may not adequately reflect the complexity of the phenomenon. For instance, any putative emotional response obtained at the subjective level needs to be associated with concomitant responses on a cognitive, behavioral, or physiological level to be fully considered as a true emotional response (see [40.34] for a discussion on this topic). Adopting methods derived from the multicomponential approach (see description below) that consists of measuring simultaneously behavioral, physiological, cerebral, and subjective reactions to the odors is consequently an extremely valuable avenue of research.

Although there is widespread agreement on the multicomponential nature of emotion, the question of its theoretical and mechanistic underpinnings lacks such consensus. A majority of studies that have investigated the relationship between olfaction and emotion have been conducted within the framework of one of the two major approaches in emotion research: basic emotion theories and dimensional models.

40.2 Basic Emotions

The basic emotions theory postulates the existence of a small number of so-called *basic* emotions, such as anger, disgust, fear, enjoyment/happiness, sadness, and surprise [40.35]. Basic emotions are supposed to share a common set of characteristics that consist of specific neural, bodily, expressive, and feeling components [40.35, 36].

In the olfactory literature, there is no consensus regarding the number of basic emotions elicited by odors. When authors ascribe to the strict definition of basic emotions as described above, their smallest number appears to be six (anger, disgust, fear, sadness, surprise, happiness) [40.10, 37]. Broader definitions can lead to the enumeration of as many as 22 distinct basic emotions (shame, jealousy, fear, anger, sadness, pride, hope, relief, boredom, contempt, admiration, disgust, desire, disappointment, love, dissatisfaction, amusement, stimulation, satisfaction, unpleasant surprise, enjoyment, and pleasant surprise) [40.38, 39].

According to the tenets of this approach, basic emotions are based on phylogenetically stable neuromotor programs and are characterized by emotion-specific response patterns [40.40–42]. In this framework, a perceived event will be automatically evaluated via what is essentially a database lookup. A positive match with a specific stored scheme will automatically trigger a prototypical affect program that includes a specific action tendency, physiological response pattern, motor

expression, and feeling state. This evaluation process or appraisal is characterized as *immediate, unbidden, opaque, unconscious, and automatic* [40.35, p. 70]).

Olfaction studies that adopt the framework of basic emotion theories focus on response patterning, that is, trying to determine whether specific odors elicit specific basic emotions with a specific set of physiological responses [40.10, 11, 19, 37, 43, 44]. To the best of our knowledge, this research trend never explored the causal mechanisms underlying differences in emotion elicitation. Thus, in accordance with this theory, *the perception of the smell of feces may match the scheme of contamination in the emotion schema database, triggering the emotion of disgust* [40.35, p. 70].

However, many studies have underlined that emotional responses, states, or descriptions in response to odors do not match basic emotions categories, such as anger, fear, or sadness [40.1, 2, 10, 11, 14, 19, 38, 39, 45–50]. It is also difficult to reconcile the assumption that basic emotion systems are impermeable to modification by learning and throughout lifespan [40.35] with the profound hedonic plasticity observed empirically. The hedonic perception of an odor changes as a function of associative learning contexts [40.51], in perceived intensity [40.52], and familiarity [40.15, 53]. It is also modulated by the perceiver's physiological and psychological states and the context of perception. For example, verbal labels can greatly affect the hedonic perception of ambiguous smells, such as cheese versus smelly feet [40.54]. Olfactory preferences are also modulated by individual needs, goals, values, and decision-making processes [40.55]. In sum, a large set of empirical data demonstrates that the emotional perception of odors is far from being fixed, rendering difficult any interpretation in terms of basic emotions elicitation based on a fixed look-up database.

Even in instances where basic emotions would be reliably triggered by olfactory cues, we still believe that the basic emotion account may paint an incomplete picture. In particular, the context in which the odor induces the reaction is rarely accounted for. This may be problematic if the selection of basic emotions depends on other environmental cues and not on the odor itself [40.38]. Yet, in studies on the relationship between olfaction and emotion, specific situations are not usually provided to the participants when assessing their feelings or physiological reactions in response to odors. The possibility that basic emotions require an appropriate – possibly motivational or social – context to be elicited via odors remains to be tested. However, if the odor presentation is contextualized, it cannot then be ruled out that the emotion is also caused by situational factors rather than by the odor alone. If the situational factors play a role, then this would question the uniqueness of the mapping from odors to basic emotions that would be claimed by the theory. Future research may shed light on these questions by manipulating the context of the emergence of odor-elicited emotions. For instance, a crucial question would be to investigate to what extent specific patterns of physiological and subjective responses are influenced by the manipulation of the context of delivery.

A recent trend in chemosensory research – the investigation of human communication skills via social emotional chemosignals [40.56] – is also permeated with concepts derived from basic emotion theories. This line of research evolves around the following question: Are humans able to express affective information via body odors? If so, what kind of affective information is conveyed by body odors: specific emotions or valence? While many of those studies are interested in anxiety or stress-related chemosignals that could be emitted [40.57–63], others have proposed that more specific basic-emotions-related chemosignals may be linked to induced emotions of happiness, fear, sadness, and disgust [40.64–68]. Globally, this approach suggests that certain basic emotions felt by the emitter are associated with the emission of specific chemosignals, which can in turn be perceived by recipients. In this emerging field, an alternative way of thinking could be rather to question which emotional component is conveyed by the signal rather than if the signal corresponds to a particular basic emotion. For instance, do emotion-related chemosignals reflect a feeling (i. e., disgust or joy) or any action tendency (approach or avoid) experienced by the emitter of the signal? Does the receiver expresses the same feeling or action tendency as the emitter (i. e., mimicry and/or emotional contagion) or any complementary aspect to adapt to the situation?

40.3 Dimensional Approaches

Dimensional approaches assume that all affective phenomena are essentially described via positions in a two-dimensional valence-by-arousal space, or sometimes a three-dimensional space that includes an additional dimension of dominance or potency [40.69–74]. Authors embracing this approach in olfaction characterize individual feelings mainly using a pleasure, arousal, and dominance (PAD) questionnaire. In this case, the

affective terms are chosen to characterize the extremes of the underlying two or three bipolar dimensions (happy/unhappy, excited/calm, powerful/without power [40.12–14, 17, 18, 20, 75–80].

This model is the one of most frequently used in olfaction research because pleasantness is one of the major if not the primary perceptual dimensions of odors [40.6, 81]. Moreover, many studies emphasize the relation between the intensity of the odor and the subjective and/or physiological arousal. Whether the intensity and arousal dimensions of odor perception are independent or not is still debated (see [40.28] for a recent discussion on this topic). Historically, arousal was defined as a short term increase in excitatory processes resulting in an increase in behavior or physiological activity, mainly linked to the activity of the sympathetic nervous system [40.82, 83]. This definition emphasizes the physiological aspects of the arousal dimension [40.84]. Congruently, olfaction research using dimensional models has investigated physiological differences associated with verbally reported pleasantness and arousal produced by an odor [40.12, 13], as well as the underlying brain structures associated with these dimensions [40.28].

For experimental applications, adopting dimensional approaches has practical appeal, as reporting an emotion on scales of pleasantness and arousal is easy to implement, and allows using continuous measures for data analysis.

But what does this approach tell us about the elicitation and differentiation of the resulting feelings of valence and arousal? Unfortunately, dimensional theory remains largely agnostic about the processes underlying emotion elicitation and differentiation in general [40.85]. Research in olfaction research is no exception. According to the tenets of dimensional theory, the perception of an event (either an actual or an imagined odor) results in a primitive core affect, constituted by valence and arousal, on the basis of which the participant will construct the emotional meaning. The resulting feeling is dependent on both situational and sociocultural factors. However, the theory does not propose specific production mechanisms for the genesis of the core affect.

Some of the work in olfaction postulates that hedonic/valence coding may be (at least partially) innate in nature, the probability of an odor being pleasant or unpleasant being dependent of its physico-chemical properties (molecular complexity; [40.86]). These properties can allow an artificial nose to categorize the odors according to their pleasantness with high accuracy [40.87]. These accounts suggest a potential direct mechanism for valence determination in core affect elicitation. However, this does not account for the high variability observed in odor valence perception, except if one accepts the highly flexible nature of this pre hardwired mechanism. Imaging techniques have revealed differential valence activations at the early stages of odor processing in humans (in the anterior piriform cortex [40.28]. However, neuroimaging techniques do not have the fine grained temporal resolution that would be required to tease apart whether these differentiations result from a bottom-up processing of valence or top-down influences from high-order structures.

In sum, to date, most of the work in olfaction that investigates the neural and cognitive mechanisms leading to odor-elicited emotions adopts dimensional approaches. Unfortunately, these approaches do not propose clear elicitation mechanisms for valence or arousal determination.

40.4 Beyond Valence: The Case of Feelings

In any experiment on the relationship between emotion and olfaction, the measurement of the monitoring component of emotion, the subjective affective experience or feeling is constrained by the choice of the theoretical model adopted by the researchers. In most cases, participants are requested to characterize their perception of the hedonicity/valence of the odors using a visual analogue scale varying from unpleasant/dislike/negative to pleasant/like/positive. Recently, several results have seriously questioned the theoretical grounding for restricting the description of feelings to the unique scale of valence, liking, pleasantness, or acceptability [40.1, 2, 7, 39, 45, 48, 80, 88]. The underlying assumption of these studies is not to question the valence as a fundamental aspect of odor perception. It is rather argued that describing odor-elicited feelings with one dimension of valence (or two dimensions of valence and arousal or tri-dimensions of valence by arousal by dominance) loses most of the important qualitative differences between the affective effects of different types of odors. Even if every feeling could be positioned in an uni-, bi-, or tri-dimensional affective space – as feelings associated with basic emotions can be – the use of these simple representations may not be sufficient to answer relevant questions related to olfaction [40.47]. This point was particularly emphasized by *Rétiveau* et al. [40.7]. *Rétiveau* et al. observed that some fra-

grances that were similarly evaluated in a valence by activation space were clearly differentiated when participants evaluated them with several mood adjectives [40.50].

This is why research aiming at establishing a systematic, empirically derived taxonomy of olfactory-induced feelings was developed. For instance, acknowledging the strong influence of culture on hedonic perception of odor, a series of studies were conducted in six different countries (French-speaking part of Switzerland [40.45]; United Kingdom and Singapore [40.48]; USA, China, and Brazil [40.2]). The two main objectives of this project were: (1) the identification of cultural invariants and differences in odor-elicited feelings and, (2) the investigation of whether these feelings are well predicted by the dimensional and basic emotions theories. In each country, a list of terms selected for their relevance to describe affective feelings induced by odors was assessed while participants were exposed to a set of odorant samples. The data was submitted to a series of exploratory and confirmatory factor analyses to reduce the set of variables to a smaller set of summary scales and to get a sense of the differentiation of affective feelings elicited by odors. The originality of the methodology adopted in each country was threefold [40.45]:

1. The initial list of affective terms included (among others) the terms of the different versions of the pleasure/arousal/dominance questionnaire and the most exhaustive list of terms derived from the basic emotions approach applied to olfaction.
2. The choice of the relevant feelings terms was based on a data-driven approach derived from respondents' evaluations; the authors did not impose a strong theoretical framework in the selection of the relevant terms.
3. The selected odorants covered a large range of everyday odors that tend to reflect different odor-related contexts (56 different odorants: sweet aroma, savory aroma, cosmetic-household odors, woody-earthy odors, fruity odors, floral odors, spicy odors, animal odors, and medicinal odors).

These odorants were complex mixtures, not monomolecular compounds, not specifically selected for being extreme in valence but rather to be familiar and evoke as many associations as possible. In this respect, this line of research is not subject to the issue underlined by *Mohanty* and *Gottfried* [40.28], which is why a lot of studies could have favored the dimensional models since the stimuli they used were sampled as extremes in the valence scale.

The resulting feeling landscape that emerges from these studies [40.1] is represented by several affective categories that were recurrent in all the countries examined: disgust/irritation, happiness/well-being, sensuality/desire, energy, but also soothing/peacefulness and hunger/thirst. Practically, a new set of scales was created and freely distributed (emotion and odor scales: EOS [40.89]). Each category of feelings is characterized by three representative relevant affective terms and is evaluated with the help of a feeling intensity scale varying from *not experienced* or *not intense at all* to *strongly experienced* or *intense* [40.45, 50]. It is worth mentioning that similarities and differences in odor-elicited feelings are influenced by the geographic proximity (the closer the countries, the more similar the feelings elicited by a set of odors). In addition, culture-specific feeling categories also emerged [40.2]. These two last results reflect again the strong influence of cultural aspects on odor-elicited feelings. But the main result of this line of research was that the emotional categories observed in many different cultures go well beyond those described by the traditional basic emotions or dimensional approaches applied to olfaction.

This line of research also permitted a formal evaluation of which of these sets of terms (i. e., derived from basic emotions, dimensional, or EOS approaches) is optimally suited to verbally measure the feeling associated with odor perception. This point was statistically tested both during each step of the EOS constitution [40.45, Table 5] and in a supplementary validation study [40.47]. Psychometric approaches revealed that by using the valence by arousal (by dominance) dimensional approach, one misses crucial qualitative differences in odor-elicited feelings. For instance, *Porcherot* and colleagues [40.50] have demonstrated that fine fragrances that did not differ in valence were differentially evaluated in terms of sensuality/desire feelings [40.7, 80].

Research using verbally reported valence or more complex feelings should consequently acknowledge that it does not capture the whole emotional phenomenon (though this may not always be necessary for answering the question of interest). Researchers should carefully consider the sense in which the chosen affective terms represent or capture true emotions, and the usefulness of the chosen framework in the context of their research question. This is complicated by the fact that although there is a general consensus that emotions are multidimensional (i. e., emotions are composed of a subjective feeling, action tendencies, a physiological arousal, appraisal processes, and expressions), there is no generally accepted agreement on what an emotion exactly is [40.90]. Thus, when using a particular set of terms to characterize emotional reactions to odors, researchers can at present merely assert that they used *potential emotions*, as the

differentiation on the feeling level would need to be confirmed by differentiations on the cognitive, behavioral, or physiological level to be fully considered as true emotions [40.34].

40.5 From Traditional Models to Appraisals

Given the limitations of the two classical models of emotions described above, we would like to argue that an alternative theoretical model of emotion may be needed to better predict a wider range of olfactory phenomena. We suggest that the theoretical models of emotion based on appraisal theory [40.91] may be more appropriate to explain and predict – two major functions of a theory – the processes underlying emotion elicitation in general and through olfactory stimulation in our particular case.

The basic premise of appraisal theories is that the elicitation and the differentiation of emotions are determined by appraisals, which are continuous, recursive evaluations of events. These appraisals can occur at different levels of information processing, that is, sensory-motor, schematic, and conceptual [40.92] and they correspond to the cognitive component of emotions. Appraisals should not be seen exclusively as high level, and conscious conceptual processes, since they include automatic, low level and unconscious ones [40.93] and can be investigated by neuro-scientific techniques [40.16, 94–96].

Although there has been some mild controversy among theorists regarding the precise number of the classes of appraisals they believe to be most important in emotions elicitation and differentiation, these disagreements concern details, and are small in comparison to the wide overlap of commonly held conceptions. Appraisals are organized into major types or classes of information that an organism needs to process to adaptively react to a salient event [40.31, 33, 93]. These types of information are:

1. The relevance of the event (how relevant is this odor for me?).
2. The implication of the event (what are the implications or consequences of this odor being around and how do these affect my well-being and my immediate or long-term goals?).
3. The coping potential (how well can I cope with or adjust to the consequences of this event?).
4. The normative significance of the event (what is the significance of this odor with respect to my self-concept and to social norms and values?).

Each of these four classes of appraisals is itself more finely organized in subevaluations. For instance, the relevance evaluation includes the novelty detection, the intrinsic pleasantness, and goal/need relevance appraisals as subevaluations. A complete description of all evaluations and subevaluations in the different appraisal models is beyond the scope of this contribution (for a more complete account [40.85, 91–93]. Appraisal models propose that emotion elicitation and differentiation result from the temporal unfolding of these different evaluations, their resulting values and consequences. Once an evaluation is completed, the outcome changes the state of all other components of emotion. Moreover, changes produced by the result of a preceding evaluation are modified by a consequent evaluation. The unique combination of values for these appraisal criteria determines which specific emotion is elicited and with which intensity [40.33]. Recent work has directly investigated the unfolding of cognitive appraisals [40.95, 97] and their consequences on peripheral responses [40.16, 94]. Together, they revealed sequential effects of novelty, pleasantness, and goal congruency appraisals, in this order.

A recent review of neuroimaging research investigating the processing of major appraisals [40.96] underlines that many appraisal mechanisms (e.g., novelty detection, intrinsic pleasantness, or goal-relevance) have been the focus of intense empirical research in cognitive and affective neuroscience, but typically without links being made directly to emotion elicitation [40.84]. In line with a growing number of investigations [40.94, 95, 98–100], we would like to emphasize the utility of using appraisal theories as a framework of research. More particularly, an appraisal-based approach consists in experimentally manipulating different appraisal criteria (i. e., novelty, goal relevance, coping potential . . .) and measuring their efferent effects over time and the resulting outcomes on the different emotional components (e.g., measuring the reported feeling with questionnaires or the autonomic support with physiological indicators). By directly manipulating the putative processes underlying emotion elicitation and differentiation, this approach allows us to characterize more precisely which and to what extent any process is crucial in emotional response to odors [40.16].

Applied in olfaction research, appraisal models may also reconcile both the immutability and the flexibility of odors hedonics as reported in the literature [40.28, 55]. Let us consider relevance detection again. Organisms constantly scan their external and internal

environments for the occurrence of events (or the lack of expected events) requiring further information processing, and to eventually adopt an adaptive reaction. During this relevance detection process, the first subevaluation is related to novelty detection – any change in the ongoing flow of processed stimuli could require attention and demand further processing (novelty evaluation). In the second step, the organism evaluates, with the help of genetically fixed schemata or over learned associations, whether a stimulus event is likely to result in pleasure or pain (intrinsic pleasantness evaluation). What is described in the appraisal literature as *genetically fixed schemata* could refer to what is presented in the olfaction literature as the innate nature of hedonic coding – through the physico-chemical structure of the odorant molecule [40.86, 87]. In contrast, according to appraisal models, this intrinsic pleasantness could also be the product of over-learned associations, leaving the door widely open to influences due to learning, exposure, contextual, and cultural factors. As already mentioned, hedonic variability linked to these factors is a key characteristic in olfaction perception [40.28, 55].

Appraisal models have not been extensively used in olfaction research but we feel that, in contrast to traditional discrete emotion or dimensional theories, appraisal models could provide a highly differentiated and flexible framework that can explain both the elicitation and the reaction patterning in a dynamic perspective. Moreover, they could account for the changeability and high degree of qualitative differentiation of emotional experience, as well as individual differences in emotional reactions.

40.6 Functions of Emotions in Olfaction

Emotions are viewed as intelligent interfaces that mediate environmental input to adaptive output [40.101]. Emotions are thought to allow an adjustment or to solve survival-relevant problems, such as forming attachments, maintaining cooperative relations, or avoiding physical threats (for a review about the functions of emotions [40.102]. In this way, emotions motivate organisms to respond in an appropriate way. This view is well accepted, whatever the theoretical point of view adopted, from an evolutionary perspective adopted by the majority of the basic emotions theorists [40.35] to the constructionist viewpoints mostly adopted by the advocates of the dimensional theories [40.85].

As mentioned before, emotions motivate organisms to adapt their behaviors to the changing situations [40.103]. The most fundamental forms of these motivational states are the approach/avoidance tendencies [40.104]. These states are coherent with a bidimensional view of odor-elicited emotions for which the reported feeling of pleasantness is assumed to be associated with approach tendencies. Conversely, unpleasantness is assumed to be associated with withdrawal tendencies. However, motivational states can be associated with much more action tendencies than approach or avoidance solely [40.105]. They encompass various intended behavior including freezing, attack, nurturance, or exploration among many others [40.105]. Odour-elicited feelings depicted by more complex approaches (EOS, [40.1]) could account for this variety in action tendencies. The categories of feelings (disgust/irritation, happiness/well-being, sensuality/desire, energy, soothing/peacefulness. . .) depicted by the EOS may represent the way respondents' feelings are related to the different functions of olfaction. We claim that experiencing these feelings will motivate the individual to adopt the optimally suited reaction to an odor's perception.

In a review on the functions of human olfaction, *Stevenson* [40.106, p. 3] defined three major classes of functions related *to ingestion (detection/identification prior to ingestion; detection of expectancy violations; appetite regulation; breast orientation and feeding), avoiding environmental hazards (fear related; disgust related), and social communication (reproductive [inbreeding avoidance, fitness detection in prospective mates]; emotional contagion [fear contagion, stress buffering])*. In this respect, most of the terms gathered under the EOS categories of feelings called *disgust-irritation* could reflect the unpleasant subjective affective experience associated with the detection of *expectancy violation* or *environmental hazards*, described as key functions of olfaction. These unpleasant feelings could, for instance, motivate a withdrawal behavior. The terms from the *well-being-happiness* category could reflect the feeling associated with the fulfilments of *expectancies* or *food intake*. We can also mention that the terms gathered in the *sensuality* category could reflect the feelings associated with many situations of *social communication*. The terms gathered in the *energizing-refreshing* and *soothing-peacefulness* categories could depict the feelings that motivate responses in relation to many functions of olfaction, such as being energized prior to ingestion to enhance the search for food, or feeling relaxed after smelling the known odor of a partner.

In summary, while adopting a valence by arousal space as a representation of feelings would not fully account for many of odor-elicited action tendencies, more complex representations of feelings as the one proposed by EOS constitute a better picture of the way individuals adjust to solve olfactory-linked survival-relevant problems, representing a key function of emotion [40.2, 45, 47].

40.7 Conclusion

In this contribution, we have introduced the major psychological theories of emotion and their current applications to olfactory research. This field is dominated by the two classical approaches corresponding to basic emotions theories and dimensional approaches. Despite their wide use in olfactory research, these classical models have difficulties to explain two main characteristics of emotional reactions to odors: (1) to account for fine-grained physiological, motor, motivational, cognitive response pattern, and feeling states due to intrinsic chemosensory quality differences between odors and (2) to account for the highly flexible nature of the response determined by the preferences, needs, goals of an individual at a particular point in time, and contextual and sociocultural conditions. In this chapter, we have tried to emphasize the need for grounding fundamental research activities in an ecologically and theoretically plausible model of the emotion process. We have introduced, as an alternative model in olfactory research, the appraisal model of emotion that possesses the potential to overcome the generally atheoretical stance of research in this area. This model requires specific experimental methodologies designed to better understand the olfactory emotion elicitation at different levels (unconscious processing, subjective feeling and verbalization) using a multicomponential approach (consisting in measuring behavioral, subjective, physiological, and brain reactions to these odors).

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41. Aversive Olfactory Conditioning

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The mammalian olfactory system is intertwined with emotional and memory centers of the brain, thus providing an ideal model to study olfactory-based fear conditioning, a behavior lying at the intersection of perception, emotion, and cognition. In the present chapter, we first outline a brief overview of the olfactory system's anatomy, and then, we define the structural and functional changes induced by aversive olfactory conditioning with a clear focus on rodent and human models. In detail, we discuss aversive experience-dependent modulations at each level of the olfactory pathway, differentiating between experimentally presented (shock) and naturally occurring aversive pairings (toxicosis). Whenever possible, developmental trajectories are reported. The description of aversive olfactory conditioning mechanisms are finally used to provide insights on psychiatric and medical conditions characterized by aversive odor memories which may open up future possibilities of developing novel treatment options.

> *But as present pleasures are tremendous reinforcers, and present pains tremendous inhibitors of whatever action leads to them, so the thoughts of pleasures and pains take rank amongst the thoughts which have most impulsive and inhibitive power. James* [41.1, pp. 550]

Valence – the affective evaluation centered on liking – is arguably the dominant dimension in olfactory perception [41.2, 3]. Although lately it has been suggested that odor valence is hard-coded into its chemical properties [41.4], perception of odor valence is commonly thought to be predominantly derived from a learned association with the emotional context in which an odorant is encountered [41.5–7]. The emotional context can be related to positive and negative experiences, and the ability to discriminate between the two is an adaptive trait that promotes reproductive fitness [41.8]. Indeed, organisms who correctly distinguish safe from threatening stimuli can more strategically guide perception and attention to the environment, and as a consequence, anticipate and escape harmful events [41.9].

Pavlovian conditioning is the prototypical form of all types of learning [41.10, p. 103], resulting *from exposure to relations among events in the environment. Such learning is a primary means by which the organism represents the structure of its world.* [41.11]. It is such a fundamental form of learning that it is expressed between species (from invertebrates to humans) as well as within species, in all individuals [41.12].

Aversive olfactory conditioning is a specific form of Pavlovian learning. It involves an unpleasant unconditioned stimulus (US), that produces a vigorous negative response irrespective of training (or unconditionally) and a neutral cue that acts as a conditioned stimulus (CS) [41.13]. The CS is a stimulus that at first induces only a minor orienting response. However, fol-

lowing contingent associations with the US (such that the CS predicts the occurrence of the US), the CS can acquire aversive properties itself and evoke an aversive conditioned response (CR). Imagine a rat wandering in his cage and suddenly receiving a foot shock. Without any previous exposure to this aversive stimulus, the rat freezes. Freezing, the behavioral counterpart of acute stress responses in many prey animals, is a typical measure for unconditioned responses (UR) in rodents. If the same rat is exposed to a stimulus neutral in valence, such as a colored light, an auditory tone, a tactile stimulation, a flavored solution, or a smell, no negative unconditional reaction (no freezing) is expected. However, if the presentation of the neutral stimulus – such as rose – is paired with the foot shock in such a way that a clear association can be established between the two events, the neutral stimulus will acquire aversive properties. In other words, after a certain number of pairings between rose and foot shocks, the sole presentation of the rose odor will trigger a freezing reaction in the rat. Thus, the rose odor has become a conditioned stimulus, a stimulus whose response has acquired an aversive power by the repeated pairing with the stressful event (foot shock). However, by virtue of their rich affective connotation, odors can additionally play the role of US. When odors are perceived as markedly unpleasant – such as, for instance, in the case of rotten eggs for humans – odors can act as an aversive stimulus.

Pavlovian aversive olfactory conditioning is a unique source of information for unveiling the rules and functions underlying sensory-mediated learning processes for several reasons. For example, olfaction is the most archaic sense, thought to be the earliest to appear during ontogeny and the oldest sensory informant from a phylogenetic perspective [41.14, 15]. In virtue of this, the olfactory sense has evolved to a complex and highly sensitive organ [41.16]. In all vertebrates, olfactory information is rapidly distributed to multiple central targets, which are confined to the anteromedial temporal and posterior orbitofrontal lobes rather than being widespread across the whole brain [41.17, 18]. In fact, the first central brain area that process odors is situated only one synapse away from the olfactory receptor body, a distinctively short pathway between the periphery and the central sensory brain [41.17]. Furthermore, olfactory information does not require a mandatory thalamic relay from the periphery to the cortex, as for our other sensory modalities [41.19]. In addition, even if ancient, olfaction can be considered the most *dynamic* modality. Neurons in the olfactory epithelium uniquely regenerate on a monthly basis [41.20] and experience-dependent morphological and functional changes in the adult olfactory system have been revealed at many different stages of the pathway [41.21, 22]. This flexibility is essential to sustain the highly complex and versatile representation of odors in the brain [41.22].

As reviewed in Chap. 38 as well as elsewhere [41.23], cerebral areas in the inferotemporal and frontal lobes are linked to lower and higher order emotional and memory processes and are profoundly related to odor processing. It will suffice to mention the famous Proustian effect – in which smells have the power to unleash a flood of emotional memories, present in the life of almost every normosmic person [41.24]. To add biological credence to the anecdotal connection between olfaction and emotions is the fact that neural representations of odors with different valence are separable in the olfactory structures also implicated in emotional processing [41.25]. The nature of this architectural feature, that again makes olfaction a special sense as compared with other modalities, raises interesting questions about the peculiarity of olfactory-mediated aversive learning.

Olfaction also offers an exclusive prenatal-postnatal sensory continuity, which allows for the development of adaptive behavioral and neural mechanisms in utero [41.26–28]. Prenatal olfactory learning favors adaptation to the postnatal environment and the development of the neural structures supporting that learning. Critically, within the early postnatal period when altricial neonates are entirely dependent on the mother for food, warmth and protection, odor learning is heavily biased to produce an attraction to the maternal odor, regardless of whether the mother causes the infants pain or not [41.29, 30]. Thus, at a crucial period during early development, aversive olfactory conditioning is suppressed to prevent infants from learning an aversion to an odor their life depends on.

Olfaction is further the only sense that allows the receiver to have a dual experience of a unique stimulus. On the one hand, when an odor is smelled orthonasally, both animals and humans are able to make sense of it at a relative distance from its source. In an aversive context, this distal feature of the system allows for the implementation of actions that more successfully will attain the goal of avoiding the threat. On the other hand, food odors can access the system through an additional route – the retronasal pathway, which is also characterized by an internal (or proximal) evaluation of the stimulus. Humans experience this retronasal smell as flavor. Perceptual as well as neural underpinnings of the two routes are not completely overlapping and therefore can contribute differently to olfactory learning mechanisms [41.31–33].

The functional role of olfaction is differently expressed in mammals at different levels of the phylogenetic scale. Although humans have been demonstrated

to outperform many nonhuman species in odor sensitivity [41.34], certain animals, such as rodents, depend heavily on olfactory information to navigate the world, whereas humans are considered to be less reliant on orthonasal olfactory cues [41.35], though may be expert at retronasal olfaction. A comparative perspective can offer the opportunity to study the impact of this aversive olfactory information in the full behavioral context and account for the variability that can specifically be found within species. As an example, animal models are critical for the definition of the molecular and physiological mechanisms of aversive olfactory conditioning, whereas humans offer the possibility to directly assess how the participants evaluate the nearby stimuli [41.3]. If on one hand, defining how mice pair the smell of banana with gastric malaise using single cell recordings can help us determine the neuronal mechanisms of aversive olfactory learning [41.36], it would prove fairly difficult to acquire a verbal report of the animal's preference of said odor. On the other hand, one can simply ask human participants to gain information about odor pleasantness; however, obtaining information from single neurons using the same gastric malaise paradigm as for the mouse would face rejection by most ethical committees.

From a theoretical standpoint, defining how specific olfactory memories are created and stored represents an opportunity to better define cognition in rodent models, which show impressive odor-based memory abilities [41.37]. From the clinical (human) perspective, aversive olfactory conditioning represents the key to unveiling mechanisms that promote and maintain certain pathologies, such as post-traumatic stress disorder (PTSD) [41.38, 39], multiple chemical sensitivity (MSC) [41.40] and pretreatment chemotherapy nausea [41.41]. Historically, the scientific community has primarily relied on animal models to uncover etiopathogenetic and maintenance mechanisms of fears and phobias. However, given that humans exhibit behavioral vulnerability to odors in certain instances, it is timely to validate the functional anatomy of human olfaction and olfactory memory to unveil how aversive olfactory conditioning contributes in humans to the etiology and the treatment of fear-related disorders [41.42], in which odors might play a critical role.

Considering the nature of the olfactory system's architecture and its close anatomical connections to emotional and memory brain centers, it seems to us that olfaction is the sense allowing for the most reductionist and naturalistic study of aversive conditioning processes. It is worth noting, however, that this observation should not be interpreted as derived from the principle of Occam's razor. As Occam stipulated, in the absence of certainty among competing options, the one with the fewest assumptions [aka the simplest] should be selected. However, complexity has its intrinsic value and alternatives with a greater number of assumptions may ultimately prove correct. Indeed, olfaction is a simple, yet not a trivial system. Instead, it is characterized by a level of complexity that needs to be valued and manipulated to extend the boundaries of our actual knowledge on this system, whose potential is still underappreciated. Aversive olfactory conditioning is particularly interesting because it allows us to join sensory, cognitive, and emotional information to provide an effective model to study where and how these pieces of information are encoded and, importantly, integrated in the brain.

To better account for this holistic view of olfactory aversive experience, in the next section we will first broadly sketch the anatomy of the olfactory system as well as known fear circuits in mammals; then we will review how each structure along the olfactory pathway alters the signal based on experience-dependent dynamic plasticity, according to functionally different types of aversive olfactory conditioning. As we will review, important aspects of this plastic system are age-dependent; therefore, for the sake of completeness, a developmental perspective will be included.

41.1 The Anatomy of Neural Circuits Involved in Mammalian Aversive Olfactory Conditioning

The functional and organizational structure of the olfactory system is conserved across the animal kingdom [41.43]. Whether this is the result of homology or it reflects an independent evolution due to similar constraints has yet to be clarified. However, the organizational and structural similarities are an opportunity to explore the neuronal processing of aversive olfactory conditioning between species. The goal of this section is not to provide a complete review of the neural systems involved in olfactory perception and aversive conditioning; rather, it is to outline the primary factors underlying olfactory-dependent aversive conditioning. For a more complete overview, please see Chap. 38).

As depicted in Fig. 41.1, olfactory perception is initiated in the periphery of the olfactory system with an interaction between volatile odorant molecules and

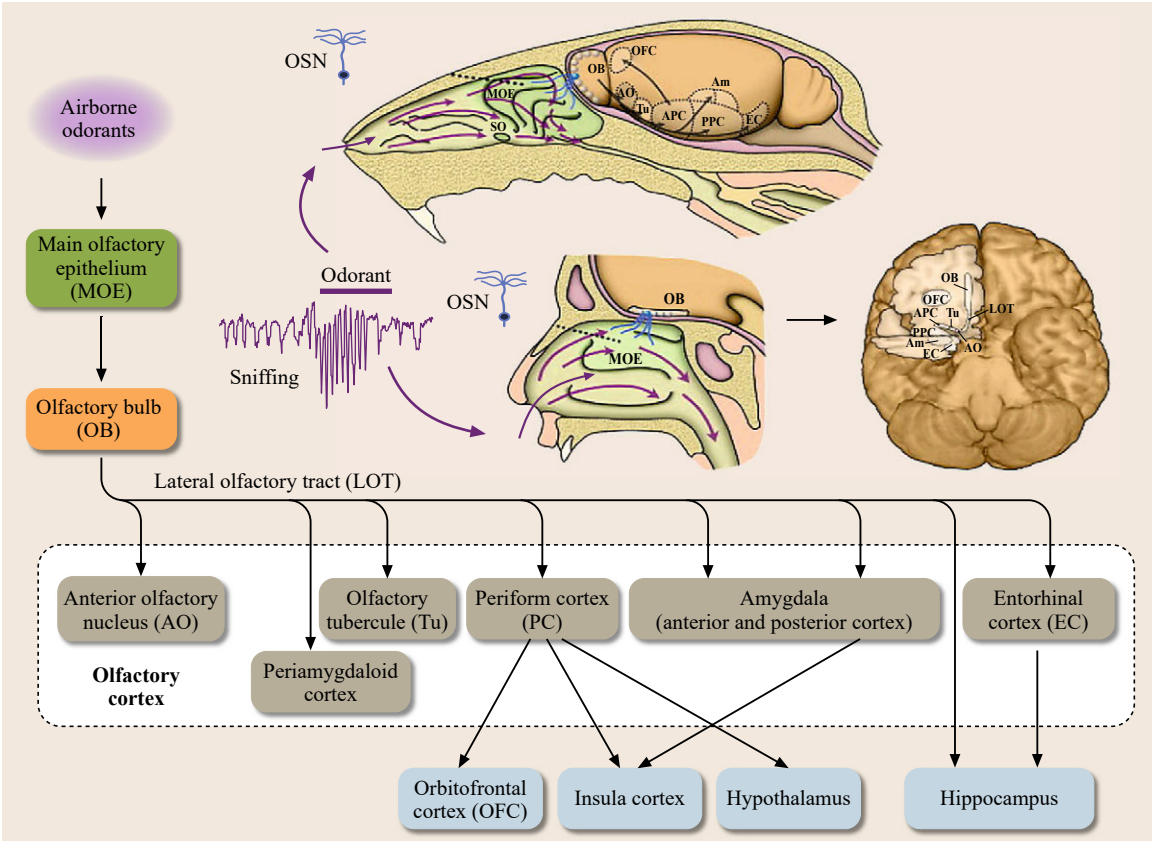

Fig. 41.1 Neural organization of the mammalian olfactory system. Sensory pathways in the main olfactory system of mammals are illustrated for mouse (*upper image*) and human (*lower images*). Volatile odorant molecules enter the nasal passages as a result of phasic sniffing, and interact with olfactory sensory neurons (OSNs) in the main olfactory epithelium (MOE). OSNs project into the olfactory bulb (OB). Discrete sets of glomeruli in the OB are targeted by the OSNs expressing the same receptor, forming odotopic spatial maps. Dozen of mitral/tufted (M/T) cells project from each glomerulus to the lateral olfactory tract (LOT), which distributes the information to the olfactory cortex. Major recipients of OB input include the piriform cortex (PC) with anterior (APC) and posterior (PPC) subdivisions, the olfactory tubercle (Tu), the anterior olfactory nucleus (AO), the hippocampus, the amygdala, the periamygdaloid cortex and the entorhinal cortex (EC). From here, odor information is routed to higher-order centers such as the orbitofrontal cortex (OFC), the agranular insula, and the hypothalamus. Adapted with permission from [41.44]

receptors expressed on the cilia of olfactory sensory neurons (OSNs) located in the main olfactory epithelium (MOE). Individual olfactory sensory neurons express a single olfactory receptor gene from the large family of olfactory receptor protein encoding genes. The axons of these bipolar neurons project to a single glomerulus, or module, situated in the olfactory bulb (OB). Each glomerulus is odorant-receptor specific in that it receives input from olfactory sensory neurons all expressing the same receptor gene. This creates an odotopic spatial organization in the OB, similar to what occurs in the visual or somatosensory systems [41.43, 44]. Dozens of mitral/tufted (M/T) cells constitute second-order projections that transmit via the lateral olfactory tract (LOT) information to the primary olfactory cortices. This group of cortices is dominated by the piriform cortex – structurally and functionally divided into anterior (APC) and posterior (PPC) regions – and it is complemented by the olfactory tubercle, anterior olfactory nucleus (AO), hippocampus, anterior and posterolaternal amygdala, periamygdaloid cortex, and entorhinal cortex (EC). Subsequently, the olfactory information is processed in other regions such as the orbitofrontal cortex (OFC), agranular insula, mediodorsal thalamus, and the hypothalamus [41.23, 45].

This system constitutes a powerful tool to model the functional neurocircuitry of fear. In fact, topograph-

ical representations of the olfactory sensory inputs exist only one synapse away from the amygdala, which is considered a key structure enabling aversive conditioning. The main olfactory bulb directly targets the cortical nucleus of the amygdala, which in turn targets the basolateral amygdala (BLA) [41.46]. The central nucleus of the amygdala (CeA) acts as an output regulator for fear responses, by targeting midbrain and brainstem structures that initiate and control the expression of fear or reactions to threat, such as freezing. This neural circuit is identified as the medial hypothalamic defensive circuit [41.47, 48]. Although it is not involved in cue fear conditioning per se, the hippocampus is a fundamental limbic structure involved in adult contextual aversive olfactory conditioning [41.49].

In the present chapter, we will consider only the structures critically contributing to the learning of representations processed in the context of aversive olfactory conditioning. For the sake of cross-species comparison, we assume that structural changes in the brain have functional correlates, whether behavioral or physiological.

41.2 Aversive Olfactory Conditioning–Induced Structural and Functional Plasticity

After repeated pairings with an aversive stimulus (US), a neutral stimulus (CS) develops an emotionally salient response (CR). Electrical shocks or nonlethal levels of toxins are commonly the stimuli that artificially or naturally induce aversive olfactory conditioning. However, in virtue of the hedonic trait of odors, unpleasant odors (and tastants) have been used as behaviorally salient US with unconditioned aversive properties [41.50]. Although few direct comparisons have been reported, the potency of the different aversive stimuli has been deemed equitable [41.25, 50]. However, provided that the acquisition of olfactory object perception is a prerequisite for aversive olfactory conditioning to occur [41.51], the involvement of different brain structures depends on the task used to elicit aversive reactions. We will therefore explore the neural plasticity shaped by the use of different aversive stimuli separately. To this end, we will initially focus on experimental (arbitrarily chosen) stimuli used in pairings or associations, which do not naturally occur in the ecological niche of an organism (electrical shock-odor coupling) with the goal of providing the basic theoretical and mechanistic principles of aversive olfactory conditioning. We will then focus on more ecologically relevant and naturally occurring phenomena of the odor-induced aversive conditioning (toxin effects odor coupling). For the sake of brevity and clarity, we will limit this review to studies using odors that are experimentally paired with aversive stimuli or unpleasant (aversive per se) odors and food-related biologically relevant stimuli (toxicosis). For odor aversion induced by predator chemosignals, please refer to the thorough review by *Staples* [41.52]. Since anatomical development is not always matched with functional development [41.53, 54], whenever possible, we will report how the mechanisms change across maturation to approximately define at which age a brain area is involved, in rodents and humans alike.

41.2.1 Somatosensory Stimulation US

Different noxious somatosensory stimuli have been used to induce threat associations with an odor (CS). In adult rodents, the use of strong tail pinches – whose limits rely on the inability of maintaining a constant stimulation over time and across subjects [41.55] – have been attempted. However, the by far most utilized stimuli in both rodent and human studies are electrical shocks of different intensities [41.56–59].

In the following sections, we will consider aversive olfactory conditioning-induced plasticity at different levels of the olfactory system, beginning from the periphery and moving towards the more central cerebral substrates.

Olfactory Sensory Neurons

The olfactory sensory neurons constitute the first order of synapses of the olfactory system and they represent the interface between the environment and the central nervous system. Experience-dependent structural plasticity has been revealed as early as at this level during the first phases of development [41.60] and during adulthood in mice [41.61, 62].

For example, mice trained in a fear conditioning paradigm with the odor acetophenone as the CS, an odorant specifically activating the M71 olfactory receptor (M71OR), demonstrated an increase in number of M71 specific sensory neurons within the olfactory epithelium and a related increase in size of the targeted glomeruli within the OB of adult mice [41.61]. This aversive learning plasticity does not appear in untrained mice, mice trained to a non-M71 activating odorant, or mice exposed to nonassociative pairings of

acetophenone, therefore confirming the link to the aversive experience. This hypothesis has also been tested with other odors and demonstrated that synaptic output of olfactory sensory neurons (OSNs) is modulated by odor-shock conditioning [41.62]. In other words, aversive learning-dependent modulation occurs already at the level of the receptor neuron.

Moreover, neonatal exposure to acetophenone in association with aversive olfactory conditioning promotes glomerular refinement, revealing not only experience-dependent structural changes but also modulation of the speed of glomerular development [41.60]. At present, it has not been conclusively settled whether the association between the CS+ and the US takes place in the epithelium or whether it is the result of a top-down link. As demonstrated by Kawai and colleagues [41.63], it is possible that an instantaneous increase in adrenaline in the olfactory epithelium (as a result of the US) might mediate an increase in receptor activity.

Although it is assumed that the human brain is able to similarly adapt its structure following aversive experiences, the technical challenges have so far prevented us from empirically testing this hypothesis, especially at such an initial level of the olfactory system. Nevertheless, it seems plausible that the regeneration of olfactory neurons throughout a lifetime [41.64] will support enhanced olfactory experience.

Olfactory Bulb

The axons projecting from the olfactory sensory neurons terminate within specific glomeruli in the OB, and synapse with M/T cells and juxtaglomerular interneurons [41.65–67]. Considering that each glomerulus represents input regarding a single odorant receptor, the glomerular and M/T layers of the OB constitute odorant receptor maps [41.68], which can be molded by experience. Studies conducted in rodents demonstrated that early in postnatal development, the repeated, daily exposure to odor-shock pairings induces fear responses in the pups. It also intensifies the CS+ odor-evoked brain activity as measured by an increase in focal uptake of glucose (precisely, 2-deoxy-D-glucose, 2-DG) in the OB glomerular layer, as well as changes in M/T cell responses [41.69]. In adult rats, even a single exposure to an odorant paired with shock will result in subsequent CS-evoked freezing and increased odor-evoked 2-DG uptake in OB glomeruli [41.70].

These aversive olfactory conditioning-induced changes in OB response to the CS may be mediated by long-term potentiation-like mechanisms within the olfactory bulb [41.71, 72], as well as learned changes in olfactory sensory neuron input [41.61, 62]. Importantly, the olfactory bulb also undergoes continued neurogenesis of inhibitory granule cells throughout life, and several studies have demonstrated that the incorporation of these newborn neurons into the OB circuit is shaped by odor experience and learning, and the inclusion of these new neurons significantly contributes to the odor memory [41.22, 73, 74]. This effect is true for both appetitive odor conditioning and aversive olfactory conditioning [41.75]. Although aversive conditioning does not reorganize the glomerular representation, mice exposed to the odor-shock pairings demonstrate an increase in the size of glomeruli within the specific odor representation [41.61, 76]. Moreover, the activation of glomeruli that were originally only weakly activated by the CS pre-training increased after conditioning [41.76]. Together, these data suggest that aversive olfactory conditioning, even in immature animals, helps tune the olfactory bulb to an increased and more specific representation of the CS odor. The tuned representation is expressed at the level of both glomerular layer spatial activity patterns and M/T output activity, and includes neuroanatomical changes such as local addition of granule cells.

At present, noninvasive neuroimaging techniques that can be applied in human studies do not allow for the characterization of the functional plasticity of the OB in an aversive olfactory conditioning paradigm. However, it seems plausible that humans do also show some sort of learning-dependent OB plasticity after aversive olfactory conditioning. This area deserves further study.

Piriform Cortex

Second-order projections bridge the information from the OB to the primary olfactory cortices, by means of the lateral olfactory tract (LOT) [41.77]. In contrast to the odor-specific spatial maps expressed in the OB, both connections in the olfactory cortex [41.78, 79] and odor-evoked activity are distributed in the piriform cortex [41.80–82], revealing the presence of diffuse projections and an internal system of long association fibers. In other words, as theorized by *Haberly* [41.83], the olfactory cortex acts as a *content-addressable memory* in which each site in the system contains information about the entire input. The anterior piriform cortex (APC) receives strong afferent inputs from M/T neurons and is hypothesized to play a primary sensory cortical role in olfaction [41.84–87], as well as having some functions in odor memory. For example, single-unit recordings showed post-conditioning changes at [41.87–90] the level of the APC in awake rats [41.91, 92]. In animals trained to fear a specific odor (CS+) and not fearing a similar odor that was not paired with shock (CS−), APC expressed more selective odor coding. In contrast, rats trained to have a generalized behavioral fear response demonstrated impaired odor discrimination at the level of APC single units [41.92].

Contrary to the APC, the anatomy and the functionality of the posterior part of the piriform cortex (PPC) are reminiscent of higher-order associative areas and are thought to be responsible for odor quality and categorization. The PPC receives a relatively strong input from the basolateral amygdala, while the APC does not [41.93, 94]. As postulated by *Li* [41.95], this may be the primary locus of representation for olfactory aversive stimuli in humans. Since early development (post natal (PN) day 7–8]), odor aversion learning is associated with PPC activity [41.96]. This is maintained around the weaning age (PN12–13) with shocks of different intensity (0.5 and 1.2 mA). Post-conditioning plasticity in this area has been repeatedly demonstrated via different techniques. Animals trained with odor-shock conditioning display stronger local field potentials [41.97, 98], higher synaptic plasticity as measured via the expression of brain-derived neurotrophic factor (BDNF) [41.99], increased gamma-aminobutanoic acid (GABA) and glutamate concentrations that resist approximately for 30 minutes [41.100]. Also, lesions at the level of associative olfactory cortices (PPC included) one month after training eliminate the CR [41.101]. Taken together this indicates that the piriform cortex is an essential structure for long-term storage and retrieval of odor-paired threat. In other terms, the synthetic function of the piriform cortex as a whole complements the specificity of elaboration found in previous stages of the olfactory system (OB) and promotes a more comprehensive and stable representation that serves as the base of the holistic perceptual experience of the odor object [41.102]. Furthermore, convergent support of this statement comes from the study of the slow-wave sleep (SWS) activity in an aversive olfactory conditioning paradigm. Even if PC is usually hyporesponsive to odors during SWS, during post-conditioning its activity is enhanced and significantly correlates with subsequent memory performance [41.91]. In other words, it is possible that the PC's reduced reactivity during SWS indicates a preferential mechanism to facilitate memory consolidation of relevant information (threat) while external noise can remain unattended [41.91].

In agreement with findings from the rodent literature, the human piriform cortex has been identified as a key structure in aversive odor conditioning. PPC, but not APC, exhibits odor-paired specific plasticity [41.103] that seems to be based on response enhancement following prolonged exposure to an odor [41.104]. Critically, the PPC post-conditioning odor plasticity is evident also when the initial neural (and perceptual) representation of the odor to-be-paired with the shock (CS+) and the odor to-be-unpaired with the shock (CS–) are not discriminable [41.103]. Associating one of two entianomers, odors with chemical mirror non-superimposable images, with a shock significantly increases discrimination performance of the paired odor [41.103]. This elegantly suggests that human aversive olfactory conditioning is based on sensory augmentation processes that are independent of attention [41.103]. In line with the above-mentioned studies demonstrating that aversive olfactory conditioning-plasticity is present already at earlier stages of the system, *Åhs* and colleagues [41.105] demonstrated in human participants that the increase in discriminatory performance after aversive olfactory conditioning was based on an odorant-dependent shift in absolute sensitivity rather than on a change in discrimination performance per se. Interestingly, eight weeks after the documented aversive olfactory conditioning-dependent increase in sensitivity, the increase was no longer present [41.106]. This indicates that these effects of rapid plasticity may be of a transient rather than permanent nature.

At present, while aversive olfactory conditioning-induced changes can occur throughout the piriform cortex (especially in rodents), the site of most likely anatomical convergence between threat signals and the odor CS is the posterior part of the piriform cortex. Accordingly, the PPC is the structure that most reliably demonstrates odor-shock induced plasticity in humans.

Hippocampus

A key player in the formation and storage of memories across mammals is the hippocampal formation [41.106]. The hippocampus is thought to be responsible for the acquisition of the association between stimuli, and among stimuli and the context in which they are presented during aversive olfactory conditioning [41.106]. Critically, the development of the hippocampus is responsible for the emergence of contextual aversive olfactory conditioning that appears in rat pups at PN24, but not before that developmental stage [41.49]. Neural and immunohistochemical correlates indicate an increased activity evoked by CS odors after aversive olfactory conditioning in several subregions of the hippocampal formation, such as the Cornu Ammonis (CA) 1, CA3, and the dentate gyrus (DG). This learned odor-evoked activity presumably reflects the fact that the DG receives strong afferent input from the entorhinal cortex, which is a highly multisensory cortex including serving as a direct, monosynaptic target of the olfactory bulb and piriform cortex (Fig. 41.1) [41.49]. The DG in turn projects to the CA3.

The $GABA_A$ agonist muscimol infused into the hippocampus, which silences hippocampal activity, prevents rat pups from coherently representing the stimuli in the environment and therefore inhibits contextual

aversive olfactory conditioning [41.49]. These results are in line with most literature on adult contextual aversive olfactory conditioning.

In human participants, it has been demonstrated that the representation of an object becomes fully consolidated by involvement of the hippocampus [41.107]. In aversive olfactory conditioning, the sensory (piriform) cortex plays a key role in supporting the long-term storage of the representation [41.108], as evident also for other sensory modalities (audition) [41.109]. Recent data also indicate that the strength and precision of aversive olfactory conditioning memories can be modified by sleep [41.110, 111]. Re-exposing adult participants during the sleep stage to the odor associated with threat during the previously wake state induced a reduction in hippocampal activity (as well as a reorganization of neural patterns in the amygdala) prompted by stimulus-specific extinction. Therefore, the extinction of the feared odor can be favored during sleep, simultaneously avoiding the traumatic conscious re-exposure of the threatening odor [41.110].

Amygdala

The amygdala has historically been thought to be the key structure for initiating and controlling fear reactions. However, more recent data indicate that the amygdala codes for the biological significance, intensity, or salience of sensory stimuli [41.112, 113]. It projects outputs to the sensory cortices, which may enable perceptual analysis of potentially threatening stimuli [41.112, 114]. Although this is not the only possible pathway enabling threat perception [41.115], the amygdala remains a central area for aversive olfactory conditioning given its role in emotional, memory, and olfactory processes.

Among the amygdalar nuclei, the basolateral complex (BLA), which includes the lateral, basal and accessory basal nuclei, is the area most strongly linked to aversive olfactory conditioning processing [41.116]. The lateral nucleus has been implicated as the primary site of acquisition and consolidation of aversive memories, given its increased spike firing and long-term potentiation [41.117]. This has been confirmed specifically for aversive olfactory conditioning [41.118] as early as in the first stages of development [41.96]. Learning-associated changes in the BLA are impacted by the strength of the shock, but only from PN23–24, when BLA is involved at all intensity levels of electric shock (0.5–1.2 mA) [41.96]. Immunohistochemistry measures further confirm that amygdala involvement critically mediates the development of aversive olfactory conditioning at PN10 [41.119–123]. This structure, although sufficiently mature to respond to aversive odor stimuli during the sensitive period [41.124], is involved in the mechanisms in interaction with corticosterone levels [41.119, 121, 125–127]. Further, attenuated aversive olfactory conditioning in adulthood has been demonstrated to be associated with a deficit in CS odor-evoked 2-DG uptake in the cortical nucleus of the amygdala and the PPC, effects that have been linked to reduced local inhibition, as assessed by means of electrophysiological techniques [41.128]. In other words, odor-shock pairing experienced early in life induces functional changes in areas beyond those involved in infant learning and they are potentiated by contingencies [41.128].

In adult rats, the amygdala is critical for threat acquisition and consolidation. Pre-training lesions or pharmacological inactivation or inhibition of BLA as well as post-training lesions prevent the full formation of a CS-paired odor aversion [41.129–131]. Increased expression of BDNF [41.99] and heightened concentrations of GABA and glutamate [41.100] in trained rodents are biomarkers of the BLA synaptic plasticity following aversive olfactory conditioning.

Learning-dependent responses in the amygdala are also revealed in human studies showing time-dependent post-conditioning plasticity. In other words, the amygdala response is maximal in early conditioning trials and a progressive decay of activity in this area is subsequently seen [41.103]. This finding is in line with the exponential decay in activity observed in imaging studies using visual stimuli in association with shocks [41.132, 133] and provides an indication of how the aversive experience shapes perception. More recently, it has also been shown that odors paired with painful (trigeminal) stimulation (carbon dioxide, CO_2) elicits an enhancement of functional activation of the amygdala during conditioning [41.134].

In summary, the amygdala, especially the BLA, is strongly implicated in aversive olfactory conditioning in both humans and rodents. The BLA expresses localized changes in network function that contribute to stored memory and subsequent CR behaviors. Furthermore, BLA output to target areas, such as the PPC, may contribute to learned changes directly within the sensory cortex, a mechanism that can contribute to odor-evoked fear.

Orbitofrontal Cortex

The orbitofrontal cortex (OFC) is a vital part of the neural olfactory network in both nonhuman and human animals. In rodents, it receives olfactory input through both reciprocal connections with the piriform cortex [41.135] and via projections from the mediodorsal thalamus [41.136, 137]. The OFC is important for odor-taste multisensory integration [41.138, 139] as well as for olfactory reward evaluation and odor-guided be-

haviors [41.140]. The OFC and its connections with primary olfactory areas [41.135] demonstrates experience-dependent plasticity [41.141, 142] and this may contribute to learned odor behaviors. Important for this discussion, the prefrontal cortex, including the orbitofrontal, is also a strong modulator of amygdala activity and is involved in emotional regulation and anxiety [41.143]. However, to the best of our knowledge, the OFC has received very little attention in aversive olfactory conditioning paradigms in either humans or nonhuman animals.

41.2.2 Chemosensory Stimulation

The extent of the literature covering the use of chemosensory stimuli as the US in aversive olfactory conditioning paradigms is scant when compared to the previous section. Therefore, we present a more integrated view and we merge our discussion of the various neural structures involved. In rodents, although predator odors can be used as US in contextual conditioning paradigms [41.144, 145], we are not aware of any published odor US – odor CS conditioning data. Perhaps the closest example of such work in rodents is the recent work demonstrating transgenerational odor fear [41.146]. Female rats were conditioned (odor-shock) to fear an odor. They were subsequently bred and allowed to have litters. If the mothers were exposed to the CS odor in the presence of her pups, her fearful response to that CS induced odor-specific fear in her pups. The alarm odor she emitted in response to the fearful CS was sufficient to train her pups to fear the CS themselves [41.146]. This form of transgenerational odor fear is amygdala dependent [41.147, 148].

In humans, unpleasant odors have been used as aversive stimuli to produce aversive reactions comparable – although not identical – to those dependent on electrical shocks [41.25, 50, 147]. For instance, odors are stimuli that can induce emotional reactions closer to disgust rather than fear. In fact, simple odor exposure does not induce the same brain activations as revealed by the pairing of a neutral visual stimulus and an aversive odor [41.50]. *Gottfried* and collaborators [41.50] used neutral faces as CS and paired half of their presentations with 4-methyl-pentanoic acid, a pungent cheese-like odor acting as US and consistently rated as unpleasant. Functional magnetic resonance imaging limits the extent to which we can spatially zoom in on task-related activations and therefore does not allow assessment of learning-dependent plasticity before the information arrives in the piriform cortex, and it does not enable the analysis of single nuclei within subcortical structures. However, the PPC seems to play a critical role in salient associative activity, thus confirming using a different type of US that this area cannot be considered a strictly unimodal cortex, but rather an associative brain center [41.50]. Amygdala responses were not, however, reported [41.50]. This lack of amygdala activity could potentially be attributed to an insufficient arousal magnitude by the unpleasant odors [41.149], some of which might have triggered disgust rather than fear [41.150].

Further, an interesting aversive olfactory conditioning-dependent effect has been demonstrated in the orbitofrontal cortex (OFC), an area that has been attributed to higher-order complex processing and by some labeled as the secondary olfactory cortex [41.149]. Both the medial and the lateral portion of this cortex have been related to learning-dependent computations that outperform simple odor processing [41.50]. In other words, OFC is an essential contributor to the creation of stimulus-reward associations that are used to organize odor-guided behaviors.

41.2.3 Naturally Occurring CS–US Associations

The prevalence of object learning in the environment makes it a useful heuristic for identifying CS-US pairings in nature [41.51]. An interesting case is represented by conditioned odor aversion (COA). COA is a robust and long-lasting odor association that generates avoidance of the odor stimulus due to the contingent association of an ingested tasteless solution (CS) rapidly followed by toxicosis (US) [41.151]. COA is a phenomenon that has been shown to be present in intrauterine life and throughout development [41.70, 152–160] [41.161]. Although it has been suggested that COA learning depends exclusively on the taste modality [41.162], it is now clear that retronasal olfaction has a significant impact on post-ingestive outcomes [41.36, 163]. In fact, the distal exploration of the food via its odor acquires predictive value of the sensations experienced while consuming the food [41.36].

In the following sections, we will report the characterization of the neural activity associated with olfactory-induced malaise, following the olfactory pathway. It is worth reiterating that for many other paradigms, the investigation of the lower levels of the olfactory hierarchy has yet to be performed on human participants. Researchers working on the topic are in the process of adjusting current techniques or developing new tools to resolve the issues preventing the online neuroimaging of the OSN and olfactory bulb. Nonetheless, a direct comparison between the human and nonhuman literature is often difficult due to the necessity of experimentally inducing the malaise paired with the olfactory stimuli; a practice that is ethically difficult to perform in humans due to the long-term aversive out-

comes [41.164]. For this reason, this section will focus on knowledge derived from experiments in nonhuman animals. Observational information regarding specific cases of odor-malaise associations will instead be provided in the next section addressing the role of aversive olfactory learning in clinical conditions. To the best of our knowledge, the effects of naturally occurring odor CS-US associations on olfactory sensory neurons have not been explored. Therefore, we will start our hierarchical report at the level of the olfactory bulb.

Olfactory Bulb

In the rodents, COA has been deemed possible to induce as early as in fetal rats [41.70, 152, 157–161, 165–169] and it relies on the contribution of the OB, at least until the pup approaches weaning age. However, data gathered in older pups and adult rats [41.170] indicate that the plasticity of the OB is reduced as compared to the first phases of development. Critically, in COA paradigms, the contribution of the OB lasts longer as compared to aversive olfactory conditioning paradigms involving shock [41.49, 158, 171]. Considering that COA relies on the olfactory retronasal stimulation of a food item, the nutritional state of the animal is a critical variable in modulating the activity in the OB. As an example, single-cell recordings demonstrate that the activity of the mitral cells increases when an animal is satiated and receives an odor previously paired with malaise [41.172]. Taken together, these findings indicate that the activity in the OB reflects the coding of the conditioned relevance of a stimulus induced through natural exposures.

Piriform Cortex

Although relatively unstudied, the impact of odor-malaise learning on the APC does not seem to be robust during early development, whereas PPC seems to play a critical role [41.96]. By capitalizing on Fos immunoreactivity, a technique that allows for the quantitative analysis of the neurons activated following stress and pain by visualizing the expression of the c-Fos protein product, it was evident that the odor-evoked trans-synaptic neuronal activity increased in PPC during the retrieval of taste potentiated odor aversion (TPOA) [41.173, 174]. In line with this evidence, *Chapuis* et al. [41.36] demonstrated that COA learning clearly modified transient oscillations reflecting synchronous activities in large-scale neural assemblies. In detail, odor-induced fast oscillations in the beta frequency (15–40 Hz in rats) of local field potentials registered at the level of PPC predicted the aversive behavior to the odor in concert with the emergence of a strong beta oscillatory activity in the OB, OFC, and BLA. Altogether, these pieces of evidence seem to support the idea that specific neuronal populations in PPC respond to odors paired with aversive stimuli and have enhanced temporal coherence, allowing for experience-dependent odor representations specific to the way the aversion was acquired [41.36].

Amygdala

The amygdala contribution to COA arises post-weaning in rat pups [41.96, 158] and is maintained in adult life [41.175]. The BLA, in particular, has been described as the critical structure involved in the acquisition, consolidation, and retrieval of the COA [41.170, 176–179]. *Chapuis* and colleagues also provided evidence of experience-dependent modulation of BLA oscillatory activity in response to odors [41.36]. In line with the idea that this structure integrates the affective salience of chemosensory stimuli [41.180, 181], it is plausible that it plays a role in the representation of a general aversive connotation of the olfactory signal.

Insula

The insula, also known to be a prime processor of gustatory information [41.182], is critically involved in aversive paradigms involving tastants [41.183]. With reference to the COA paradigm, the more ventral agranular zone of the insula (an area labeled by some as primary gustatory cortex) is a particular target of projections for primary olfactory areas [41.184, 185]. *Chapuis* et al. [41.36] described how the pattern of beta oscillatory activity in both the agranular and the granular division of the insular cortex are modulated in response to the learned odor cue, but only when ingested before the animal experienced malaise. This would represent *the signature of a network supporting odor representation as a consequence of the animal experience* [41.36].

Orbitofrontal Cortex

The OFC is known to integrate the inputs of various food-related sensory stimulations and has been suggested to play an important role in flavor perception in rodents and humans [41.139, 186, 187]. *Dardou* and collaborators have shown that both olfactory and taste cues activate this structure during taste potentiated odor aversion retrieval [41.174]. Several studies have shown the existence of both anatomical and functional connectivity between the PC, the BLA, and the OFC [41.135, 188], and that these pathways are capable of learning-dependent plasticity [41.141, 142]. Thus, this network is a good candidate for the integration of both sensory and affective signals about food odor cues.

It has been suggested that retronasal stimuli are more effective than orthonasal stimuli in modulating the gustatory or flavor neural code. As an example,

De Araujo and colleagues [41.138] measured brain response to retronasally delivered odors in combination with a taste and demonstrated selective activity in an anterior region of OFC, suggesting that the region was therefore important in integration of taste and smell. Moreover, *Small* and colleagues [41.187] determined that the modulation of preceding experience (whose valence was not, however, taken into account) affects OFC, as well as the insula and anterior cingulate cortex.

41.3 Clinical Applicability

Up to this point, we have reviewed the neural changes associated with aversive olfactory conditioning across different stages of the olfactory pathway and across specific developmental ages. These modifications have a striking impact on the individual's physiology, whether nonhuman or human animals, and on the manifested behavioral correlates. Independently of the sensory modality considered, the fear-conditioning paradigm has proven to be one of the more prolific and valuable experimental models for assessment of human psychiatric disorders associated with abnormally heightened fear, anxiety, and other dysfunctional behaviors [41.189–196]. The natural response to sensory cues, the acquisition of aversive memories, and the extinction of the already acquired fear memories are mechanisms that have characterized the study of aversive olfactory memory and set the base for explaining a range of pathologies and psychopathologies. Besides etiopathogenetic explanations, behavior therapy has used aversive olfactory conditioning to reduce a variety of dysfunctional or unwanted behaviors [41.197]. Below, we will analyze some of the most relevant applications.

41.3.1 Anxiety and Trauma-Related Disorders

The range of fears is immeasurable, and some clearly present an olfactory component. Besides phobias triggered by odors of animals (for instance, dog odor for cynophobics) [41.198], post-traumatic stress disorder (PTSD) has a strong olfactory connotation [41.192]. PTSD is a mental sequela that may occur after a traumatic event, such as war, assault, or a natural disaster [41.199]. Experimentally, studies reported in the literature have used combat-related stimuli in different sensory modalities. Odors have long been noted in the clinical practice to have a strong emotional and memory component that seems to precipitate or trigger the individual to re-experience the traumatic event [41.200]. The characterization of the mechanism of aversive olfactory conditioning constitutes a model for threat perception and reaction that can be useful in the explanation of the general principles underlying these types of fear-related clinical issues. The amygdala and the hippocampus, both of which are central to the mechanism as previously outlined, are structures that have been consistently included in neural models of anxiety and mood disorders [41.201]. Specific to PTSD, a positron emission tomography study revealed a bigger change in cerebral blood flow in the amygdala and odor cues retrieved more memories of life events [41.39]. Furthermore, aversive olfactory conditioning can either induce very stimulus-specific fear (selective to the CS) or generalized odor fear depending on the events during the conditioning (presence of a CS− and a CS+ [41.92]) and experiences during consolidation of those fearful odor memories (during post-training SWS [41.110, 111]). These different features of aversive olfactory conditioning experience can change the precision with which the olfactory system encodes the learned odor. Failure to have precise stimulus control of our learned fears can contribute to PTSD and other fear-related disorders. Thus, understanding how traumatic events affect sensory coding itself will significantly contribute to our understanding of these disorders and will open up the possibility of expanding treatment options.

Well-established treatments for both specific phobias [41.202] and PTSD [41.203] are exposure-based behavioral strategies, which involve the experience of the feared object or situation in a nondangerous, controlled environment. Exposure therapy encourages the systematic confrontation of stimuli associated with fear, with the goal of reducing the person's fearful reaction [41.204]. Confrontation of external (feared objects, activities, situations) or internal feared stimuli (feared thoughts, physical sensations) can occur in imagination or in vivo [41.202–205]. Nevertheless, positive therapeutic results are critically dependent on the emotional engagement of fearful memories, [41.206] whose replay is often actively contrasted by patients [41.206]. Smells, in virtue of their automatic emotion-producing feature, are ideal candidates to increase emotional engagement [41.207] and reduce the frequency and the intensity of episodes of anxiety, flashbacks, and dissociation triggered by odors. Attempts have been made to increase the usability of exposure therapy and

the compliance to the therapy by reducing its side effects. As an example, technological advancements offer the opportunity to control odor presentation by exposing patients to sensory rich virtual reality (VR) environments [41.208, 209]. As another example, techniques have been proposed to replay the traumatic event in states of faded consciousness. Besides the scant evidence on hypnotherapeutic olfactory conditioning [41.210], which helps the hypnotized patient to develop new (positive) olfactory associations with the traumatic event, interesting potential comes from the study by *Hauner* and colleagues [41.110]. The authors exposed their participants to face images and electrical shocks in the presence of an olfactory background. During an afternoon nap, the olfactory cue paired with the aversive association was reproposed. In the ensuing wake period, arousal, amygdala, and hippocampal activations were reduced for the feared face. In other words, these findings suggest that odors are sensory cues able to target which memories can be reactivated during sleep and favor fear extinction. Although these results were reported for a group of healthy young adults, they trace a pathway for research including patients with anxiety disorders and, in general, everyone who would like be relieved of an unwanted fear.

41.3.2 Multiple Chemical Sensitivity

Another pathology that heavily relies on olfactory-based conditioning issues is multiple chemical sensitivity (MCS) [41.211], also defined as idiopathic environmental intolerance [41.40]. MCS incorporates a range of chronic polysymptomatic conditions among which low levels of common environmental chemicals (pesticides, solvents, etc.) have been reported to cause disabling problems in the sufferers. The high comorbidity with affective disorders [41.212] and the findings from the animal literature of links to the mechanism of kindling (or neurogenic sensitization), support the idea that olfactory-based conditioning and/or sensitization mechanisms might be the base of the disorder. Aside from maladaptive cognitive products (i. e. beliefs, expectations), repeated associations between multi-componential aversive responses (e.g. psychophysiological, motor) and low levels of inhaled and ingested chemicals, may condition aversive response symptomatology [41.40, 213]. In fact, it has been demonstrated that healthy participants exposed to harmless odors (i. e. butanoic acid) in association with CO_2 show post-conditioning somatic symptoms like altered respiratory behavior that can be reduced in a typical Pavlovian extinction paradigm [41.214].

The olfactory-limbic mechanisms previously described, and in particular the use of odors as US and odor-malaise paradigms, are mechanisms that offer testable hypotheses, which are urged to be explored to better characterize this still controversial syndrome [41.215]. In addition, in rodents it has been reported that norepinephrine, released in response to arousal, can prevent habituation and/or induce dishabituation of odor evoked neural [41.90] and behavioral [41.216] responses. Thus, cognitive factors could differentially maintain or heighten olfactory responsiveness to odors in individuals predispositioned to MCS via elevated norepinephrine levels. This should be the target for future studies.

41.3.3 Pretreatment Chemotherapy Nausea

The odor-malaise condition type is an optimal model to explain the phenomenology of anticipatory nausea in patients undergoing cancer chemotherapy. Many cancer chemotherapy drugs provoke nausea and stimulate the emetic reflex [41.217], resulting in approximately 25% of patients developing anticipatory nausea and vomiting [41.218]. The most common cause of pretreatment nausea has been found in the odor that the patients associate with the clinical environment or odors previously associated with a vomiting experience [41.219]. Given the repetitive aversive association with the treatment and the emetic episodes, the patients might refuse or experience the treatment as disgustful. Therefore, the patient's compliance in the treatment, essential for the recovery process, is threatened and quality of life impaired [41.40]. At present, antiemetic drugs acting on the neurochemical control of vomiting are primarily used to treat chemotherapy nausea [41.220]. However, some types of administrations, such as intramuscular delivery, prove to be painful and carry a plethora of side effects (e.g. erratic absorption of drug, sterile abscess formation, fibrosis of the tissues, etc., [41.221]). Therefore, understanding the learning mechanism of the conditioned response offers the possibility of making behavioral recommendations [41.218, 222].

Systematic desensitization processes contrast the maladaptive learned responses of anticipatory nausea and vomiting with relaxation under the assumption that fear and relaxation cannot coexist at the same moment [41.205]. This process, which often happens in imagination, facilitates the creation of an alternative response to the dysfunctional behavior. Specifically to anticipatory nausea and vomiting, olfactory stimuli associated with the chemotherapy sessions can be presented to the patient who has reached a deep stage of relaxation, promoting the counterconditioning of the unwanted response. This behavioral approach has been proven more effective than other treatments such as counseling [41.223] and relaxation alone [41.224].

41.3.4 Addiction and Substance-Related Disorders

Conditioning mechanisms involving olfactory stimuli can be used, as just reviewed, to reduce nausea and vomiting but they also have often been used to produce them in order to avoid other sorts of unwanted behaviors. Classically, aversion therapy has been an effective approach to reduce excessive drinking in alcoholics [41.225]. Pairing the odor of alcohol with electrical shocks as US has been one of the techniques [41.226], although not the most successful. Indeed, chemically based aversions, appropriately pairing the smell (or the taste) of alcohol with other chemical cues such as ammonia-like smelling salts [41.227] or with emetic drugs [41.225] resulted in longer-lasting alcohol avoidance. Aversion therapy lived its golden age between the 1940s and the 1950s, and then progressively declined. Recently, few to no uses of this technique have been reported [41.228].

Odor-based aversion therapy was not only confined to alcoholism issues but it proved useful in reducing other types of addictions [41.228, 229]. For instance, isovaleric acid, a cheese or sweat-like foul smell, presented during covert sensitization, namely the mental rehearsal of the undesired behavior, discouraged cigarette and marijuana smoking as well as paint and glue sniffing [41.230]. It successfully reduced both manifest and implicit behaviors (thoughts) [41.230]. A naturally occurring aversive therapy has been described in smoking pregnant women who after associating the cigarette odor with nausea reactions will temporarily and spontaneously terminate their smoking [41.229]. Interestingly, *Arzi* and colleagues [41.231] offered an opportunity for a broader number of smokers. In a group of nicotine addicts who wanted to quit smoking, *Arzi* et al. [41.231] presented the cigarette's odor paired with two nonarousing, yet disgusting, odors (ammonium sulfate and rotten fish) during short-wave sleep. In the following wake period, participants exposed to the negative conditioning during their natural sleep reduced the number of reported cigarettes smoked (as compared to baseline) without any additional aid. Smoking cessation persisted for a week. These promising findings open up future research endeavors to fully disentangle the physiological process and evaluate the feasibility of sleep learning as a therapy.

41.3.5 Inefficient Therapeutic Use of Olfactory Aversive Conditioning

Besides the important insights that olfactory aversive conditioning has brought to the understanding and treatment of a variety of mental health disorders, the cases in which aversive therapy using olfactory stimuli has been attempted have not always been successful. As in the case of excessive drinking, overeating is a dysfunctional behavior that has often been treated with aversion therapy involving olfactory stimuli. *Cole* and *Bond* [41.232] paired appealing foods with noxious odors and temporarily succeeded in facilitating weight loss in a group of obese patients. They also demonstrated that at the end of the eight-week program, olfactory aversion therapy produced the highest weight loss as compared to two control groups (placebo and waiting list) not exposed to the foul odors [41.232]. Measurements at the eight-week follow up revealed, however, no differences with pretreatment weight levels, thus suggesting that olfactory aversion therapy is not efficient in targeting obesity long term [41.232].

Even considering the momentum gained by olfactory aversive therapy throughout a series of behaviors considered dysfunctional, it is striking that such a technique has been widely applied to limit noncanonical sexual practices [41.197]. Sadistic sexual arousal [41.233] and homosexual behaviors [41.234], have been routinely treated by behavioral therapists by associating the unwanted behavior with unpleasant olfactory stimuli such as ammonia or sulfurous compounds (rotten egg smell). In none of these studies can the therapy be considered as successful, either short or long term. Only in a single case study of child harassment tendencies did olfactory-induced aversions, in concert with other simultaneously proposed approaches, reduce the dysfunctional behavior [41.235].

41.4 Conclusions

The ensemble of the evidences reported here leads us to conclude that the olfactory system in rodents and humans alike is dynamically regulated at all (testable) levels of the olfactory system, starting from prenatal development and lasting throughout adult life. Indeed, tentative evidence even suggests that aversive odor learning transcend generations via epigenetic modulation [41.148].

The same core brain areas (PPC, amygdala) are responsible in all types of olfactory-based aversive learning mechanisms for mediating the association between the odor and the negative emotional experience. The

changes induced in the piriform cortex by the aversive experience have a cascading impact on a large network of cerebral areas, including the reciprocal ascending connections to OB and the descending connections from OFC [41.188]. As a result, the rich experience-dependent and plastic responses have the ability to associate memories with emotional content. As recently proposed by *Li* [41.95], a sensory-cortex-based threat perception model would explain the constitution of aversive experiences. Life experiences will form negative odor-threat associations that will first be stored at the level of the olfactory cortex, enabling encoding of threat cues as early as the first stages of sensory processing. Subsequently, as the representation of the odor-threat association is consolidated in the amygdala and elsewhere, the piriform cortex may undertake both rapid and long-term plastic changes, ultimately resulting in modified neural response patterns underlying the association. Unfortunately, limits to now available methods and usability in respect to ethical standards prevent research from extending this model to the lower levels of the olfactory pathway in humans, such as the olfactory sensory neurons. Moreover, as we highlighted above, whether the stimuli to be associated with the threat (either a shock, or a toxin) are presented orthonasally or retronasally will differently impact the neural network underlying the experience both in animals and in humans.

From a developmental perspective, the literature indicates that different conditioning methods trigger different neural pathways for odor aversion learning, each demonstrating different developmental trajectories [41.96]. The amygdala is a structure whose activation is highly sensitive to the specific learning protocol involved (aversive shock as compared to odor-malaise associations) and the reinforcement condition being assigned. Unfortunately, insights on developmental trajectories of human aversive olfactory conditioning are currently lacking.

Finally, a thorough characterization of aversive olfactory conditioning mechanisms constitutes a model for threat perception that can be useful in the explanation of the general principles underlying a variety of mental health disorders and their treatment. Specifically, a detailed knowledge of these mechanisms will be vital to solve problems related to memories triggered by odors in a variety of psychiatric and medical conditions, thus opening the possibility of developing novel therapies, even directly involving aversive olfactory conditioning. Considering the peculiarity of odor-based aversive learning mechanism, an experimental model with a high ecological relevance, it is our belief that there is an urgent need to fully uncover the behavioral psychophysiological and neural underpinning underlying aversive olfactory conditioning and that the field is set for major discoveries in the near future.

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42. Odor-Based Context Dependent Memory

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Even though rarely thought of, all environmental spaces contain odor information. It has been proposed that the preconditions for episodic olfactory memory may not be optimal. For example, environmental olfactory information often goes unnoticed and barely evokes attention in humans and semantic activations that are a prerequisite for optimal episodic memory functioning are typically restricted. Still, it is highly likely that olfactory information will become part of a memory representation that is linked to a specific event. This implies that an event-congruent exposure of an odor carries the potential to trigger all, or parts of, a previous episode. Indeed, available evidence shows that odors may serve as powerful reminders of past experiences. This is demonstrated by studies exploring the nature of odor-evoked autobiographical memories and by controlled experimental paradigms where odors have been embedded in a learning context and later reinstated at retrieval where an increased memory recollection for the target information is often observed. These observations converge on the notion that odor memories are retained over long periods of time.

In this chapter, we will highlight olfactory cueing of memory and how odors may act as reminders of the recent and distant past.

42.1 Odor-Based Context-Dependent Memory

Our memories of events are often a result of experiences that occurred within a physical context. Memory scientists have shown that one way to increase the access to memory information is to reinstate the original learning context, that is to maximize the congruence between retrieval and the conditions that prevailed during acquisition [42.1]. Episodic memory consists of many different types of information (spatial, temporal) originating from several different senses. These features may act as memory cues, or *triggers* for the original learning event to eventually promote retrieval of the target information. Context-dependent memory (the encoding specificity principle; [42.2]) refers to the fact that retrieval of specific episodes or information improves when the context present at encoding (learning) and retrieval are the same. Hence, it is assumed that environmental features (e.g., an ambient odor) are merged with target information during learning and that presence of the same odor at testing functions as an additional retrieval cue to the target event. A related theoretical notion is that of *transfer appropriate processing* [42.3] where retrieval is regarded as being mediated by the extent to which the cognitive processes required at testing overlap with the cognitive processes that occur at encoding. As noted above, the study of environmental context-dependent memory and the related concepts has gained empirical support from work that has used a range of different ambient and environmental contexts [42.1]. For example, context effects have been documented for background music [42.4–6], noise [42.7], and location [42.8, 9]. Still, it should be noted that positive retrieval effects following context reinstatement is not a rule [42.10]. A number of studies have failed to show beneficial context effects on retrieval performance [42.1]. Possible explanations for the lack of context effects will be highlighted below.

Olfactory stimuli can serve as effective contextual cues in the recollection or recognition of information encountered in the recent or distant past. Although available evidence is scarce, the overall pattern of findings suggests that reinstatement of olfactory contextual information that was present in a learning phase affects retrieval of the original information. Odor-based context effects have been observed for a variety of different

learning paradigms including recall, recognition, and relearning performance. Focusing on incidental verbal learning, *Schab* [42.11] presented a list of common adjectives with instructions to write down the opposite to each word with or without the smell of chocolate present. The participants were not aware of that their memory for the words they had written would be tested for the following day. Those individuals who were exposed to chocolate both at encoding and retrieval recalled more words than participants who were exposed at learning only, recall only, or did not receive any odor at all. To investigate whether odor valence could have influenced the context effect, a follow-up experiment using the smell of mothballs was performed. The results from this study mimicked the one obtained for chocolate, suggesting that odor pleasantness is of minor importance for olfactory context-dependent memory. In a similar vein, *Pointer* and *Bond* [42.12] measured prose recall performance using a context-dependent memory paradigm. Here, university students learned a prose passage in the presence of a visual or an olfactory stimulus. Specifically, the prose passage was presented on paper that was impregnated with a peppermint odor or on yellow paper. After a short distractor task, participants were asked to recall the text either with the original context reinstated or not reinstated. The results indicated a context-dependent effect such that prose recall was selectively higher following olfactory reinstatement whereas no memory benefits were observed for the visual congruent condition. *Smith* et al. [42.13] used a relearning-savings technique as a measure of memory and reported that relearning in the same odor environment improved memory performance reliably. Further, using a recognition paradigm, *Cann* and *Ross* [42.14] presented faces to a group of younger adults in the presence of an ambient odor. Two days later, an old-new recognition test was presented where either the same or a different ambient odor was present. The results showed that recognition performance was higher when the same odor was present at both encoding and test than when a different odor was used.

It is well established that there is a fast and strong connection between brain structures processing emotion, memory, and olfactory information [42.15, 16]. Given that the olfactory sense is an emotional system, it is of particular interest to investigate whether odors also are more efficient reminders of emotional information than other modality cues. *Herz* [42.17] examined whether an ambient odor is more effective as a retrieval cue of verbal information if experienced in an emotional (pre-exam state) than in a neutral state. The results indicated that odor cueing in emotional individuals resulted in higher word recall than a neutral state. She concluded that heightened emotion experienced during encoding can enhance the effectiveness of odors as cues to memory. It is well established that stress influences learning and memory processes. Stress induces an activation of the hypothalamic-pituitary-adrenal (HPA) axis leading to release of glucocorticoids that primarily act in the hippocampus, amygdala, and prefrontal regions; key structures for the emotional memory processes [42.18]. In this vein, *Toffolo* et al. [42.19] let participants watch an aversive film while being exposed to olfactory (blackcurrant), auditory (music), or visual information (colored light). Blackcurrant was chosen as olfactory information because of its supposedly neutral odor. One week later, their memories were evaluated following one of the triggers. The results showed that odor-evoked memories of the aversive events were more detailed, unpleasant, and arousing than memories evoked by the auditory cues. However, no difference was observed between the olfactory and the visual cues. Relatedly, a recent study investigated whether olfactory information can serve as an effective retrieval cue for memories of a stressful episode [42.20]. The stress condition involved a public speaking task in front of a committee and the control group was exposed to a well matching but not stressful situation. For both conditions, an odor (methyl benzoate) was present and a set of visual objects that were either bound and central to the stressor (committee; e.g., pencil, stop watch) or peripheral (e.g., dustbin) were arranged in the experiment room. Overall, central details were better remembered than peripheral and provision of a congruent odor cue in combination with stress led to enhanced memory performance relative to the non-stressed group. Exposure of a non-congruent odor (bornyl acetate) did not produce any performance difference between the stress and control group. Hence, retrieval of information experienced in a stressful event comprising a smell benefited from reinstatement of the olfactory source.

In contrast to studies manipulating olfactory contexts to explore effects on verbal and visual information, a recent study investigated whether episodic odor recognition memory might be affected by physical context [42.21]. Here, participants encoded odors and words after which recognition memory and word recall was assessed in the same or in a different location that differed in the color of the light (neutral versus red-colored). The results indicated no effects of context change on recognition memory for odors and words or on word recall. As noted, absent context effects on memory performance are not rare [42.22, 23]. In their meta-analysis, *Smith* and *Vela* [42.1] stated that context effects are less likely to occur in tasks that promote suppression of the subjects' immediate environmental surroundings. For instance, learning paradigms that

put heavy demands on attention or association formation (learning a set of new odors) will eventually reduce the role of context (see below) on memory recollection.

Few studies have investigated context-dependent memory with odors outside the laboratory. However, *Aggleton* and *Waskett* [42.24] asked a group of young adults to recall details of a visit to a museum that had occurred about seven years previously. At the museum seven distinctive smells related to a Viking's life (e.g., burnt wood, apples, earthy) had been dispersed. Recollection for the museum exhibits was more accurate when participants recalled memories in the presence of the same distinctive odors that were present in the museum during the original visit, as compared with novel odors (e.g., coffee, peppermint, rose) or no odors. In the experimental setting, the odors were presented in bottles and the participants could smell them as long as they wished. Hence, the museum odors appeared as effective retrieval cues for information that was acquired incidentally in a real-world episode. In contrast, a recent study found no evidence of odor-based context effects. *Roos af Hjelmsäter* et al. [42.25] investigated the influence of physical odor reinstatement at retrieval on memory in a group of children in the real world. Here, children experienced a magic show where a vanilla odor was present. One week and six months after the magic show, children were interviewed about their memory of the event. During the interview, half of the children were exposed to the vanilla odor at recollection. In contrast to previous work on adults, no odor reinstatement effects were observed. Specifically, children who did not re-experience the vanilla odor during the interview recalled the same amount of information and were as accurate as children who did re-experience the odor. A less accurate and reduced level of information was also recalled after six months compared to the performance observed one week after the event. In contradiction with the hypothesis, the reinstatement of the vanilla odor at the six-month follow up for half of the group neither affected the amount nor the accuracy of the recalled information.

It has been proposed that absent context reinstatement effects may relate to the status of the contextual cue in relation to other cues. According to the *outshining hypothesis* [42.1, 26], contextual information can cue memory when better cues are absent at retrieval. If there are other, stronger cues, as is often the case for recognition but not recall paradigms, the contextual cue can be completely *outshone*, implicating that the cue does not contribute to any more recollected information. Further, in their *overshadowing hypothesis*, *Smith* and *Vela* [42.1] argued that if contextual cues are suppressed at encoding, due to, for example, a high focus on the information to be remembered, context reinstatement effects are less likely to occur. Hence, the absent context effect in *Roos af Hjelmsäter* et al. [42.25] may be due to the fact that the interview questions offered enough cues to memory so that the vanilla odor cue was superfluous. Alternatively, the children may have found the magic show more exciting than the odor.

To summarize, the overall pattern of experimental well-controlled findings shows that olfactory cues may act as significant reminders of recent events. Also, some evidence indicates that olfactory cues may be especially apt to trigger emotionally laden information.

42.2 Autobiographical Odor Memory

As noted above, most of the studies targeting odor-based context-dependent memory have been performed in the laboratory with highly controlled encoding and retrieval conditions. In related work targeting autobiographical memory (AM), reinstatement of contextual information that was present in a learning episode is also the typical method used. AMs are personally experienced events that may be located in time and space [42.27]. Here, the most common cueing procedure is the Galton–Crovitz method, where individuals are given unimodal cues (e.g., words, pictures, or sounds) and asked to retrieve any AM for each cue [42.28]. With successful retrieval, a short description of the event is provided along with ratings of experiential factors (e.g., vividness of the evoked memory, emotionality) of the recollected event. Typically, when all cues have been presented, the participant is asked to go back to each evoked event and date it (e.g., to indicate the age-at-event). Hence, in contrast to laboratory-based context-dependent memory assessments there is a lack of control regarding the veridicality of the retrieved memories [42.29]. It is also unknown to what extent odors are vulnerable to the generation of false memories.

A central theme in AM research concerns the age distribution of memories across the life span. The age distribution of memories evoked by verbal and visual information follows a specific pattern involving three components: childhood amnesia, the bump, and recency [42.30]. Childhood amnesia refers to the dramatic reduction of memories reported from early childhood. In contrast, a substantially larger number of

memories are recalled from the ages of 10–30, a phenomenon that has been termed the *bump*. The third component, recency, reflects a generally good retention of events occurring in the last 10 years [42.31]. A common explanation for the bump is that the age span in question, 10–30, typically involves significant life experiences (identity formation), which in combination with a highly functional nervous system promote an elaborated and distinct encoding of events [42.32, 33]. However, in sharp contrast to what has been thoroughly established for our primary senses, AMs cued by smells originate from the first decade of life [42.34–36] and not from young adulthood. Hence, associative odor learning begins very early in life and may become accessible in old age through exposure to event-congruent olfactory information. *Arshamian* et al. [42.37] explored the neural correlates of olfactory-evoked AM in a group of young adults. When brain activity between evoked AMs from childhood (3–10 years) and young adulthood (11–20 years) were compared, differences were observed. Specifically, odor memories clustered in childhood were related to a stronger activity in the secondary olfactory cortex (i. e., orbitofrontal cortex), whereas odor memories originating from young adulthood were associated to a more pronounced activity in the left inferior frontal gyrus, a brain region that supports semantic memory processing. Tentatively, it may be hypothesized that early childhood olfactory representations may be more perceptually and imagery based, that eventually shift to a more semantically driven consolidation with increasing age.

Odor-evoked AMs also differ with regard to phenomenology. For instance, olfactory-evoked memories are described as more emotional than verbally and visually evoked memories ([42.38–40]; but [42.19, 36] for different outcomes). They are also often reported as more vivid [42.35] and accompanied by stronger feelings of being brought back in time to the occurrence of the events [42.36]. Further, they are thought of less often, which most likely relates to our difficulties in imagining and recreating olfactory information in our minds [42.41, 42]. The key features of olfactory-evoked AMs were recently summarized in the LOVER acronym – *l*imbic, *o*ld, *v*ivid, *e*motional, and *r*are [42.43].

The nature of olfactory consolidation and why our odor-based personal memories show a distinct clustering in childhood remains unclear. We are regularly exposed to odors (e.g., orange, coffee, gasoline) in our everyday lives, but when we experience a distinct AM from a smell, it appears that we recall the first association where the odor was encoded in memory, and not any of the subsequent events where the particular odor was processed. It has been proposed that the longevity of odor memory could be understood through a resistance to interference in the olfactory system. Retroactive interference is a phenomenon that occurs when new learned information impedes retrieval of previously learned information, and is a common cause for why visual and auditory information is often forgotten [42.44]. Little is known regarding interference in odor memory, although an early study reported that after a two-week retention interval, the first of two associations to an odor was better retained [42.45]. Similarly, when *Dempsey* and *Stevenson* [42.46] let individuals learn new word associations for the same set of odors, results showed that the new learning did not affect the retention of the previously learned material. In contrast to these findings, *Köster* et al. [42.47] reported evidence for retroactive interference in odor memory, but only among individuals who were unable to name the target odors. A more recent study indicated that an odor's pleasantness also influenced its persistence to interference, such that interference was lower for unpleasant odors [42.48]. Overall, available data indicate that odor associations are difficult to alter once they are acquired, although odor knowledge and the emotional valence of the olfactory information may act as moderators in the degree of interference resistance.

It is noteworthy that most of the knowledge on odor-evoked AM is based on unimodal cueing (i. e., cues pertaining to one modality). A unimodal retrieval procedure entails that sensory information from different modalities are treated as separate entities rather than as components of integrated multimodal representations. However, in everyday life, we experience a multimodal environment, and it is still unknown to what extent sensory modalities may differ in their relative efficiency to trigger recollection of autobiographical information [42.49]. In a recent study, *Willander* et al. [42.50] randomized participants to three unimodal (pictures, sounds, odors) and one multimodal condition (picture + sound + odor). To maximize ecological validity, the three unimodal conditions were presented simultaneously in the multimodal condition, whereas in the unimodal conditions cues were presented separately. The unimodal cues were selected so that they could be combined into a multimodal naturalistic context. For example, a context of an indoor swimming hall could be represented by a photo of a swimming hall, sounds of water splashes, and laughter, and the smell of chlorine. The unimodal cueing of AM replicated previous findings by showing a significant clustering of odor memories to the first decade of life, whereas memories following visual and auditory cueing peaked in young adulthood [42.51]. However, when individuals were presented with multimodal information, the age distribution clustered in young adulthood, sug-

gesting that multimodal retrieval is mostly driven by visual and auditory information and to a lesser extent by olfactory information. Similarly, by modeling the semantic content of the retrieved memories, *Karlsson* et al. [42.49] found that the multimodal content differed from odor-evoked content but not from visual content and marginally from auditory-evoked events. This outcome suggests a hierarchy among the sensory modalities that are represented in multimodal cue information and that the subordinate role played by the sense of smell may underlie the rare occurrence of odor-evoked AM. Also, the results outcome favors the notion of visual cue dominance in multimodal contexts.

Taken together, the overall pattern of findings indicates that odors can serve as significant reminders of both the recent and the distant past. Olfactory stimuli embedded in a learning context and later reinstated at retrieval often produce an increased recollection of the target information. Likewise, exposure of event-congruent olfactory information in adult or old age enables accessibility of distant personal events experienced in our first decade of life.

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43. Infants and Children Making Sense of Scents

Benoist Schaal

This chapter summarizes research on the development of human olfactory skills to rely on different cues conveyed by odorants, such as odor quality, intensity, position in space, novelty/familiarity, and hedonic value. The sensory, neural, and psychological dimensions at the root of these early aptitudes remain poorly explored in humans, but one can safely affirm that any weak odor to which the infant has previously been nonadversely exposed will have a higher reinforcing value than any novel odor. Developmental differences in odor discrimination and appreciation are certainly causally multiple and may depend on general or olfaction-specific cognitive factors which can be traced back to prenatal or neonatal olfactory exposure effects. But some odors may also be unconditionally attractive or aversive from birth due to genetic or epigenetic factors.

Increased and systematic research efforts on olfactory development during the neonate, infancy, and childhood periods are important for several basic and applied reasons. First, they can illuminate general issues on the biological, psychological, ecological, and sociological mechanisms underlying human perception. Second, findings suggest that infantile experience with specific odors can canalize lifelong perceptual abilities. These long-term effects of early chemosensory experience relate to notions, such as sensitive periods, cerebral plasticity, and memory, and also to the early *programming* of food liking, addictive habits, and affiliative choices.

Research on human adults has repeatedly shown that odorants can mediate a wealth of informative cues that depend either on the stimulus (perceived quality and intensity, complexity, variety, etc.), on the perceiving individual (gender, physiological status, psychological age, motivational state), or on the perceiving subject's prior interactions with the stimulus [the rate of exposure to it (familiarity/novelty), expertise, hedonic valence (pleasantness/unpleasantness), or affordances conveyed by it (eatability, wearability as perfume, toxicity), position in space]. However, these properties are interdependent and their processing rests on the cognitive and semantic abilities of perceivers, which linguistic abilities facilitate some aspects of perception but may render other aspects suboptimal (memory). For example, the perception of odor quality is linked with the perception of odor intensity, odor-intensity detection favors the analysis of odor position in space, and hedonic valence relates with intensity and/or familiarity [43.1–4].

To which extent are these cues conveyed by odor percepts that are relevant in early development? How are meanings extracted from the innumerable percepts that odor objects or contexts produce, and how are they segregated into nested categories of known versus unknown, pleasant versus unpleasant, eatable versus noneatable, fruit versus meat, etc., to assist the developing brain to command adequate actions? In an attempt to synthetize *some* of the research conducted on the development of human olfaction, we will assess some of the above *dimensions* as they are processed by fetuses, newborns, infants, and children.

43.1 Nasal Chemosensation in the Fetus

It is a recently accepted fact that the functional onset of olfaction traces back to the fetal period. Long ago anatomical knowledge had shown that the chemosensitive structures of the nose are differentiated during early embryogenesis and reach stages that seem compatible with function before birth (Table 43.1; reviewed in [43.5, 6]). The three subsystems of human nasal chemoreception can then be clearly visualized at the periphery:

1. The main olfactory system shows adult-like ciliated neuroreceptors by gestational week 11.
2. The trigeminal system, which free nerve endings can be seen in the nasal mucosa by gestational week 4, responds to tactile stimulation by week 7.
3. Finally, the accessory olfactory system, composed at the periphery of the vomeronasal organ, initiates its development by gestational weeks 5–8 and reaches its maximum development at the central projection region (the accessory olfactory bulb) by week 20, but it later seems to decline or reorganize [43.7].

More recent evidence from neuroanatomy and brain imaging indicates that the peripheral structures of the main olfactory system and those of the trigeminal system are functionally connected with higher structures in the brain [43.8–11]. Despite recognized anatomical precocity at all levels of these chemosensory tracts, nasal chemoreception was long thought to be impossible due to nasal passages obstructed by mucus plugs or filled with fluid. The discovery by William P. Smotherman's group [43.12, 13] that young rats can retain odors to which they were merely exposed or conditioned in utero changed these views. Subsequent research proved the generality in vertebrates, including humans, that embryos and fetuses are capable of acute responsiveness to intra-amniotic infusions of odorants or of sensing and remembering odor from the prenatal environment [43.14, 15].

43.1.1 Amniotic Odorants

Amniotic fluid is the most obvious substrate mediating chemical stimulation to the fetus. Its composition changes along gestation due to the changing ratio in maternal/fetal contributions to its synthesis and due to its constant turnover due to fetal uptake and emission of fluid. The fraction of this fluid that contacts nasal chemosensory receptors is indeed constantly replaced due to frequent inhaling and swallowing activities of the fetus. Fetal nasal chemoreceptors may also be stimulated by odorants conveyed by the blood stream, as these may be passed from the capillaries that are adjacent to olfactory sensory neurons. But so far, this hematogenic route of stimulation has only been experimentally ascertained in adults.

The amniotic fluid composition changes along pregnancy and, in late stages, it depends strongly on fetal micturition and maternal intake of foods or any other odorous or sapid agents. The gestational increment in placental permeability may elevate the placental transfer rate of dietary odorants. It is well known by midwives that the amniotic fluid can be odorized by the food that mothers ingested shortly prior to delivery [43.16]. Odorants from a wide spectrum of foodstuffs were indeed shown to be transferred into the fetal compartment in either animal [43.17] or human pregnancies [43.18–20], including garlic, cumin, anise, carrot, mint, fenugreek, or curry. This cross-placental transfer of odor-active metabolites into the amniotic fluid can be fast. For example, in the ewe, odorants of cumin are detected in the fetal bladder 30 min after an intravenous injection of the maternal animal with cumin extract [43.21]. But much shorter placental transfer times were noted for compounds, such as nicotine or various drugs, and it is, thus, not excluded that some odorants can pass to the fetus quickly after the mother's ingestion, inhalation, or percutaneous absorption.

43.1.2 Fetal Chemoreception

From the third trimester of gestation, all chemosensory systems of the nose appear functionally ready: the nostrils are patent and nasal pathways convey flows of amniotic fluid-borne stimulations set in motion in the nasal passageways by the pseudorespiratory activity of the fetus. So far there is no direct, stimulus-response demonstration of fetal olfaction in the human species. However, studies in animal models indicate that such responses are clearly effective [43.15]. In the fetal rat and sheep, for example, intranasal infusions of odorants induce marked heart rate changes, while a control solution has no such effect. In addition, different odor qualities can elicit opposite autonomic responses indicating the discrimination of odor qualities [43.14, 22].

Indirect demonstrations of functioning olfaction in the human fetus are derived from tests on infants born prematurely. A sample of such infants whose age ranged from less than 28 up to 36 gestational weeks was screened for responses to the odor of mint [43.23]. The infants tested in the gestational age ranges of < 29, 29–32, and 32–36 weeks responded in 16% ($n = 6$), 73% ($n = 11$), and 100% ($n = 15$) of the cases, respectively. The 32–36 week group of premature infants

Table 43.1 Anatomical and functional findings on the antenatal development of chemoreceptive subsystems in the human nose

Gestational age (in post-ovulatory weeks)	Structural and functional events
3.5–5	Formation of the olfactory placodes
4–14	Trigeminal nerve endings in the respiratory mucosa, including olfactory mucosa
4.5–6	Formation of nasal grooves
7–8	Mesenchymal proliferation forming a miniature nose with two nasal cavities, with anterior nostrils and posterior choanas
4.5–7	Formation of the olfactory nerves
5–8	Formation of the vomeronasal grooves
5–13	Presence of sensory cells in the vomeronasal regions
5.5–7.5	Reactivity to touch on the surface of the nose (receptive fields innervated by the maxillary division of trigeminal nerve)
5–8	Terminal-vomeronasal pathways differentiated
6–6.5	Formation of the main olfactory bulbs (MOB)
6.5	Formation of the accessory olfactory bulbs (AOB)
7.5–9.5	Trigeminal nerve differentiated (ophthalmic and maxillary divisions)
8–10	Reactivity to touch on the main olfactory mucosa (receptive fields innervated by the ophthalmic division of trigeminal nerve)
8.5–18.5	Clearly localizable AOBs, but regressive or reorganizational changes occur during this period resulting in a rudimentary structure in older fetuses
11	Evidence of ciliated olfactory neuroreceptor cells
11–18.5	MOBs visualized by MRI. Clearly delineated mitral cell layer in the MOBs
25	Erratic and instable motor responses to mixed olfacto-trigeminal stimuli (in premature newborns aged 25–28 weeks)
29	Reproducible motor responses to mixed olfacto-trigeminal stimuli (in premature newborns aged 29–36 weeks)
30	Discriminative motor and brain (NIRS) response to purely olfactory stimuli (in premature newborns aged 29–36 weeks)
32–35	Olfactory marker protein (marker of functional maturity) in the olfactory neuroepithelium, the olfactory nerve, and glomerular layer within the MOBs

For details about references, see [43.5, 6]

did not differ in the rate of responding subjects from a group of term newborns. Thus, at least during the last gestational weeks, nasal chemoreception can sense an odorant, such as mint, which carries both olfactory and trigeminal properties. Subsequent tests on preterm infants showed that other odorants devoid of the trigeminal effect (nonanoic acid, anethole, vanillin) are efficient stimuli at high and low concentrations [43.24–26]. Thus, olfactory abilities of premature infants tested under aerial conditions right after birth may predict comparable abilities in fetuses of equivalent gestational age.

Another source of evidence for human fetal olfaction is derived from studies on responses of term-born neonates to stimuli which they could only have encountered in utero. For example, choice tests opposing the odors of amniotic fluid differing in familiarity (own versus *alien* fluids) and a control odorant (water) indicated that infants respond positively to any amniotic fluid. But when simultaneously exposed to the odors of their own amniotic fluid and of the amniotic fluid from another fetus, they preferred the most familiar stimulus [43.27, 28]. Thus, amniotic fluids from different individuals are endowed with idiosyncratic odor qualities that 3-day-old newborns can process selectively, indicating their perception in late pregnancy (or their very rapid encoding right after birth).

The nature of the prenatal odor cues that mediate such responsiveness in the neonate is uncertain. The idiosyncratic odor of amniotic fluid may be encoded either as a unique, momentarily dominant odor quality, as

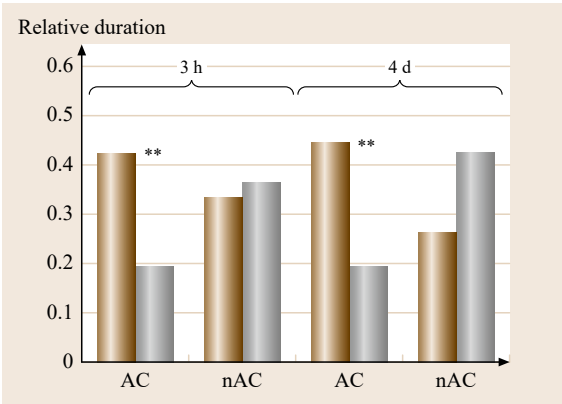

Fig. 43.1 Relative duration of head (nose) orientation of 3 hour-old (3h) and 4 day-old (4d) newborn infants exposed to a simultaneous choice between the odor of anethole (anise) (*yellow bars*) and the control stimulus (*blue bars*) as a function of their mother's consumption (AC) or not (nAC) of (nonalcoholic) foodstuffs containing anise during the last 10 days of pregnancy ($n = 12$/group; **: $p < 0.01$; after [43.29])

a complex chemosensory pattern giving rise to a unique odor blend, as a given intensity of chemosensation, or as several of these possibilities concurrently. These amniotic odor cues may be derived from various sources in maternal or fetal metabolisms, such as the mother's diet, inhaled aromatic compounds, or percutaneously transferred compounds, genetically determined odortypes, or fetal acute reaction to stress (often inducing fetal urine release). One confirmed effect is that odorants introduced into the fetal compartment through the pregnant mother's diet affect subsequent odor responses in newborns. For example, the frequency of maternal alcohol consumption during pregnancy is linked with the 1–2 day-old infants' increase in motor activation when alcohol odor is presented to them [43.30, 31]. Likewise, the maternal ingestion of anise-scented foodstuffs during the last 10 days of pregnancy induces clear acceptance responses to the diluted odor of anethole in the newborns tested 3 hours post-birth [43.29]. In contrast, 3 hour-old infants born to mothers who never consumed anise expressed the rejection of this odor. When retested later on postnatal day 4, the infants that were exposed to anise in utero displayed a clear preference for anethole in a choice test opposing it with a control stimulus, whereas nonanis-exposed infants behaved indiscriminately under these conditions (Fig. 43.1). A convergent postnatal effect was found when mothers ate garlic during gestation [43.32]. Thus, fetal experience with given odorants can foster selective odor responses in neonates, demonstrating that fetal olfactory encoding is active and connected to stable memories for at least several days post-birth (but see in the following, for much longer-lasting reminiscence of fetal odor experience).

In sum, the above results indicate that chemoreception does support the qualitative discrimination of odorants:

1. In fetuses tested in or ex utero (best established through animal research [43.14]).
2. In infants born prematurely up to 2 months before gestational term.
3. In term-born infants exposed to odorants they have experienced previously under prenatal conditions.

Research on animal models (rat, sheep) also demonstrates an early ability to differentiate odorants in terms of intensity and/or trigeminal potency [43.14, 22]. The mere fact of introducing an odorant into the amniotic fluid subsequently appears to increase its perceptual saliency, generally in the sense of reducing its aversive value or of increasing its reinforcing value. However, there are exceptions to this *familiarity-breeds-preference* rule. A recent investigation of human neonates' response to androstenone, an odorous steroid detected in the amniotic fluid, showed indeed that it elicits negative responses, even at levels within the range of concentrations measured in amnio [43.33]. Thus, pure, highly diluted androstenone odor appears to carry negative valence for newborns, as it does in most adults because of irritative effects and/or of intrinsic unpleasantness. In amniotic fluid, androstenone may not be perceived on its own, but as blended into a mixture which perceptual effect differs qualitatively from the percepts of its elementary compounds. Thus, the odor of pure compounds may sometimes be processed as qualitatively different from the odors carried in original mixtures, so that their separate perception can be received as reflecting novelty and, hence, tends to convey negative hedonic valence. Taken together, the above data reveal that nasal chemoreception may be already perceptually sophisticated in the fetus.

43.2 Newborn Infants

When empirical interest in infant olfaction begun in the mid-19th century, it was falsely (see Sect. 43.1) believed that this sense was not stimulated before the first breath and that the fetal brain was a Tabula rasa in terms of odor experience. Neonatal olfaction was then investigated, along with the other senses, in order to

trace the original state of the human mind and consciousness [43.34]. This research was initially driven by adultocentric questions using methods and nonecological stimuli aiming to establish when infants attain adult performance in terms of acuity and discrimination. But more recently, under the impetus of European ethology and American developmental psychobiology, infant olfaction was considered on its own value in fostering adaptive responses that are specific to the early challenges of normal development [43.5, 20, 35, 36]. Accordingly, the interests of initial psychophysical research based on odorants devoid of ecological validity to human newborns progressively shifted toward the use of biological odorants associated with the maternal organism or of artificial odorants previously experienced under rewarding conditions.

43.2.1 Quality

It is often claimed on the basis of differences in the responses elicited by distinct odorants delivered against water or other reference odorants that nasal chemoreception can differentiate odor qualities from minutes after birth. This detection ability was, in fact, a central issue of the very first investigations on olfaction in infants. Since the 1850s, more than 80 distinct odor qualities were delivered to newborns by numerous experimenters [43.34, 37–39], yielding unsure conclusions about the functional status of human olfaction after birth because of unsatisfactory control of stimuli. Often, these stimuli were indeed delivered undiluted, conveying pungency and irritation, and leading to the prevailing assumption that newborns mostly react to the trigeminal side effects of these stimuli.

This situation changed when experimental psychologists investigated neonatal perception using better controlled stimulations and better defined and more objective responses. Using such objective variables, *Engen* et al. [43.40] monitored body and leg movements (using a crib transformed into a kind of actimeter), as well as variations in respiratory rhythm, of 20 newborns aged 32–68 hours in two tests presenting odorants on Q-tips while they were sleeping. From the first test showing that acetic acid was more reactogenic than phenylethyl alcohol, they assumed stronger behavioral potency of trigeminal or unpleasant stimulants. In a second test, the same authors presented the odors of anise and asafetida, two weak stimulants of (adult) trigeminal sensors, which increased neonatal responses. But the latter stimulus was more potent than the former to activate the infants, confirming that an unpleasant odorant appears more potent than a pleasant one to elicit neonatal response. *Engen* et al. [43.40] showed that newborns could differentiate trigeminal from nontrigeminal stimulants by the fact that their responses to the former (acetic acid and phenylethyl alcohol) did not habituate at the same rate and magnitude than their responses to the latter. In a later study, *Self* et al. [43.41] tested infants in their first 3 postnatal days using complex odorants presumed to be free of trigeminal effects, such as anise, lavender, valerian, or asafetida extracts, and water as control. The recorded responses relied on respiratory rate variations and various motor reactions from the face and body. From day 1, two-thirds of infants reacted to most odorants, but also to the water stimulus (nearly 70% on days 1 and 2). Those infants' responses to the odorants increased over 3 days, and decreased to the control stimulus, showing rapid developmental changes in odor reactivity (see also [43.42], in terms of an increase in olfactory reactivity in newborns followed up longitudinally between days 1 and 4). Interestingly, in these studies, newborns could already be sorted into low, moderate, and high responders, but without further causal understanding of such early individual differences.

Surprised by the newborns' high response rate to the scentless water stimulus, *Self* et al. examined that case in two later studies. They first found that sleeping 3-day-old newborns failed to differentially respond to orthonasally presented odors of anise, asafetida, lavender, valerian, and water as the control (for example, in the light sleep state, the newborns ($n = 36$) responded as much to water (54%) than to valerian and anise odors (54% each), and more than to asafoetida odor (27%) [43.43]). In a second study, 3-day-old newborns were habituated to distilled water until they ceased any response on two consecutive trials; subsequently, they were dishabituated to the sensation triggered by water in being presented anise, lavender, valerian, or water stimuli. The rate of subjects responding to these stimuli was 30, 17, 22, and 25%, respectively [43.44], showing again that a scentless humid Q-tip was efficient to elicit responses, and even more efficient than certain odorants delivered at clearly suprathreshold values. This result raised the authors' doubts about the functionality of neonatal olfaction. Alternatively, it may be that some unnoticed parasite stimulus contingent with testing was ongoing (uncontrolled odor released by humidified cotton or from experimenters' hand; noise or air flux associated with experimenter's movements) or that newborn infants are chemosensitive to the hygrometry of inhaled air. In the first 3 days following birth, infants are indeed in a state of relative dehydration, as they can lose physiologically up to 10% of their body weight, and they may accordingly exhibit increased chemoreactivity to stimuli conveying humidity. The possibility of water sensing in newborns is open to further investigations, although recent data using near-

infrared spectroscopy (NIRS) did not find water-related activation of the orbitofrontal cortex in newborns aged 6 hours to 8 days [43.45]. But this may not exclude differential responses to the vapor of water at peripheral levels of the olfactory and/or trigeminal tracts.

In contrast to Self et al. mentioned above, *Engen* and *Lipsitt* [43.46] demonstrated the neonatal discrimination of odor qualities in applying the habituation paradigm to odor mixtures and their components. They first habituated respiratory disruption to a mixture of anise and asafetida, and then showed the recovery of the initial response to asafetida, but not to anise. As similarity assessments by adults indicated that the anise compound was dominant in this mixture, they inferred that newborns could not differentiate it from the whole odor of the mixture. They repeated then the same habituation procedure with a mixture (heptane-amyl acetate) the odor of which differed from that of its components. In this case, both components were efficient to dishabituate the infants' respiratory disruption, but the magnitude of the dishabituation of each compound was proportional to its dissimilarity with the mixture. Engen and Lipsitt concluded that 3 day-old newborns can not only discriminate odors, but they also can detect similarities between odors much like adults do. Later investigations focusing on odor *preferences* (see below) further confirmed the ability of human neonates to differentiate odor qualities. These studies generally indicated convergent odor-quality evaluation in infants and adults, although some studies reported notable differences [43.47].

Several psychophysiological investigations also support the notion that odor qualities are discriminated in the first postnatal days, and even before as attested by preterm infants' responses. Although some earlier studies indicated indiscriminative electro-encephalographic responses to the odors of coffee, citral, vanillin, and pyridine in less than 6 day-old infants [43.48], others found differential EEG responses in neonates for the odors of orange versus mother's milk [43.49]. In more recent studies using NIRS imaging, the odors of vanilla and of mother's colostrum were shown to elicit patterns of oxygenated hemoglobin (indicative of local vascular activation) over the left orbitofrontal region that were different in both magnitude and time-course [43.45]. A related NIRS study showed differences in oxygenated hemoglobin in the orbito-frontal cortex caused by the odors of the mother's milk and formula milk, the former causing on average a larger response than the latter [43.50].

43.2.2 Intensity

Research on the early capacity to process intensity cues from odor stimuli (i. e., differentiating stimuli triggering a weak odor sensation from stimuli triggering a strong odor sensation) has so far ended in inconsistent results. *Rovee* [43.51] thought it possible in 3-day-old infant because of the enhanced amplitude of their motor response with increasing concentration of aliphatic alcohols delivered orthonasally. The increasing mobilization of defensive responses (negative orientation, increased fussiness and crying) due to the increased recruitment of trigeminal chemoreceptors is probably responsible for this effect (see review in [43.52]). But in another study, increasing concentrations of vanilla, an odorant considered free of the trigeminal effect [43.53], or of butyric acid in low concentration levels, did not greatly affect latency, amplitude, or duration of variations in neonatal respiratory or facial responses [43.47]. Thus, one can only suggest here that much more research is needed on the infant's perception of odor intensity and on the interactions of the trigeminal and olfactory subsystems in explaining the early processing of chemical stimuli.

43.2.3 Position in Space

The ability of nasal chemoreception to sense spatial cues has long been discussed to occur through inter-narinal temporal differences of olfactory [43.54] or trigeminal stimulation [43.55]. *Kobal* et al. showed, for example, that odorants bearing trigeminal effects could be laterally localized, but that odorants devoid of trigeminal effects could not. Trigeminally mediated orientation was noted in newborns, which, on average, turned away from pungent ammonia vapor [43.56]. But such negative head-turns were easier to elicit when the ammonia odor was presented from the left side, that is, when the irritative/aversive nature of ammonia corresponds with the strong rightward bias of neonatal head-turns. Another study on odor-induced head turning of neonates used nontrigeminal odors chosen to be hedonically positive or negative (as determined a priori by adults). Positive vanilla and almond odors elicited significantly more *toward* than *away* head-turns when they were delivered to the left nostril, as compared to when they were delivered to the right nostril [43.57]. A similar trend was seen for the negative rotten egg or sour milk odors. Thus, the nature of the odor and the stimulated side of the nose (and brain) interact in determining an infant's response.

Under experimental conditions where the movement of the whole organism is considered, human adults were shown to be able to creep in following an odor-track left on the soil through bilateral sampling actions (active sniffing) along this track [43.58]. Infants' performance in localizing odor intensity gradients may be similarly facilitated by sampling movements, such as those involved by newborns' bilateral rooting move-

ments toward nipples. But it is not clear whether active sniffing co-occurs with neonatal rooting actions. As will be further described, such infants' abilities to turn their head (and hence nose) toward natural odorants has been largely used to examine their discriminative and hedonic performances.

43.2.4 Familiarity/Novelty

The detection of familiarity or novelty is increasingly thought to be the very first *dimension* involved in the perceptual sequence of olfactory analysis, even before the hedonic screening of the stimulus [43.59, 60]. The faculty of sensing familiarity/novelty in odor stimuli depends on the functionality of detection and of all steps of memory from encoding to retrieval. In adults, familiarity with an odor noticeably increases its detectability and discriminability [43.61], and similar processes may operate in early development. But almost no research has been conducted on this topic in early development.

However, familiarity effects have been reported from the neonatal period onward as infants orient or mouth more insistently toward stimuli encountered previously during reinforced interactions with the mother [43.62–64] or as mere contextual cues. Odorants can indeed be recorded as familiar by neonates when merely diffused into the sleep environment for several hours [43.65]. Even preterm infants can already encode and retain an odor introduced in their incubator's atmosphere [43.26] and olfactory familiarization can occur even earlier in the fetus (see earlier). The above-mentioned research with newborns shows indeed that they are more attracted to the odor of their familiar amniotic fluid than to the odor of unfamiliar amniotic fluid, both stimuli being attractive against water.

Odor familiarity effects are well illustrated by everyday or experimental situations that consist in deodorizing or in odorizing given contexts/objects, namely, in disrupting an infant's expectation of olfactory continuity. For example, when the natural odor of mother's breast is adulterated by adding a novel odorant, newborns refuse to latch on the strangely odorized nipple, often becoming fussy and crying, while they immediately accept the other, intact nipple [43.38, 66]. Likewise, 3 day-old neonates manifest less frequent appetitive oral movements after the odor of their mother's breast is abolished by covering it with a scentless alimentary plastic film [43.67]. Thus, newborns react to violations of odor cues habitually associated with the mother, and probably also with the wider environment (especially the sleep environment or food). But newborns are also highly plastic under these conditions as they rapidly learn novel odor cues introduced in their environment [43.64, 68], especially when these novel odors are brought together with a familiar odor background and with sensory inputs from the other senses [43.69].

Multiple ways of olfactory familiarization operate in newborns, including nonassociative processes, such as exposure repetition (habituation) or more or less prolonged mere exposure (which we here call *familiarization*), and associative processes, such as evaluative, classical, or operant conditioning, social learning or individual learning by trial and error. Such processes by which the brain acquires an odor as familiar or *known* may operate more easily under certain conditions (e.g., suckling at the breast) or at certain times in development, delimiting so-called sensitive periods. For example, an exposure period of only 30 min after birth is sufficient to shift an odorant from novelty to familiarity, but when this 30-min exposure is made 12 h after birth, this shift is not effective, and the odor does not become especially attractive [43.70]. This result, already known in neonates of other species [43.71], has been related to a special neurochemical stage associated with the normal birth process and which eases the encoding and/or retention of odors. Thus, studies of perception, perhaps especially in early development, should always be precisely situated in the background of an organism's prior or concurrent physiological or affective states.

43.2.5 Hedonic Valence

Numerous studies have sought to determine whether odorants have an inherent affective value, and thought resolving this issue by testing newborn infants because they were considered as devoid of any prior odor experience. In addition to this latter false assumption, the testing of newborns was, moreover, mostly run in comparison with adult performance, leading to assess neonatal response to odorants known to evoke feelings of pleasantness or unpleasantness in adults. From the first postnatal hours, odors considered to be pleasant for adults (e.g., orange, geranium) were indeed reported to evoke appetitive oral activation in infants, whereas odors considered as unpleasant for adults (e.g., asafetida) were reported to cause facial and oral expressions of disgust and head-turning away from the odor source [43.72]. *Canestrini* [43.39], and later, *Pratt* et al. [43.73], established that odorants (acetic acid, valerian, and clove) elicit facial, oral, and sucking responses in 1−11-day-old infants. *Stirnimann* [43.74] also established differential orofacial expressions emitted by newborns, including a premature infant born at 6 gestational months, in response to odorants that are pleasant or unpleasant to adults.

These initial studies were systematized by *Steiner* [43.75, 76] who exposed newborns less than 12 h of

age, before any postnatal experience of ingestion, to cotton buds soaked with odorants known to provoke pleasant (banana, vanilla, milky) or unpleasant (shrimp, rotten egg) sensations in adults. These stimuli were presented at high concentrations, via the orthonasal route, while the infants' facial reactions were photographed at their apex. The pictures of the infants' facial response were then evaluated by a panel of adult judges who ignored the nature of the presented odorant and had to guess whether it was pleasant, unpleasant, or indifferent. Generally, the odorants considered pleasant by adults induced facial responses read as expressing pleasure and acceptance (relaxed facial muscles, raising of mouth corners, licking and sucking), whilst unpleasant odorants elicited expressions read as reflecting dislike and disgust (lowering of mouth corners, lip and tongue protrusion, gaping) (Fig. 43.2). Steiner assumed that facial expressions that were morphologically similar in neonates and adults imply the same underlying affective functioning, and accordingly that some odors are more pleasant than others for newborns as they are for adults. He proposed that neonatal facial reactivity to odors depends on a hard-wired *hedonic monitor* that he located in the brainstem after having tested an infant lacking cerebral cortex and who responded to banana, fishy, and rotten egg odors by facial expressions similar to those of normal infants. He concluded the *olfacto-facial reflex* to be innate [43.76]. However, this study used high-intensity stimuli [43.76, p. 274] reporting that some of them were *offensive*, so that trigeminal percepts might in part account for the responses. In addition, a firm conclusion on the *inherent* hedonic value of odorants would have needed to ensure that such odorants were never experienced in utero. Finally, the term reflex means a high level of automaticity and inter- and intra-individual repeatability of the response, which are difficult to deduce from a response captured at only one time on a single-shot picture.

In an attempt to replicate and extend Steiner's study, *Soussignan* et al. [43.47] used highly diluted odorants intensities of which were matched (by adult noses) to the natural odor of several biological substrates infants encounter in their everyday life, such as amniotic fluid, human milk, and infant formula. The infant's facial-oral responses were videotaped in the same time as their respiratory rate was recorded. The 3 day-old newborns were exposed (in different states of arousal) to 12 stimuli, including 4 unfamiliar biological odorants (amniotic fluid, human milk, and two infant formulas), 4 dilutions of vanillin and of butyric acid matched by adults on the intensity of these biological odorants, and 4 odorless controls. The infants' videotaped oral–facial responses were then decoded in using a quantitative coding system devoid of any a priori hedonic construal,

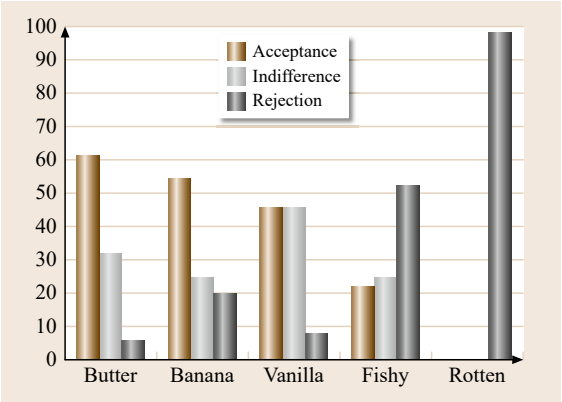

Fig. 43.2 Distribution (in %) of hedonic judgments (in terms of acceptance, indifference or rejection) by 6 adult judges based on photographs of the facial responses of infants ($n = 25$) exposed to 5 undiluted odor stimuli presented on Q-tips. The infants were aged less than 12 h at the moment of testing and had no postnatal ingestion experience (after [43.76])

Ekman and *Friesen*'s [43.77] Facial Action Coding System that itemizes the movements of each of the 44 facial muscles. The patterns of newborn response to the odor stimuli were then interpreted using prior data from newborns and infants exposed to stimuli of known affective valence (sometimes in other sensory modalities, such as taste, where stimulus-related hedonic responses were more clearly established). Under such conditions, neonatal facial actions to the different odorants were highly variable between individuals and could thus not be considered *automatic*, as predicted by Steiner's olfacto–facial reflex model. However, the infants' facial responses were differentiable (from that elicited by the control) as a function of the hedonic value of the odorant, but in an asymmetrical way. While unpleasant butyric acid odor elicited more negatively valenced responses than vanilla odor, the pleasant-to-adult odor of vanilla did not elicit more positive facial responses than butyric acid. Both odorants were very low and matched in subjective intensity (at least for adult noses); the negative response could only be due to the neonates' detection of qualitative and/or hedonic cues in the odors, thus partly confirming Steiner's view. Although clearly unpleasant to adults, unfamiliar infant formula odors did not elicit negative facial responses in newborns. Therefore, at least in these cases, the hedonic cues that newborns and adults detect in odors did not appear to overlap.

Odorants affect various responses controlled by the autonomic nervous system (i. e., heart and respiration rates, release of endocrine and exocrine secretions in the digestive tract). Conversely, the metabolic state

(hunger/satiety) influences hedonic responses to odors and this bottom-up regulation of sensory responsiveness operates already in newborns. When 3-day-old bottle-fed infants are exposed to the odor of their usual formula milk 1 h before a feed, they expectedly display positive facial responses. But, when this same test is run 1 h after a feed, when they are satiated, their faces express clearly negative responses [43.78]. This fluctuation in hedonic responsiveness to a food-related odor in infants resembles the phenomenon of negative alliesthesia described in adults. Thus, even in newborns, aged several days, a participant's motivational state is an important factor of variation to be considered in the testing of olfactory performance.

Neonatal hedonic processing is more strongly corroborated by studies involving an active choice response between two paired odorants. When infants simultaneously face two attractive odorants, they are able to express which one they prefer most in terms of head (nose) orientation and of mouthing movements toward one stimulus more than toward the other. *Macfarlane* [43.62] was the first to use such behavioral indices in a two-choice paradigm to infer olfactory discrimination and preference in neonates. He videotaped newborns lying supine while they were exposed to two odorous gauze pads hanging alongside each cheek. In a test in which the odor of their mother's breast was paired with the odor of another lactating mother's breast, 2 day-old newborns turned their nose equivalently to either stimulus, but 6 day-old newborns oriented significantly longer toward the mother's breast odor, suggesting that the individual odor of the maternal breast is learned as rewarding during the first week. Using a less demanding indicator of discrimination (viz., the mere reduction of motor activity of head and arms), another study showed, however, that such discrimination could be effective from 2 days of age [43.63]. Subsequent series of experiments made clear that lactating women emit from their breasts an odor factor that is attractive to newborns, even when mothers and newborns are unrelated [43.79].

In the same way, human milk conveys an odor factor that is attractive for any newborn [43.80], although breastfed infants prefer their mothers' milk odor to the odor of an unfamiliar mother's milk odor [43.81]. Thus, human milk conveys multiple odor cues that are either proper to the newborns' own mother, or common to all post-parturient, lactating mothers, and it appears that newborns can differentiate between them. The preferential responses elicited by odor compounds from the lactating breast or from human milk do not seem to depend on previous direct exposure to the breast or milk, as infants exclusively fed formula (based on cow milk) from birth also express them. However, the positive behavioural effects of these biological odors on newborns cannot be excluded to depend on their exposure to (at least partly) similar odor compounds in the amniotic fluid.

These results raise the issue of whether some odorant compounds of biological origin have a special significance in early development, for example, in favoring the unconditional expression of attraction or appetence or soothing in newborns [43.82, 83]. As suggested above, the odors of conspecific milk or that of the breast during lactation may emit such inherently reactogenic compounds. Potential sources that are common to milk and breast are the secretions of the areolar glands of Montgomery, which enlarge during lactation and can give off a latescent fluid made of milk and sebum [43.84, 85]. When this Montgomerian secretion is presented separately under the nose of asleep newborn infants, it acutely elicits mouthing responses and stimulates respiration, even when these infants were never exposed to breastfeeding before the test [43.86]. This Montgomerian secretion may play a special role in the infant's attraction, coordinated oral and respiratory actions to the lactating breast [43.87].

43.3 From Preverbal Infants to Prepubertal Children

Beyond the neonatal period extending during the first postnatal month, infants and children undergo biological, psychological, and social-ecological changes, the range and depth of which are obviously considerable. Their body and more so their brain grow by several orders of magnitude, their perception becomes increasingly multisensory and embodied in action (although they already are from the newborn period; cf. [43.69]), their eating modes and digestive processes change from exclusive lactivory to omnivory, their emotional style stabilizes in temperamental phenotypes, their cognition becomes increasingly dependent on language which expands their semantic and symbolic processing abilities, and from initial selective attachments to primary caregivers they integrate more and more complex social networks. All sensory systems continue their maturational agenda along that protracted childhood period which ends with puberty (note that the olfaction-related specificities of puberty is beyond the scope of this chapter). The perceptual performance of these sensory

systems is shaped by gene–brain–environment interactions in the experiential context provided by caregiving, education, and the local culture at large [43.88]. Olfaction certainly contributes to all of these facets of development, although we only have small snapshots on how it works at given times and in different situations. In the underlying context of developmental change and exposure to multiple and complex experience, infants' and children's abilities to extract different meanings from their odor world should, in principle, expand rapidly. But, as in neonates, the sensory impact and deferred consequences of odor experience are not linear across development and appear far from being homogeneous across individuals.

43.3.1 Quality

Several lines of evidence indicate that toddlers and children are fairly reactive and discriminative toward odor qualities. First, behavioral measures indicate that preverbal children are differentially affected by distinct odorants, whether these are presented as features of objects or of contexts. When odors are presented on age-adapted objects (fluffy toys, rattles, toothing rings, bottles) in age-adapted protocols, infants react to them more or less clearly. For example, 9 month-old infants given a succession of objects scented pleasantly, unpleasantly, or neutral (for adults) failed to yield conclusive data in terms of behavioral discrimination at first sight, although a systematic fine-grained coding of related videotapes indicated that the odor quality of the handled object was subtly differentiated [43.89], especially by girls who explored the odorized toy more than the matched scentless toy [43.90]. However, this gender difference in odor *attention* was not evident in a study investigating food odor discrimination (presented on bottles) in infants followed up between 8 and 22 months of age [43.91].

Persons themselves are ecological conveyors of scent qualities through their natural body odor or artificial odors (perfumes), or their mixtures, and infants and children clearly attend to these qualities. Natural odor signatures are indeed accurately recognized by infants and children, as shown by the recognition of a T-shirt worn by their mother (in 3.5–4.5 year-old children; [43.63]), by siblings (3–7 year-old children; [43.92]), or by an unrelated friend presented among distractor T-shirts from other same-age children (4–5 year-old children [43.93, 94], 9 year-old children [43.95]). Children are also able to accurately categorize gender from body odors (9 year-old children [43.95]). They can further identify the artificial perfume used by their mother: among six odor stimuli presented in small flasks, including four perfumes (among which the mother's one was surreptitiously introduced) and two controls, 5 year-old children were required to rate their preference for each stimulus, to tell which one they would use to self-scent, and lastly to identify their mother's perfume. Although under these conditions these children failed to express reliable preference for their mother's perfume, they nevertheless expressed greater wanting for it to use as a body scent, and accurately recognized their mother's perfume among the reference stimuli [43.96].

Toddlers and young children also react to the odor quality of contexts. For example, [43.97] exposed 1–2 year-old infants to the odors of lavender, amyl acetate, butyric acid, and dimethyl disulphide (as well as water as control) while they were absorbed playing a game at a table. Although more than 48% of the stimulations did not lead to any discernible facial reaction, the remaining responses indicated differential responsiveness to the odors (more details on Bloom's study in the following section on hedonic responses). In another series of interesting studies, 3 month-old newborns had to fulfill Carolyn Rovee-Collier's operant conditioning paradigm: they had to realize that their moving leg can activate a hanging mobile to which it was attached by a string. When this association between kicking and setting the mobile in movement was established in the presence of an odor, and when this odor was delivered to assess the retention of the contingence 1, 3, and 5 days later, the infants re-exposed to the same odor remembered well the conditioned response. In contrast, those in the control group exposed to no odor exhibited only partial recall, while those exposed to a novel odor displayed no signs of remembrance [43.98–100]. Thus, contextual odor qualities are detected and encoded by infants, and on subsequent occasions, they are differentially used to facilitate memory retrieval. The better recall of the conditioning when infants are re-exposed to the odor they faced during the acquisition phase may be due to the absence of emotional and/or attentional interference caused by olfactory contextual novelty [43.60].

Research studies assessing brain response to odorants in infants and children give another argument in favor of odor-quality perception. But, so far, this approach remains inconsistent and underlines the decisive nature of the stimulus and recording conditions to reveal brain responses. While some studies reported undifferentiated EEG responses to food odors in wake infants [43.101], others revealed differential brain activation to distinct odor qualities. For example, olfactory evoked response potentials (OERP) were recorded in 3.5–5 year-old children in response to odorants administered orthonasally with an air-dilution olfactometer. The late positive peak of OERP increased in latency

with age, a change which was provisionally interpreted as reflecting more in-depth perceptual and semantic processing in older, as compared to younger, children [43.102]. But much more psychophysiological research is needed with infants and children.

Some attempts were made to standardize psychophysical testing of olfaction in very young children. But these efforts were generally based on methods designed to test older children or adults, and they revealed to be globally inadequate before the age of 4–5 years [43.102]. The application of psychophysical approaches to children older than 4–5 years was more frequent, when language mastery is sufficient to fully understand procedures (An attempt was made to devise an olfaction test for 3-year-olds [43.103]). Several tests were used to quantitatively assess children's olfaction, firstly for clinical purposes. (e.g., University of Pennsylvania Smell Identification Test (UPSIT) [43.104]; Match to Sample Odor Identification test [43.105], Sniff Magnitude test [43.106], Biolfa test [43.107], San Diego Odor Identification test [43.108], Sydney Children's Hospital Odor Identification Test [43.109], Sniffin Sticks test [43.102], Lyon Clinical Olfactory Test [43.110], and NIH Toolbox Pediatric Odor Identification Test [43.103]). Each of these tests has its advantages and limitations (see [43.111], for more details).

In most children's studies, odor-quality perception is generally conceived in terms of an individual's knowledge of the accurate identity of an odor source. Related research used various tasks involving suprathreshold identification and naming in children [43.112–115]. In simple discriminative tasks where the verbal identity of the stimuli did not matter, 5–8 year-old children's performance nearly equalled adult performance (in similarity judgments [43.116]), or were slightly below it (oddity test [43.117]). But when the verbal recognition of odors was required, children were clearly lower as compared to adults, with the magnitude of this disadvantage depending on the task, children's age/gender and familiarity of odor stimuli. Some normative tests were implemented to characterize how odor identification develops over wide age spans. For example, the University of Pennsylvania Smell Identification Test (UPSIT), which is composed of 40 suprathreshold odorants each cued by 4 response alternatives as words or icons to lessen age/individual variations in mnesic proficiency, was applied cross-sectionally over the lifespan. The performance of smell identification followed an n-shaped curve, the average recognition score of 5–9 year-old children being equivalent to that of adults in the 7–8th decades, when olfactory abilities appeared to sharply decline (Fig. 43.3). But by the age of 10–19 years, children's UPSIT scores reached as high as adults' highest levels of performance [43.104]. Otherwise, across the 6–21 year age-range, females had better UPSIT recognition scores than males [43.104], suggesting female advantages in either sensitivity, cognitive abilities relevant to language or memory, or familiarization with odor qualities. Although such standardized approaches allow for the establishment of norms of olfactory performance that may have some validity in clinical settings, they certainly underestimate the abilities of olfaction in the everyday life conditions of infants and children (and even adults).

As younger children are clearly disadvantaged in the identification of odor qualities that require naming of a veridical source, some researchers investigated odor-quality recognition in terms of wider categories or affordances of source objects (e.g., eatability, wearability as perfume) [43.113, 118]. This strategy revealed children's much more elaborate odor cognition than was thought earlier, even under cultural conditions which do not favor training and teaching of olfaction (as in Western or Westernized general lifestyle). For example, when 8–14 year-old American children had to tell the identity of 17 odorants and whether they belong to edible/nonedible categories, they were expectedly lower than young adults by accurate naming of the odor source, but equalled them by the edibility criterion [43.113]. A related study asked French 5–6-year-olds to classify 12 odorants along criteria, such as origin (in-/outdoor, natural/artificial, vegetable/animal) and subjective function (edibility, wearability) [43.119]. While these children categorized the odorants accurately for edibility, and moderately ac-

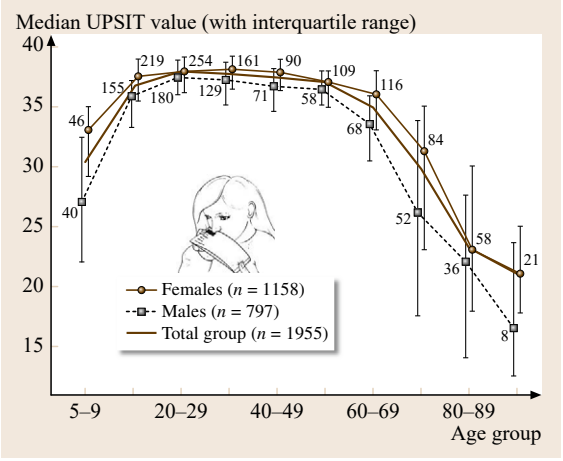

Fig. 43.3 Olfactory scores in a heterogeneous group of subjects submitted to the University of Pennsylvania Smell Identification Test (UPSIT) along the lifespan, presented by age in decades and by gender (after [43.104])

curately for wearability, they could not use the more abstract (or less useful) classificatory criteria. Another important study required 4–11 year-old French children and adults to rate 6 fruit and 6 flower odors in terms of typicality, familiarity, liking and edibility, and to classify them as members of either fruit or flower categories [43.118]. Results indicated a high classificatory agreement from age 5 years for some odorants in the series. But, interestingly, these children did not overlap in their semantic (fruit versus flower) and hedonic categorizations, suggesting that while processing odors, young children can separate semantic from emotional operations. From age 8 years, most odorants were systematically classified in the expected category, as well as assigned a name in the correct semantic category. Finally, when children were required to assign an odor one color (selected among several colors in a chart), they came out with nonrandom odor–color associations that reflected the underlying knowledge of the color of the odor source [43.120, 121]. Thus, long before the ability to accurately name odor sources is acquired, the ability to attribute self-centered functional or semantic meanings to odors prevails in infant and child cognition.

43.3.2 Intensity

As noted above for newborns, our understanding of children's perception of the subjective intensity of odors remains limited. We know that children are as accurate as adults to express quantitative judgements about odors. When participants aged 6–94 years had to evaluate the subjective intensity of 7 dilution steps of propanol in displaying simple finger actions (index–thumb distance) commensurate with their intensity estimates, psychophysical functions were stable at all ages [43.122]. Furthermore, in 7 year-old children as in adults, the intensity judgments correlate negatively with preference judgments for many odorants [43.1]. However, other sources suggest that children may diverge with adults in intensity judgements. For example, when 5–6 year-old French children were compared with a group of young adults for their rating of the subjective intensity of a set of 6 odorants, children rated the perceived intensity of the odors higher than the adults. This may be explained in terms of higher olfactory sensitivity in children or in age-related differences in the use of rating scales [43.123]. Thus, sensing of intensity cues in odors is an important part of olfactory development expectant of further investigation.

43.3.3 Familiarity/Novelty

Infants and children are clearly able to extract familiarity or novelty information from odor impressions, and such abilities are basic to their olfactory attention and attraction or avoidance responses. The so-called *Proust effect* is an extreme demonstration of this ability to acquire and retain over long periods of time – sometimes life-long – familiar odor imprints of given contexts, foods or persons, or of given internal states. For example, *Mennella* and *Beauchamp* [43.124] and *Delaunay-El Allam* et al. [43.125] assessed the behaviour of infants (aged 6–13 and 5–23 months, respectively) while they handled identical objects carrying different odors, one of which had been associated with the mother's breast. The infants expressed more positive facial and bodily reactions toward the objects that carried the odor to which they were familiarized earlier. Another study [43.126] introduced olfactory novelty to assess odor detection in 7–15 month-old infants; videotaped in consecutive sessions while playing the first time with scented or unscented versions of visually/tactually similar, attractive toys, these infants manipulated and mouthed the unfamiliarly scented toy less and for lower duration (than the nonscented toy), indicating relative withdrawal from olfactory novelty against all other highly attractive objects' features.

In a loose sense, the notion of familiarity means that one recognizes that something has been encountered previously, without knowing precisely what and when: *I already smelled that before*! A contrario, infants and children react to the irruption of olfactory novelty into familiar situations, a circumstance that has rarely been exploited in developmental studies of olfaction. For example, it is a common observation that soft objects (blankets, clothing, fluffy toys, etc.) are carried around and used as a *secure base* by a great proportion of toddlers in Western cultures at the age of 1.5–2 years when they move away from the mother [43.127, 128]. But the use of such *attachment objects* goes far beyond toddlerhood. A recent survey in 6–10 year-old French children showed that 96% of them owned or still own one such object, and at the moment of the study between 82% (at 6 years) and 52% (at 10 years) were still fond of them, and especially of the odor they themselves, or their mother, impose on it [43.129]. Among these children, 27% reported to react to the odor change of the attachment object after its washing, some in negative terms, others in positive terms. In infants and younger children, the odor constancy of such attachment objects may be more important than in older children, however, and their importance as a *transitional object* [43.130] has been recognized by many behaviour scientists or clinicians to manage disturbances caused by mother–infant separation [43.5, 128, 131–133]. Infants and children do also very easily pick up contextual olfactory side-effects of emotionally arousing events and implicitly label them in negative or positive terms

depending on the emotional nature of the situation (see in the following). When such odor cues are subsequently re-encountered, they can re-evoke the affective states in which they were acquired [43.134, 135].

In stricter acception, the notion of familiarity was often operationalized in terms of the ability to accurately name an object, person or context, or categories to which they may belong (see above). Numerous studies have sought to assess odor identification and naming in children of various ages [43.104, 110, 112, 113, 117, 119, 136–139]. Odor-identification tasks were variable across this set of studies, generally based on the free remembrance of an accurate name or, to make mnesic effort more easy, on a choice among 4 or 5 alternative words or images. Under these conditions, younger children had generally lower identification and denomination performances than older children and adults, who themselves have generally low abilities to name accurately the exact source object of an odor. Difference with adults tended to fade when children were semantically primed by a name/picture to the identity of the odor, indicating that early limitation in odor-recognition memory may depend on producing/remembering explicit verbal associations [43.60].

The general effect of age further interacts with gender, girls being generally better than boys in odor identification and naming. This age by gender interaction is often interpreted as resulting from superior verbal fluency or broader odor-related semantic knowledge in females, even from early ages on [43.110, 129]. But this apparent gender bias may originate in antecedent cultural factors related to the explicit or implicit education of sex roles. A questionnaire study prompting self-reports of active seeking of awareness and affective reactivity to odors of food, people, and the environment in French children aged 6–10 years revealed indeed that girls were more olfaction-oriented than boys, especially in everyday life domains related to the body, to odor cues in the general environment, and to the actual use of scents as means of self-comforting [43.129]. This gender bias was confirmed in Czech and Namibian children based on the same questionnaire approach [43.140].

43.3.4 Hedonic Valence

During infancy and childhood, odor-based preferences fluctuate until a time when they come to stabilize and more or less overlap with those of surrounding adult culture. Infants' and children's hedonic reactivity to odors and its alignment with adult preferences have been (and still are) a source of long-standing theoretical arguments on how olfactory preferences emerge and stabilize [43.1, 83, 141].

Early studies report young children's relative tolerance to odorants that are unanimously rejected by adults [43.141]. For example, based on behavioural or declarative responses of (hospitalized) Hungarian participants aged 1 month to 16 years to the offensive odors of oil of Chenopodium, trimethylamine or asafoetida, *Peto* [43.142] pinpointed age 5 years as a turning point in the hedonic appraisal of odors; before that age, negative responses occurred only in 3% of the subjects, whereas beyond that age 72% expressed dissatisfaction. A convergent pattern was noted among American children's responses sampled between 3 and 14 years age groups [43.143] with fruity (amyl acetate), sweaty, and fecal odorants: 3–4 year-old children were reported to find all stimuli equally likeable, whereas older children discerned fruity from sweaty and fecal odorants, and clearly rejected the latter. As Peto and Stein et al. were of psychoanalytic obedience, they interpreted their findings as perceptual correlates of the resolution of the anal phase. Using better controlled methods, *Engen* and *Katz* [43.144] later found that the liking response for the unpleasant odor of butyric acid drops sharply between ages 4 and 5–6 years, while the liking for pleasant odorants remains steady. In an effort to elucidate this apparent shift in the hedonic appraisal of odors around age 5 year, *Engen* [43.1] suggested the involvement of a nonspecific age-related response bias in a situation where an infant is questioned by an unfamiliar experimenter, rather than endorsing less parsimonious psychoanalytic explanations. He showed that, at 4 years of age, American children smelling an unpleasant odor answer more often *yes* than *no* to either questions *Tell me if it smells pretty/... ugly*? This tendency fell sharply after 5–6 years, when the answers *no* and *yes* prevailed to the former questions, respectively. Thus, a tendency to acquiesce to a dominant adult experimenter may best explain why younger children report positive responses to negative odorants. A similar conclusion was reached when facial responses to odors were recorded; the mere presence of an adult experimenter who presents odor stimuli to the children participants biases the communication of their hedonic feelings, and more so in girls than in boys ([43.145] see in the following).

Studies asking American or British children older than 6 years to rank series of odorants in terms of pleasantness resulted in responses roughly similar to those of adults [43.141, 146]. However, children ranked some odorants bearing fecal notes lower in unpleasantness than adults did [43.141], suggesting either that rating scales are used in different ways at different ages or that preferences assessed through relative judgments (rank ordering or pair comparisons) are more prone to age-related variations [43.147]. Finally, a study compared

6–12 year-old children (males only) from contrasted cultural backgrounds in Québec, Syria, and Indonesia for their ranking of 14 odorants on a hedonic scale ranging from very unpleasant to very pleasant [43.148]. Their responses were consensual across cultural groups for the most negative odors (although with between-group differences), while the most preferred ones were very variable between cultural groups, reflecting locally appreciated scents. Thus, verbal responses to pleasant and unpleasant odorants appear asymmetrical in infants and children, unpleasant stimuli generally eliciting more consensual responses than pleasant stimuli (a point already noted above in neonates).

Do measurements based exclusively on behavioural variables (devoid of any verbal declaration) provide more reliable developmental trends about odor preferences? In the study by *Bloom* [43.97] already mentioned above, pleasant (lavender, amyl acetate) and unpleasant odors (butyric acid, dimethyl disulphide) were discreetly sprayed in front of 1–2 year-old children who were playing at a table. Their facial reactions were rare under these conditions as most odor puffs were inefficient to cause a response detectable to hidden observers. But when analyses were restricted only to the stimuli that effectively elicited a facial response, pleasant odorants triggered more positive than negative facial actions, and conversely for unpleasant odorants. The hedonic ordering derived from these data in 1 and 2 year-old children was consistent with that predicted by adult ratings, that is, from best to least preferred, lavender > amyl acetate > butyric acid > dimethyl disulphide. When these same odorants were presented to 3, 4 and 5 year-old children, their facial reactions led to the same hedonic ordering of them. Thus, at least for these four odorants, orderly preferences begin to develop in 1 and 2 year-old children and appear stable through age 5 years [43.97]. But even earlier, by age 9 months, American infants act differentially on identical objects scented pleasantly or unpleasantly for adults (using methyl salicylate and butyric acid, respectively) or not at all (mineral oil) [43.89]. While some infants expressed reliable hedonic facial actions, others seemingly ignored the scented object. A similar test situation in 7 and 21 months-old infants came out with more positive (as indexed by object mouthing) and less negative responses for an odor encountered during breastfeeding in the first postnatal weeks [43.125]. Finally, a greater consensus about disliking odorants which are unpleasant to adults was also documented in infants tested longitudinally at 8, 12, and 22 months of age with 8 odorants (four pleasant, four unpleasant) presented in bottles [43.91]. At the three ages, and similarly for girls and boys, the duration of mouthing was shorter for unpleasantly-scented bottles compared to pleasantly-scented bottles. From this and earlier studies [43.19, 47, 76, 125, 149, 150], one may thus hypothesize more generally that negative responses of infants appear clearer in communicative value to adult observers than positive responses.

However, returning to the issue of whether behavioral responses are more reliable than declarative responses to assess odor preferences, one may highlight that both types of responses can be strongly biased by the social context of experiments. The accuracy of facial cues to reveal an infant's or child's hedonic experience can be mitigated by the social context, in terms of exaggeration or inhibition of facial responses [43.145]. For example, when the odor stimuli were presented by an unfamiliar adult, as compared to when they were self-administered by the children themselves, 5–12 year-old children responded more positively to pleasant odors and less negatively to unpleasant odors (and girls were even more sensitive to the social context than boys). A less time-consuming alternative to facial responses in vivo or *in video* was proposed through electromyography (EMG), the recording of electrical activity of facial muscles before any visually detectable response. EMG has been used successfully in 7 year-old children exposed to pleasant versus unpleasant odorants [43.151]. However, EMG responses are far from being totally immune of social constraints [43.152].

Another approach to assess the development of hedonic processes in olfaction has relied on the subject's ability to translate hedonic judgments from odors onto visual stimuli eliciting pronounced judgments of value. For example, *Strickland* et al. [43.153] required 3–5 year-old children to inhale hedonically contrasted odorants and make the dichotomous decision to place them on *smileys* displaying contrasted hedonic responses. In these conditions, the pleasant (to adults) benzaldehyde and the unpleasant dimethyl disulphide were more often assigned to the smiling and frowning smileys, respectively, but with 3-year-olds' attributions being more ambiguous than those of 4–5 year-old. In a further study, 3-year-olds and adults were asked to give nine odor bottles to one of two characters well-known to American children looking at the *Sesame Street* television show, Big Bird and Oskar the Grouch considered as desirable and undesirable figures, respectively [43.154]. Both age groups showed overall agreement in their preference pattern, although they differed in their hedonic assignments of some odorants to the positive or negative figure. These studies corroborate that young children at the fringe of language acquisition and schooling can consciously attend and distinguish odor stimuli in hedonic terms and that they can do so in relative consensus with adult judgments.

43.4 Conclusions and Prospects

The research summarized here clearly establishes the capacities of newborns, infants, and children to attend, detect, discriminate, and categorize olfactory stimuli along different cues. On top of this is their aptitude, through nonverbal or verbal means, to subdivide the olfactory environment into familiar/novel and attractive/repulsive stimuli. The sensory and psychological dimensions at the root of these early preferences remain poorly explored in humans, but one can safely affirm that any weak odor to which the infant has previously been nonadversely exposed will have a higher reinforcing value than any novel odor. This general principle has, however, its exceptions. For example, sensory specific satiety, alliesthesia, or boredom effects can induce the occasional aversion of familiar odor stimuli [43.78, 155, 156], or even initiate attraction toward olfactory novelty. Other exceptions are either odor stimuli (such as androstenone) which can remain paradoxically aversive to newborns despite they occurred in their developmental environment [43.33] or evolved chemoperceptual specializations that emerge uninfluenced (or minimally influenced) by experience (see discussion in [43.83]). But, globally, the principle of preference (or lower aversion) for familiar odors is massively verified, and, beyond the domain of preferences, children's odor discrimination, identification or categorization abilities also depend strongly on the width and depth of their prior experience with odors. Exposure creates indeed familiarity with some features of objects or contexts, possibly increasing the perceptual salience of such features and enhancing the ability to (consciously or nonconsciously) sense their alteration or to track them analytically.

Developmental differences in odor discrimination and appreciation are certainly causally multiple. They presumably involve olfacto-motor and sensory variations, such as changes in odor-sampling ability (active, volitional sniffing emerging after 2–3 years of age [43.5, 157]) or in odor sensitivity due to varying properties of olfactory structures and processes. Such developmental differences may depend on general or olfaction-specific cognitive factors. Expanding experience with odors leads not only to enhanced odor-related semantic stores without necessary link with verbal abilities, but also to increased ability to verbally label odor stimuli [43.123, 139, 158–160], longer spans of working memory [43.136], or better recognition memory [43.139, 161]. The latter psychological factors are also often evoked to explain early gender differences in olfaction. Performances in odor discrimination and preferences in older infants or in children can trace back to perinatal [43.19, 125, 162] and prenatal olfactory exposure effects [43.163], or to postnatal windows when the encoding of odors may be facilitated during sensitive periods [43.164]. All along infancy and childhood, sensory cues contingent with particular contexts can also be monitored and stably recorded. For example, 5–10 year-old children dislike alcohol-related odors more when raised in families where parents consume alcohol to cope with dysphoria, as compared to children whose parents usually drink for fun [43.165, 166]. Similarly, children whose mothers smoke to alleviate stress dislike the odor of tobacco smoke more than children whose mothers report smoking for other reasons [43.167]. The emotional tone of early exposure to food is another context of very efficient acquisition of persistent odor likes and dislikes [43.168]. In sum, children rely on odors to differentially *tag* objects, persons or contexts related to attachment figures or to (negatively or positively) challenging emotional situations.

However, some odors may be unconditionally (i. e., without the necessity of direct exposure) attractive or aversive right from birth. So far, this was best shown in several mammals (rabbit: [43.169]; mouse: [43.170]), but not yet in humans (although the odor of androstenone was reported to be aversive right from birth [43.33]). In the case of these mammals, it may be assumed that functional loops between given odor ligands, olfactory receptors, and responses are specified by evolved genetic and developmental mechanisms. Recently, an additional, nongenetic way of elaboration of selective olfactory responses in the absence of direct exposure has been suggested in the mouse [43.171] where male or female mice trained to fear the odor of acetophenone produce offspring that respond negatively to that odor up to two generations despite they were never directly exposed to it. This study highlights unexpected ways of structuration of (parts of) the olfactory system as a function of parental or grand-parental odor-related emotional experience. It complexifies the gestational role of the mother in shaping fetal olfaction in also involving both parents' pre-conceptional experience. Evidence for such transgenerational inheritance of sensory abilities and preferences acquired from ancestral experience will probably expand during the next decades [43.172].

Increased and systematic research efforts on olfactory development during the neonate, infancy, and childhood periods are important for several basic and applied reasons. First, such research can illuminate general issues on the biological, psychological, ecological, and sociological mechanisms underlying human perception: How does perception change according to chronological, biological and psychological ages?

Which biological, psychological, or social-cultural processes modulate fluctuations in odor-related preferences and cognition (conscious and unconscious attention, discrimination, categorization, memory)? Do certain early chemosensory skills result from specializations evolved to optimize adaptive responses to early transitions (birth, weaning, puberty)? Second, some rare findings suggest that infantile experience with specific odors can canalize lifelong perceptual abilities and subsequent attitudes [43.162, 163, 168, 173, 174]. These long-term effects of early chemosensory experience relate to notions, such as sensitive periods, cerebral plasticity and memory, and also to the early *programming* of food liking, addictive habits, and affiliative choices.

Overall, this chapter emphasizes the importance of a developmental approach in understanding the mechanisms and functions of human olfaction. It will be a rewarding challenge for future research programs to integrate longitudinally in single multidisciplinary studies the biological, psychological and environmental determinants of chemosensory performance during human ontogeny. But innovative ways to study olfactory function and functioning should be designed, and the proposal of *Köster*, *Møller* and *Mojet* to shift research methods toward more ecological paradigms should be taken into account to this aim [43.60, p. 7]:

> *... odors are ... not meant to be identified, but should ... be recognized as the ephemeral and unnoticed providers of feelings of safety and comfort, unless they are unknown and unexpected or out of place, we may need to devote more time to emotional effects of odor associations and to the investigation of incidentally learned situational odor memories instead of investigating how* odor objects *are constructed and changed by odor-odor and odor-taste learning under laboratory conditions with odors from bottles or olfactometers.*

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44. Olfaction and Eating Behavior

Sanne Boesveldt

The olfactory sense plays an important role in food and flavor perception, and thus in our eating behavior. This chapter provides an overview of the current status of research in this field. It describes the two-way relation between subdomains of olfactory function (detection sensitivity, pleasantness; ortho- and retronasal odor exposure) and various aspects of eating behavior (appetite, food choice, intake), and discusses the difficulties in this type of research. Findings are summarized which show that metabolic status might affect odor perception and vice versa odor exposure can affect appetite. Determination and anticipation of the type of food by odor cues may tailor the preparation of the body to the specific macronutrient composition of the anticipated foods. However, this does not automatically lead to subsequent specific food intake. Eating behavior is a complex phenomenon that entails more than simple liking or wanting of foods induced by olfactory cues, rendering the need for more research to get a better grip at understanding the underlying and interacting mechanisms to be ultimately able to customize nutrition to individual needs.

What we perceive as flavor when we eat or drink something is a combination of different senses. We integrate sensations from mainly smell and taste, but also texture or mouthfeel and irritation in our nose and mouth. All this combines together to form a unique flavor percept of food or beverage. And since taste only consists of five primary qualities (sweet, salty, sour, bitter, and umami), the olfactory sense plays an important role in food and flavor perception, and thus in our eating behavior. Moreover, smell does not only play a part in the consummatory phase of eating (flavor perception by means of retronasal olfaction) but also orthonasal olfaction has been suggested to influence our anticipatory behavior, by modifying the selection of foods, meal size, and increasing appetite,even in the absence of physiological hunger. An everyday example can be the smell of food or fresh bread from a bakery that is enough to make you feel hungry and perhaps even walk into that bakery to buy some croissants. On the other hand, when you have a cold, and your nose is plugged, you do not taste your food so good anymore, and do not enjoy eating as much, because the olfactory component is missing. In addition, early exposure to volatile flavors in breast milk or formula can shape our food preferences in later life [44.1].

Although this evidently is a vital area of research, not much work has been done so far investigating (solely) the effect of odors on food anticipation and consumption. This chapter will provide an overview of the current status of research in this field.

Unlike taste, it is commonly accepted that the olfactory sense is difficult to categorize into primary qualities [44.2, 3]. From a functional point of view however, it is possible to distinguish between food-related and nonfood-related odors. Although what constitutes food or not is culturally determined [44.4] and can be subject to change, some behavioral studies suggest that humans respond differently to food versus nonfood odors. For example, food odors are identified more accurately than nonfood odors [44.5, 6]. Moreover, from an ecological perspective, the classification of odors into the

food or nonfood category can be of eminent importance. Reaction speed is typically considered a measure of biological relevance of a stimulus: threatening stimuli are thought to be prioritized by the brain to effect faster preparation for *fight or flight* action [44.7–9]. This fast response not only applies to negative stimuli [44.7–11], but also to other biologically relevant stimuli [44.12]. More importantly, human responses to food odors (fish and orange) were also found to be faster, and more accurate than to perceptually similar nonfood odors (dirty socks and rose), regardless of their pleasantness [44.13], suggesting food odors comprise a separate category of odors, that are of ecological importance for us.

44.1 Metabolic Influences on Odor Perception

Already from the early 20th century on, there has been a keen interest in trying to relate energy homeostasis to olfactory sensitivity. Indeed, it is an intuitively appealing concept to be more sensitive to odors, signaling food, when in an energy-deprived state. Nevertheless, this hypothesis still remains subject to some debate, despite many studies that have been done ever since the first scientific work in 1928 [44.14]. In that study, *Glaze* reported on two subjects (himself included) who fasted for 5−10 days, and demonstrated an increased sensitivity to various odors during that period, as measured with a Zwaardemaker olfactometer. An additional experiment, in which subjects were more sensitive to odors before a meal than after, replicated this finding [44.14]. In concurrence, some 20 years later, using a blast-injection technique, *Goetz* et al. supported such claims and showed that diurnal variations in olfactory sensitivity were associated with the ingestion of food: meals were stated to be preceded by a period of increasing and followed by one of decreasing olfactory acuity [44.15, 16]. However, at the same time, *Janowitz* et al. failed to find any effect of time of day or lunch on odor (recognition) thresholds using the same method [44.17]. Since then, more discrepant results have been reported in the literature, ranging from an increase in olfactory acuity when hungry [44.18–21], no change in sensitivity [44.22–27], to even an increase in acuity after lunch [44.28]. It is problematic to directly compare all those studies to each other, since they used different measurement techniques (an olfactometer, a blast-injection technique, a walk-in olfactorium, method of constant stimuli), different odors (both food and nonfood: coffee, citral, butanol, iso-amylacetate, etc.), different *fasting* durations (ranging from a few hours after lunch, to up to 3 months) and different types of meal (possibly initiating *sensory-specific satiety*, see later). After a *silent period*, research activity on this topic has sparked again in recent years. Although those studies used partly similar methods (the Sniffin' Sticks detection threshold for *n*-butanol, [44.29]), their results were contradictory as well [44.30–32], both with respect to the nonfood odor as to used food odors.

The inconsistency of results in the literature mentioned earlier may be considered as a reflection of the magnitude or relevance of the effect: Effects of hunger on olfactory sensitivity may be small, and perhaps less essential for the detection and subsequent consumption of foods or for the regulation of energy intake in humans.

Though the effect of hunger or satiety on olfactory sensitivity remains to be determined, the effect of food intake on odor pleasantness is more clear. *Rolls* and *Rolls* first coined the term *sensory-specific satiety* in 1981 [44.33], and described it as

> *the decrease in the pleasantness of a food after it has been eaten to satiety, and the smaller amount of that food, relative to other foods, that is subsequently eaten in a meal.*

This phenomenon is probably not driven by postingestive effects, but more by external factors such as the sensory properties of a food. As humans are omnivores, we seek variety in our meal. Typically, the sensory characteristics (taste, smell) of a meal reflect its' macronutrient content [44.34, 35]. As noted earlier, not only does the definition of sensory-specific satiety adhere to a change in food intake, but also to a change in the pleasantness of a food and its' sensory signals. For instance, after ad libitum consumption of banana, pleasantness ratings for banana as well as for banana smell decreased significantly, compared to the pleasantness of other odors; an equivalent result occurred for chicken [44.36]. Similarly, a decrease in the pleasantness for isoamyl-acetate (banana odor), but not *n*-butanol, was found after consumption of a breakfast that contained banana [44.30]. In addition, these effects of sensory-specific satiety can be revealed in brain areas related to reward processing: by means of functional magnetic resonance imaging (fMRI), activity in the orbitofrontal cortex has been shown to decrease in response to an odor of food that has been eaten to satiety [44.37].

The change in pleasantness that occurs in sensory-specific satiety should not be confused with alliesthesia,

that the pleasantness or rewarding value of a given stimulus is dependent on the subject's internal state [44.38], which is a more general effect. Several studies have indicated that the pleasantness of food odors declines (negative alliesthesia), whereas that of nonfood odors remains stable, after food intake [44.39–41]. Moreover, this effect was more pronounced for high-energy dense food odors; the decrease in appetite and liking when satiated was greater for fatty food odors than for nonfat food odors [44.40, 41].

In conclusion, metabolic status seems to mainly impact the hedonic evaluation of odors, and appears to be intake-specific, that is, sensory-specific satiety, and may thereby guide our eating behavior toward variation in nutrient intake. The effect of hunger on olfactory sensitivity may be small and of less importance for regulating energy balance. On the other hand, odor exposure can have a profound effect on shaping food preferences and modulating eating behavior, as will be described in the next section.

44.2 Anticipation Effects of Odor Exposure

Although metabolic status can influence olfactory perception, the reverse is also true. In the presence of food, olfactory (and visual) cues are perceived before any intake, and may trigger so-called cephalic phase responses in anticipation, to prepare the body for ingestion and digestion of foods (for a detailed review of the relation between olfaction and appetite hormones, see [44.42]).

Pleasantness or liking is considered to play a substantial role in food choice and preferences, and if odor exposure affects (food) pleasantness, it may be consequently expected that odors are also able to modulate appetite, food preferences, and eating behavior similarly. In their publication, *Rolls* and *Rolls* not only described sensory-specific satiety, but also (partial) olfactory sensory-specific satiety; that the pleasantness of an odor decreased after smelling that odor for a period of time (5 min, approximately as long as it would be in the mouth during a meal), in contrast to the pleasantness of other (food) odors. The intensity of the smelled odor did not change which implies that this is not an effect of peripheral sensory adaptation [44.36]. Although they did not assess craving or appetite for the specific foods under question, later studies did. Most research on food cue reactivity, however, has focused on visual cues of foods [44.44, 45], or a combination of olfactory and visual stimuli [44.46–48]. Food cue exposure appears to lead to an increase in appetite or craving for the cued food specifically [44.43–49], and this effect is similarly found in the few studies that solely looked at

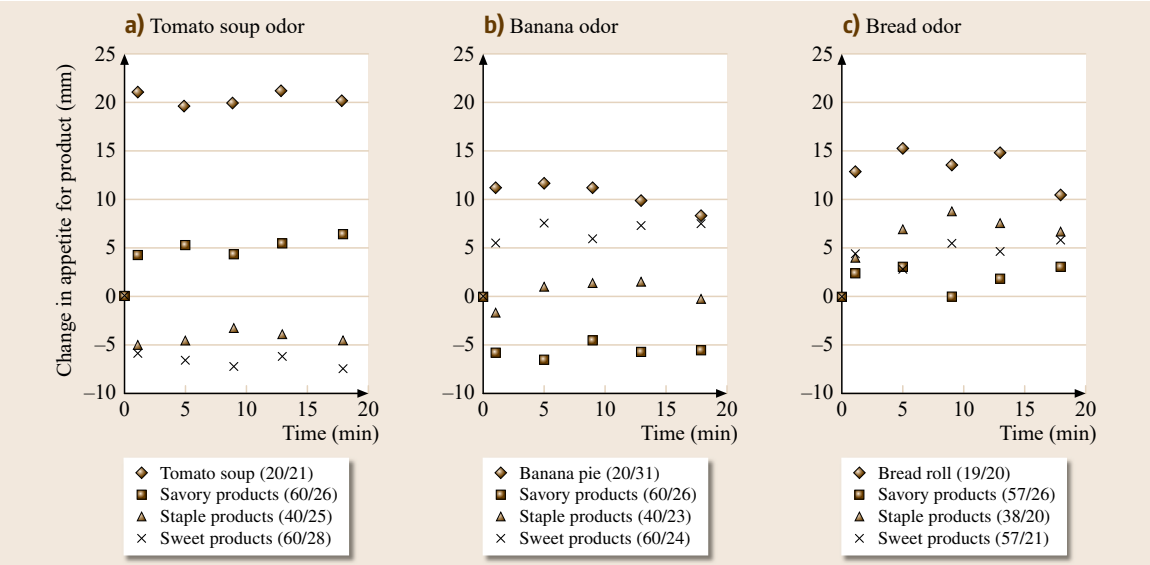

Fig. 44.1a–c Average change in appetite for odor-specific and category-specific foods, during exposure to **(a)** tomato soup odor, **(b)** banana odor, and **(c)** bread odor, measured on 100 mm VAS (visual analog scale). The numbers between the brackets represent (number of observations/average s.d.) (after [44.43])

odors [44.43, 49]. This phenomenon is termed sensory-specific appetite, the specific effect of cue/odor exposure on congruent appetite. In addition, this effect seems to *spill over* other, similar foods that share common super-ordinate characteristics, such as taste category. For instance, after exposure to banana odor, the specific appetite for banana not only increases most, but also stimulates appetite for other sweet products, such as a chocolate brownie. Moreover, exposure to food odors not only increases the appetite for congruent foods, but also decreases the appetite for incongruent foods (such as tomato soup, after banana exposure; Fig. 44.1) [44.43].

It is thus likely that food odors communicate information about the nutrient composition of their associated foods [44.35] that elicit cephalic phase responses to help the body prepare for the ingestion and digestion of that specific food/macronutrient, rendering it unfavorable to consume foods that are very different from the cued food [44.43]. Perhaps it is not so surprising then that the effect of odor exposure on general appetite or hunger is less clear. Some studies showed an increase in hunger during odor exposure [44.43, 46], whereas others found that participants experienced less appetite and felt more satiated after smelling a food odor [44.50].

Similarly, the effects of odor exposure on subsequent food choice and preference are inconsistent; some studies have shown that ambient exposure to fruity odors (citrus, pear) increased congruent food choice during a subsequent buffet [44.51, 52], while such an exposure effect was not found for other odors, or using a different method to measure food preference [44.51, 53, 54].

To summarize, odors have an anticipatory function for eating behavior, in that they are able to generate appetite, specific for the cued product, but do not consistently increase general appetite. The effect of odor exposure on pleasantness ratings can perhaps be attributed to different underlying mechanisms that include sensory stimulation and lead to a form of habituation. How food cues and appetite subsequently affect eating behavior, in terms of food choices and actual intake that both may rely on more cognitive reasoning, remains to be determined.

44.3 Consumption

The studies described so far have looked into the behavioral responses to food (odor) cues mainly using subjective ratings [44.36, 43, 48, 55]. Although subjective hunger and appetite ratings often predict food intake [44.56], they cannot be directly extrapolated; the type and amount of food people indicate that they want to eat is not necessarily equivalent to what they eventually consume [44.57–60]. Indeed, literature shows a distinction between prospective measures of eating (appetite ratings, preference) and actual food intake, resulting in conflicting findings of an increase [44.44, 46, 49, 61–64], decrease [44.46, 61, 65] or no effect [44.48, 54, 64, 66] of cue exposure on subsequent food intake. When limiting the focus specifically to odors as food cues, the results remain inconsistent. Studies by *Fedoroff* et al. [44.62], and more recently *Larsen* et al. [44.64], have shown an increase in intake of the cued food after odor exposure, but surprisingly only for restrained eaters or low-impulsive participants, respectively. On the other hand, *Coelho* et al. [44.65] found that exposure to chocolate-chip cookie odor led to a decreased food intake for restrained but not unrestrained eaters. Lastly, *Zoon* et al. [44.54] observed no difference in intake after exposure to odors signaling different types of food (high and low energy-dense) versus a non-food control odor, neither for total food intake, nor for specific foods congruent with the odor cue. These results indicate that eating behavior, actual consumption, is not an easy phenomenon to study, and that personal characteristics and individual traits can have a large impact. Other internal factors, such as hunger or satiety [44.54, 67–69], but also weight status [44.46, 54], attention or awareness of the odor cue [44.64] and similarity between the odor cue and the food to be consumed (general versus specific; [44.43, 49, 65] can affect the outcome of a study. Even though people report general hunger or craving for specific foods, this should not be taken as a direct indicator that they will also readily consume these foods. Actual food choice and intake is influenced by more factors than liking or wanting of foods; situational context, cognitive appraisals, or the focus on longer-term goals, such as health or weight concerns, can affect the current eating behavior despite the appetites induced by odor exposure.

Most of the studies stated earlier have focused on short-term effects of experimental interventions to study the influence of olfaction on eating behavior. If our sense of smell indeed plays an important role in the dietary choices we make in our daily lives, this may exert longer-term effects, such as changes in body weight or BMI (body mass index). Additionally, patients suffering from eating disorders (anorexia nervosa or bulimia) may display altered chemosensory function; alternatively, specific subgroups that experience

olfactory dysfunction (elderly, anosmics) may show differences in their food preferences and intake. It can be argued that a decline in olfactory function could either lead to a decrease in food intake (by negatively affecting food pleasure), or to an increase in food intake (in order to achieve a similar sensory experience). In the early days, *Thompson* et al. [44.70] reported no differences between normal weight and obese subjects in their intensity ratings or hedonic responses of olfactory stimuli, while later studies on (morbidly) obese adults and children have shown impaired olfactory functioning and a greater likelihood to be anosmic [44.71, 72]. *Boesveldt* et al. examined the correlation between olfactory function and BMI in a large national probability sample of older adults in United States, and found that a higher BMI was associated with higher scores on an odor identification task [44.6]. Yet this is only a cross-sectional study, from which it is difficult to draw conclusions regarding cause or consequence, or whether the effect is mediated by related underlying metabolic processes. Moreover, weight status or BMI is a crude, proximal measure of eating behavior, and is likely not directly affected by olfactory (dys)function. For example, several studies have indicated that a decreased sense of smell is related to a poor appetite, lower interest in food-related activities, or changes in food preferences and dietary patterns [44.73–78], but not necessarily to low energy intake or low BMI [44.73, 74, 76]. Moreover, research has shown no relation between (loss of) olfactory function and liking of (flavor enhanced) foods [44.79, 80] or degree of sensory-specific satiety, either measured by changes in pleasantness [44.81] or intake [44.36], indicating that changes in olfactory performance can, but do not automatically, lead to changes in dietary patterns. It is therefore suggested that when the sense of smell is absent, the pleasantness of food stimuli is judged on the basis of the remaining available sensory attributes (visual cues or taste), but possibly also by the help of mental images of pleasantness based on previously acquired product concepts, in order to compensate for the olfactory losses [44.80, 82, 83].

Patients suffering from anorexia nervosa or bulimia demonstrate disturbed eating behavior, however, it is unclear whether this can be related to alterations in their chemosensory perception, that is, their sense of smell. Several studies showed that anorectic patients had lower olfactory test scores than their healthy normal-weight counterparts, but this pattern was not consistent over different olfactory function subtests. *Schreder* et al. [44.84], *Roessner* et al. [44.85], and *Dazzi* et al. [44.86] found reduced scores on olfactory discrimination, while *Rapps* et al. [44.87], and *Schreder* et al. [44.84] found lower scores for odor identification, and *Roessner* et al. [44.85] showed a decrease in olfactory sensitivity in anorectic patients. Fewer studies have been done on bulimic patients [44.86, 88], however those also present no clear relation with olfactory functioning. Heterogeneity of patients (age, weight), and small sample sizes seem to play a role in the divergent results of all these studies. More importantly though, it is likely that, as in normal healthy subjects, the influence of olfaction on eating behavior is not based on (changes in) olfactory acuity or perception, but via the hedonic evaluation of (food) odor stimuli, leading to a difference in reward responses that may subsequently alter eating behavior.

44.4 Effect of Retronasal Odors on Eating Behavior

As discussed earlier, the olfactory sense has an important role in our eating behavior, by shaping our appetite and preferences. However, smell does not only contribute to the anticipatory phase of eating, but retronasal olfaction is considered a fundamental part of flavor perception during the consumption of foods. By chewing, oral breakdown of foods, and swallowing, volatile molecules are released into the oral cavity, which travel through the nasopharynx during exhalation and subsequently stimulate receptors on the olfactory epithelium in the nose [44.89]. Identical to orthonasal olfaction, the signals are then carried via cranial nerve I to the olfactory bulb, and from there to the piriform cortex and orbitofrontal cortex, among other structures [44.90]. Unlike orthonasal olfaction that is used to identify objects in the external world, retronasal odors refer to objects inside the body, and are thus principally related to foods [44.91, 92].

Considerably fewer studies have been done in the field of retronasal smell and eating behavior than orthonasally, most likely due to the difficult nature of stimulus presentation. Typically, the extent of retronasal aroma release during consumption appears to be a physiological feature that characterizes a person as well as depends on the physical structure of a food [44.93]. For odors to be administered retronasally in an experimental setting, a tube has to be inserted via the nose to the back of the mouth to deliver the aroma onto the nasopharynx [44.94] (Fig. 44.2).

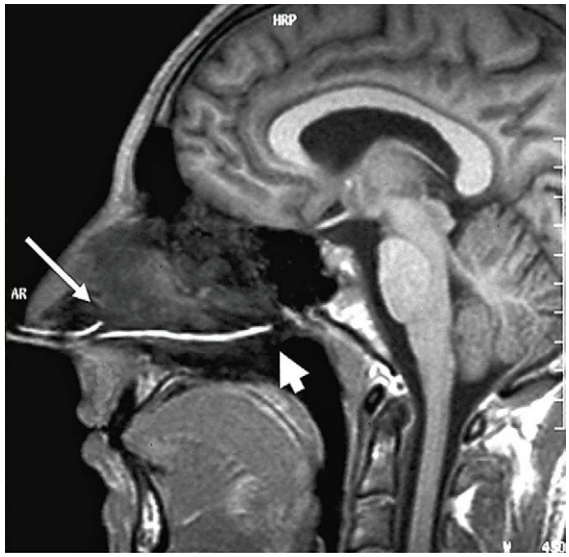

Fig. 44.2 Image (magnetic resonance imaging [MRI]) of subject's head after tubes for ortho- and retronasal stimulus presentation have been inserted into the nasal cavity. The distal end of the two tubes lies in the epipharynx (*white arrowhead*), the end of the other tubing lies in the antrum of the nasal cavity (*thin white arrow*); for this MRI scan, the tubes have been filled with a contrast agent (after [44.94])

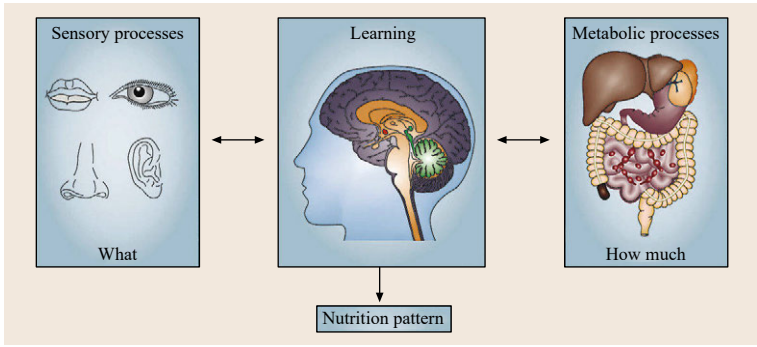

Fig. 44.3 Factors that affect eating behavior (after [44.95])

It was found that presentation of a retronasal odor stimulus increased the intensities of thickness and creaminess of a liquid (milk) in the mouth [44.96]. From this it can be inferred that enhancing retronasal odor stimulation may be used to induce satiety during consumption and ultimately may contribute to a decrease in food intake. Indeed, *Ruijschop* et al. [44.97] demonstrated that participants felt significantly more satiated and had less desire to eat congruent (sweet) foods if they were stimulated with a longer retronasal odor profile during (sweet) milk consumption. However, in that study there was no effect of retronasal odor exposure on the subsequent milk intake, while a later study did establish a 9% lower food intake when participants were exposed to a *high* retronasal condition versus other conditions with lower concentrations or shorter exposure duration [44.98]. These results indicate that changing the retronasal aroma released by concentration and exposure time may affect perceived satiety and food intake, but perhaps not in all situations or for all participants. As mentioned earlier, subjects differ in the extent of retronasal aroma due to differences in oral processing, salivation, or eating rate [44.99, 100], which may account for the differences in effect on satiety and intake. For example, the microbiota composition of saliva composition can differ between normal weight and obese subjects, and may influence the retronasal release of volatiles in a food [44.100], altering its' perception, which can subsequently lead to a change in eating behavior. Longer sensory exposure times of foods in the mouth are consistently associated with lower food intakes; sensory signals during eating are linked to the metabolic consequences and need time to inform the brain and the gastrointestinal tract about the inflow of nutrients [44.95, 101] (Fig. 44.3).

Eating rate is positively associated with ad libitum intake; liquid foods are typically ingested faster than solid foods [44.102], and also the duration of retronasal aroma release differs between various liquid and solid foods [44.97]. Indeed, higher retronasal aroma intensities resulted in significantly smaller bite sizes

in a controlled lab environment [44.103]. Accordingly, longer or higher retronasal aroma release may help the human body to associate the sensory signals from food with their metabolic consequences and may therefore lead to lower energy consumption and ultimately to weight loss. Nevertheless, *Zijlstra* et al. [44.104] were not able to confirm this idea in their study and did not see clear differences in retronasal aroma release, ad libitum intake or eating rate between normal weight and overweight subjects, nor was there a correlation between eating behavior and the extent of retronasal aroma release.

Similar to orthonasal odor exposure, the influence of retronasal aromas seems to be mainly in the subjective domain; where orthonasal odors affect the hedonic evaluation and specific appetite for foods, but remain limited in their influence regarding actual food intake, retronasal exposure is likewise able to modify the subjective feelings of satiety but does not automatically lead to a decrease in consumption.

44.5 Methodological Considerations

It is clear from the earlier given information, that combining olfaction and eating behavior is an exciting area of research, in which much work remains to be done in order to understand the intricate relationship between them. Though it is intuitively appealing that odors can influence appetite and food intake, and vice versa, that metabolic status can influence olfactory perception, the results of experimental studies are sometimes inconsistent. Understandably, methodological factors may be in part causative of this; a few of which that are common in olfactory or eating behavior research will be discussed next.

Since odors are considered difficult to categorize into primary qualities, its main perceptual dimension is valence or pleasantness ([44.105, 106]; for more information on odor valence perception, see Chap. 39). Not only the pleasantness, but also the concentration of an odor can thus play a role in the responses it elicits. Most odors that are used in studies on eating behavior are food related or edible, and are typically well-liked [44.5, 6], thereby limiting the possible role of pleasantness. Nonetheless, palatability of odor cues may depend on the food category it represents, sweet/savory, or high/low energy dense foods, and appetite and liking for fatty food odors appears to be more prone to changes in internal state than nonfat food odors [44.40, 41].

Additionally, the sensation and perception of an odor are partly dependent on sniffing behavior [44.107]. Active sniffing may lead to a higher concentration of volatile molecules in the nose and greater awareness of the odor, and hence may affect the (magnitude of) results of a study. However, *Ramaekers* et al. performed studies with similar setup except for the way of odor presentation, active sniffing or passive ambient exposure, and showed that this did not affect the development of sensory-specific appetite [44.43]. Also, adaptation to the odor may occur when exposure is prolonged over time, leading to a decrease in perceived intensity (see for instance review by [44.108]). Nevertheless, in separate studies, duration of odor exposure did not affect appetite ratings or subsequent food intake [44.43, 64]. Furthermore, it has been suggested that subconscious or subthreshold odor exposure may lead to stronger behavioral influences than when we are consciously aware of them (for review, see [44.109, 110]), perhaps due to the direct link with memories and emotion centers in the brain [44.90].

Odors appear to have an anticipatory function for eating behavior; in that they are able to generate appetite, specific for the cued product, but do not consistently increase general appetite or intake. This difference may be due to the distinction between subjective, prospective measures of eating (appetite ratings, preference), and actual consumption. The inconsistency in the results on general appetite and food intake most likely reflect small effect sizes and reveal the complexity of food cue reactivity. Moreover, different methods of measuring appetite or consumption may also have an effect on the outcome. The visual analog scale (VAS) is a commonly used approach that can serve multiple purposes, ratings on liking for the odor, liking for the product, general appetite, hunger, prospective consumption, desire to eat, appetite for a specific product, etc., [44.43, 46, 50, 111]. It is sometimes difficult for a participant to distinguish between the various questions, that relate to different underlying constructs (liking and pleasantness versus wanting and appetite, [44.112]), and it is clear that this may yield different results or interpretations. One should be equally careful when applying a food preference task, in which participants have to choose between two presented food products and indicate which one they would like to eat most at that particular moment; the preferred choice for one product may implicitly be a rejection of the other product.

Lastly, when studying food cue reactivity it is important to take into account personal characteristics such as restrained eating, impulsivity, or weight sta-

tus and internal state [44.46, 62, 64, 67, 68, 113], which may affect the way or magnitude in which people respond to food odors. Moreover, investigating the (subtle) impact of the olfactory system on eating behavior requires a tight control over the experimental conditions, which is much easier to achieve in animal studies, and may explain the sometimes small or conflicting findings in human studies.

44.6 Conclusion

Though it is clear that the olfactory sense is vital for food and flavor perception, and anecdotal evidence and personal experience suggests that odor exposure plays an important role for the anticipation and consumption of foods, experimental data is limited and sometimes inconsistent. Metabolic status seems to mainly impact the hedonic evaluation of odors, not sensitivity, and appears to be intake-specific, that is, sensory-specific satiety, and may thereby guide our eating behavior toward variation in nutrient intake. The reverse is also true, odors have an anticipatory function for eating behavior, in that they are able to generate appetite, specific for the cued product. How food cues and appetite affect subsequent eating behavior, actual consumption, remains to be determined and is likely influenced by more factors than the liking or wanting of foods that is induced by odor exposure. However, smell does not only contribute to the anticipatory phase of eating, but retronasal olfaction is also considered a fundamental part of flavor perception during the consumption of foods. Similar to orthonasal odor exposure, the influence of retronasal aromas seems to be mainly in the subjective domain, and is able to modify the subjective feelings of satiety but does not automatically lead to a decrease in consumption.

The relation between olfaction and eating behavior is complex and entails more than plain liking or wanting of food induced by odor cues, rendering the need for more research to get a better grip at understanding the underlying and interacting mechanisms to be ultimately able to guide people toward better dietary patterns.

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45. Olfaction and Sleep

Ofer Perl, Anat Arzi, Ilana S. Hairston, Noam Sobel

As far as I know, the only reason we need to sleep that is really, really solid is because we get sleepy. Coming from William C. Dement, one of the pioneers of contemporary sleep research, this statement depicts sleep as a *neurobiological black box*. One of the best ways to probe such black boxes is through exceptions, and olfaction stands out as such an exceptional sensory system during sleep. Specifically, whereas sensory stimuli presented during sleep typically wake, this is not the case with odors. In fact, odors may promote sleep. In turn, they remain processed by the sleeping brain, and provide a telling window onto sleeping brain capabilities. Here, we briefly review the foundations of sleep, and then extensively detail the literature on olfaction in sleep, concentrating on studies in humans. We speculate that the unique interplay of sleep and smell whereby odors are processed in sleep without causing wake reflects unique aspects of olfactory neurophysiology, particularly the direct projections from periphery to cortex without a thalamic relay. Finally, although the mechanisms allowing odor processing during sleep without arousal remain unclear, this phenomenon lends itself to using olfaction as a window onto sleep mentation. This approach has uncovered several aspects of learning and memory during sleep. We review these efforts, and conclude with detailing their potential application in the treatment of disease.

45.1 Sleep: Architecture and Measurement

To the observer, sleep may seem a more or less uniform state of inactivity. This is interrupted by phases where the eyes apparently rapidly shift from side to side under the closed eyelids, a phase of sleep therefore referred to as rapid eye movement (REM) sleep. Only by harnessing the power of physiological signal measur-

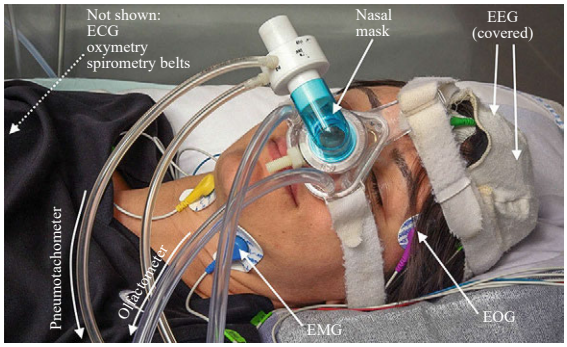

Fig. 45.1 Olfaction polysomnography setup (photo by Michal Cooper) ◄

ing techniques, however, it becomes apparent that sleep contains clearly distinguishable states that alternate in a cyclic manner throughout the night. These states that are not REM (NREM) sleep are collectively referred to as NREM. The physiological signals measured during sleep are chiefly electroencephalography (EEG), the electrooculogram (EOG), the electromyogram (EMG), and measures of posture and respiration, collectively referred to as polysomnography (Fig. 45.1). The core

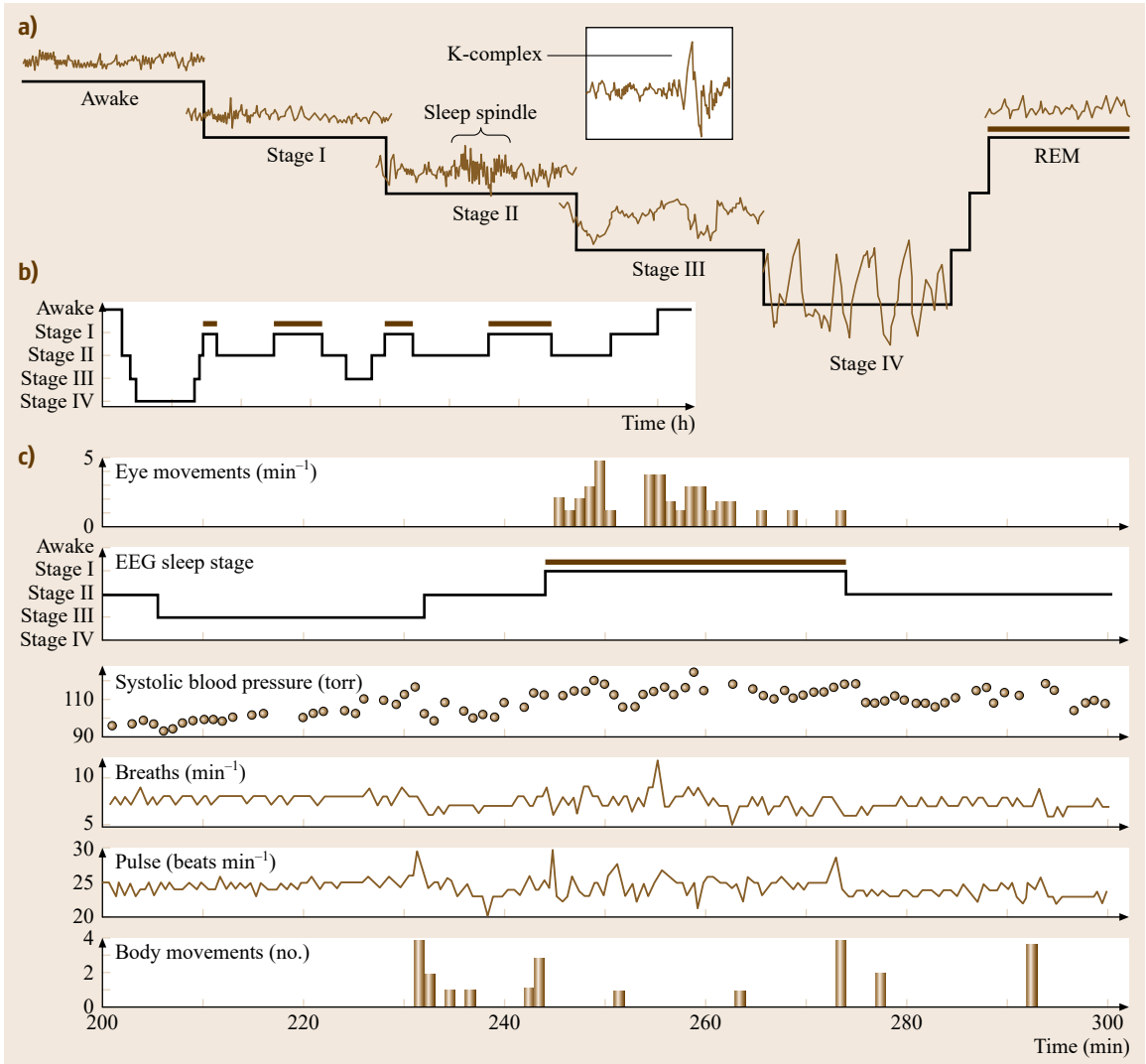

Fig. 45.2a–c An overview of human sleep: stages, cycling, and architecture. **(a)** Sleep is divided into stages according to EEG brain activity. **(b)** Progress of sleepstages over night. **(c)** Physiological and behavioral phenomena evident during sleep (after [45.1])

of polysomnography entails recording of brain activity (EEG), eye movements (EOG), and muscle tone (EMG). When required for research or diagnostic purposes, respiration, heart rate, blood oxygenation, limb movement, body position, and snoring sounds may also be sampled. Note that in this setup, designed for olfactory studies, a nasal mask delivers olfactory stimuli from a distant olfactometer. The mask is subserved with a vacuum line pulling the air from the environment of the nose to assure a temporally accurate presentation of the stimulus and a clean-air state between trials.

45.1.1 NREM

Polysomnography reveals that humans spend most of their sleeping in NREM. According to the contemporary sleep-scoring technique, NREM is further subdivided into three stages on the basis of EEG criteria [45.3]. These stages represent to some degree, a depth-of-sleep continuum, with arousal thresholds generally lowest in stage 1 sleep (Fig. 45.2b). Moreover, neuronal activity becomes increasingly synchronized as sleep stages progress. Nocturnal sleep adheres to a more or less constant scaffold, usually referred to as sleep architecture. Sleep stages orderly progress across the night in a cyclic manner, each cycle lasts about 80–120 min. Note that normal sleep onset is through NREM sleep. However, as the night progresses, time spent in REM is increased, on the account of stages 3 and 4 sleep (SWS).

45.1.2 Stage 1 Sleep (N1)

Stage 1 sleep is the stage of transition from wake to sleep. As sleep takes over, alpha rhythms (8–13 Hz) that dominate brain activity during wake diminish to less than 50%, and are often intermixed with theta (4–7 Hz) and beta (13–30 Hz) waves. Stage 1 sleep is light, thus easily interrupted by environmental cues reverting the sleeping brain back to wakefulness. Due to its resemblance to wake, stage 1 sleep is somewhat difficult to quantify or manipulate.

45.1.3 Stage 2 Sleep (N2)

Stage 1 sleep lasts only a few minutes and then quickly transitions into stage 2 sleep. The primary indication of stage 2 sleep is the appearance of sleep spindles, short bursts of 11–16 Hz activity, and K-complexes (KCs) (Fig. 45.2a). KCs are distinguished singular waveforms resembling high-amplitude slow waves, first described by *Loomis* et al. in the late 1930s [45.4]. KCs occur in stage 2 NREM sleep, both spontaneously and

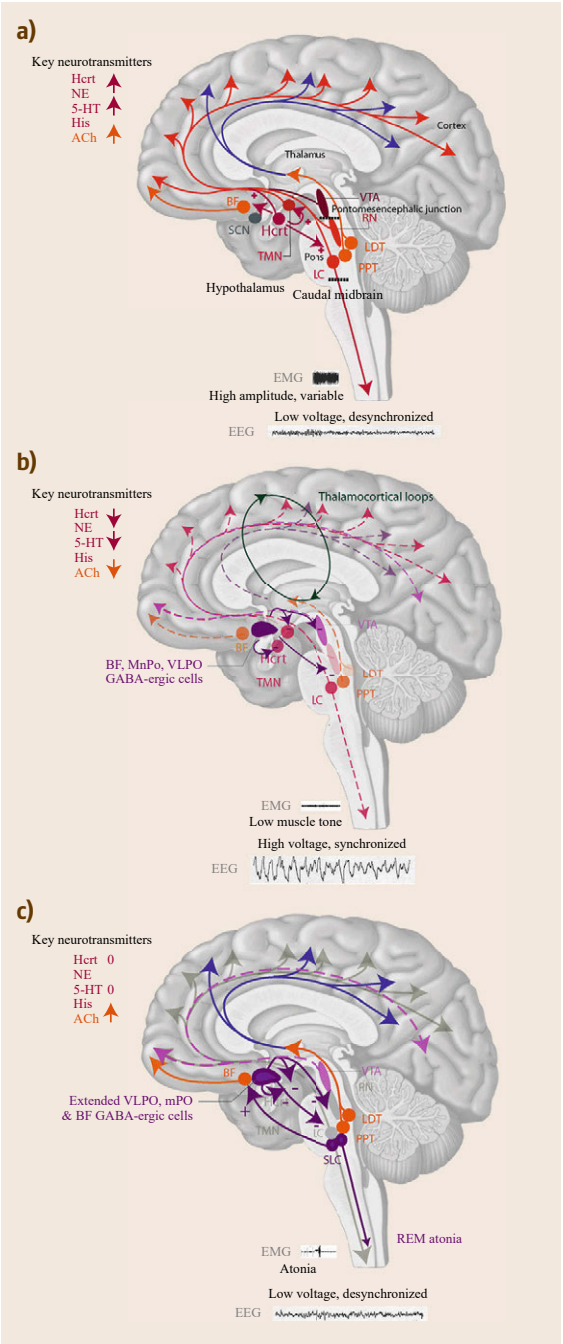

Fig. 45.3a–c Activity of major neural networks during wake, NREM sleep, and REM sleep. **(a)** Wakefulness. **(b)** NREM SWS sleep. **(c)** REM sleep (after [45.2])

in response to sensory stimulation [45.5]. KCs may have a sleep protective function as their appearance following a sensory stimulus prevents EEG characteristics of arousal [45.6]. Sensory thresholds in stage 2

sleep become elevated and thus require a more intense stimulus to produce arousal. The same stimulus that produces arousal from stage 1 sleep often results in an evoked KC but no awakening in stage 2 sleep [45.7].

45.1.4 Slow-Wave Sleep (SWS or N3)

This deepest stage of sleep is characterized by slow rhythm delta waves (0.5–4 Hz), and is termed slow-wave sleep (SWS). SWS replaced stages 3 and 4 of the original Rechtschaffen and Kales scoring criteria in the new American Academy of Sleep Medicine (AASM) sleep staging manual. Delta oscillations reflect synchronized fluctuations of neuronal membrane potentials, shifting between a depolarized upstate and a hyperpolarized downstate. They originate from both corticothalamic and intra-cortical regions. Power of delta activity is higher in adult women than men, and has right hemisphere dominance [45.8–10]. By orchestrating the coherence of rhythmic oscillations, slow waves are thought to take part in the promoting of sensory deafferentation of the cortex during sleep, thus enhancing sleep continuity and depth [45.7]. During SWS GABAergic projections are highly active. Thalamocortical loops contribute to the generation of light NREM sleep. As NREM sleep deepens, slow-wave oscillations appear on the EEG. Muscle tone is low. Experimental findings of reduced responsiveness in cortical neurons during slow-wave activity corroborate this model [45.11]. For this reason, as with the case of threshold elevation from stage 1 to stage 2 sleep, an incrementally stronger stimulus is required to produce wake from SWS than from lighter sleep stages. The first SWS episode usually lasts 20–40 min, typically phasing into REM through a short period of stage 2 sleep.

45.1.5 REM (R)

Whereas NREM sleep may be referred to as a *power-saving mode* for brain activity, REM sleep is quite the opposite. The generation of REM sleep is orchestrated by groups of REM-on cells located in the brainstem. Hypothalamic and basal forebrain sleep-on cells, as well as glutamatergic cells are active during REM. Atonia is accomplished through caudal projections. Brainstem cholinergic activation leads to EEG desynchronization similar to wakefulness. During REM sleep, EMG is low, but may occur. REMs are evident. Apart from the typical episodes of saccadic REMs after which the term *REM* was coined, these brainstem nuclei exert a neck-down muscle atonia (Fig. 45.3c). Brain activity during REM sleep resembles that recorded during wake: EEG during REM consists of low-amplitude beta frequency (13–30 Hz) mixed with theta (4–7 Hz) and alpha (8–13 Hz) rhythms. Similarly, brain metabolism during REM may reach the same levels as in wakefulness [45.12]. For these reasons, REM sleep is sometimes termed *paradoxical sleep*. Despite the seemingly active cortex, arousal thresholds during REM sleep remain high as in other sleep stages. In research, REM sleep can be subdivided into a tonic and phasic stage, according to the lack or presence of saccades, respectively. In healthy adults, REM sleep accounts for 20–25% of total sleep time. When awoken from REM sleep, subjects will often report vivid dreams [45.13] (Sect. 45.7.3).

45.1.6 Arousals and Wakefulness (W)

The typical sleep cycle sometimes terminates with a transient period of arousal linking between the cycles. Arousal (with eyes still shut) is differentiated from sleep by the presence of alpha rhythm activity for 3 s or more. If such arousal endures for more than 15 s, it will probably be remembered in the following morning and is termed *wakefulness*. During wakefulness multiple systems are active and contribute to EEG desynchronization through thalamic and cortical projections. Hypocretin cells excite monoaminergic cells. Muscle tone (EMG) is variable and high, reflecting movements. However, brief discontinuation of sleep lasting less than 15 s is referred to as *arousal* and is most likely not to be remembered.

45.2 Sleep Mechanisms

The different EEG patterns evident during sleep are the result of interplay between multiple brain regions (Fig. 45.3). During waking, the high-frequency desynchronized neural activity is the outcome of a mix of arousing signals originating from brainstem monoaminergic projections (specifically, the serotonergic raphe nucleus and the noradrenergic locus coeruleus) to the diencephalon and cortex, cholinergic projections from the pontine reticular formation and basal forebrain, and hypothalamic arousal signals including hypocretin/orexin and histamine. During NREM sleep, these systems are largely quiescent due to GABAergic in-

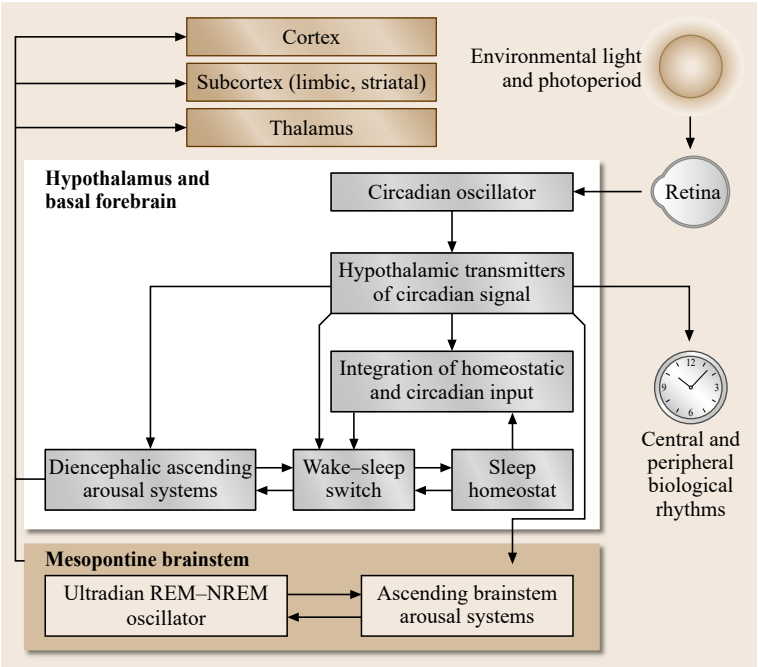

Fig. 45.4 Regulatory circuits involved in sleep (after [45.1])

hibition generated from the ventrolateral preoptic area (VLPO). This inhibition helps disengage the cortex from external stimulation, resulting in the aforementioned slow oscillations. During REM sleep both pontine and forebrain cholinergic systems are re-engaged. This leads to the activation of cortical circuits expressed as high-frequency desynchronized activity. Simultaneously, inhibition of the brainstem monoaminergic systems is augmented to maintain muscle atonia during sleep. The interplay of aminergic and cholinergic systems results in an ultradian oscillation of NREM and REM sleep, of approximately 80–120 min [45.14]. Finally, arousal from sleep is generated through a mechanism reflecting combined activity in the reticular formation of the brainstem, the hypothalamus, and the thalamus (see Fig. 45.4) [45.15].

45.2.1 Why Do Animals Sleep?

Sleep as a behavior is universal [45.16]. Most mammals spend a substantial part of their lives asleep and humans are no exception. The paramount importance of sleep is best demonstrated by its deprivation; organisms who regularly sleep cannot function normally, up to the point of death, when deprived of sleep [45.2, 17, 18]. Though obviously essential, and despite advances in brain sciences, mapping of genetic pathways, and neuroimaging, the functional purpose of sleep remains obscure.

An ecology-centered hypothesis claims sleep offers a state of energy conservation, in order to reduce energy demands in times when conditions (temperature or daylight) are not optimal. Such a metabolic perspective further suggests that sleep may have developed to allow for the biosynthesis of depleted biologically relevant macromolecules [45.19]. Indeed, there is evidence supporting a role for sleep in timing the expression of genes involved in endocrine regulation (specifically growth), immunity, and tissue regeneration processes following injury [45.20, 21]. However given that metabolic activity during some sleep stages is similar to that of wakefulness, this hypothesis alone may fall short of providing a comprehensive explanation for sleep [45.16, 22]. From a neurobiological perspective, cortical functions, such as cognition, attention, and memory are rapidly affected by sleep deprivation, with the latter being improved by subsequent sleep. Therefore, sleep is likely important for mechanisms of brain plasticity that underlie learning and memory facilitation [45.2, 23, 24]. One model for the role of sleep in synaptic plasticity proposes sleep exerts a synaptic downscaling effect for the sake of synaptic homeostasis, correcting an overall gain in synaptic strength accrued during wake [45.25].

45.3 Olfactory Stimulation, Unlike Most Forms of Sensory Stimulation, Is not Sleep-Perturbing

Administration of supra-threshold sensory stimulation during sleep will typically either wake (even if just for a short, often unrecalled arousal) or evoke EEG KCs. Studies involving somatosensory stimulation, such as touch, pain, or thermoperception have repeatedly demonstrated how salient sensory stimuli perturb sleep by inducing arousals, and disrupting the regular sleep period [45.29]. The term *salient* implies that the sleeping brain is able to distinguish between recurring, already habituated stimuli, and novel ones. Yet despite habituation mechanisms, continuous or repeated sensory stimulation impacts sleep quality. For example, when a continuous ambient acoustic stimulus was introduced, a decrease in total sleep time, particularly of SWS was evident, and frequent returns to wakefulness were reported [45.30, 31].

Although the majority of evidence favors the idea that sensory stimuli of varied characteristics disturbs sleep, some exceptions exist. One particular exception is that a rhythmic acoustic stimulation at 0.8 Hz (a frequency which falls well within the intrinsically generated slow wave activity) can effectively enhance slow oscillation activity during adult human sleep [45.32]. A much broader exception is olfactory stimulation.

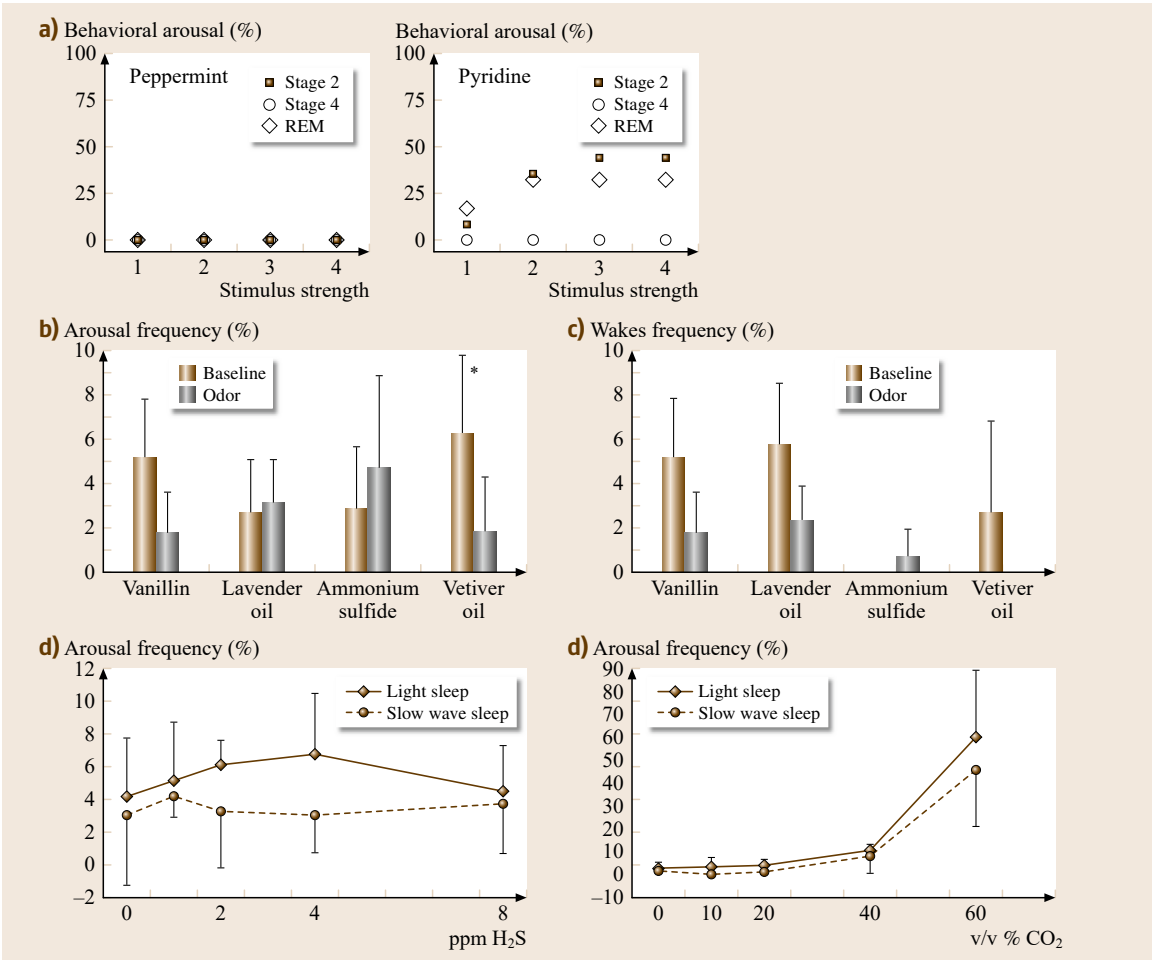

Fig. 45.5a–e Administration of purely olfactory odorants result in minimal physiological and behavioral arousal. **(a)** Arousal frequency following exposure to peppermint (*left*) and pyridine (*right*) stimulation of increasing strength in different sleep stages (after [45.26]). Frequency of arousals **(b)** and wakes **(c)** associated with odor (compared with a baseline of clean air) (after [45.27]). Arousal frequency following exposure to different concentrations of H_2S **(d)** and the trigeminal CO_2 **(e)** during light and slow-wave sleep (after [45.28])

45.3.1 Purely Olfactory and Mildly Trigeminal Odorants Do not Wake

Odorants can be divided into two main categories according to their mechanisms of detection and transduction: the first category includes *pure olfactants*, so called because they selectively activate olfactory receptors of the olfactory epithelium alone [45.33]. Examples of such pure olfactants are hydrogen sulfide (H_2S), vanillin, and phenyl ethyl alcohol (PEA) [45.26, 33, 34]. The second category includes *trigeminal odorants*, so called because these odors simultaneously activate receptors found at the end of the trigeminal nerve as well as olfactory receptors [45.35]. A large body of evidence suggests that exposure to pure olfactants during sleep (as opposed to trigeminal olfactory stimulation) does not lead to arousal or wake [45.26, 28, 36] (Fig. 45.5). Some studies suggest that this general notion can be extended to mildly trigeminal odorants as well [45.26, 27, 37].

A study by Carskadon and Herz was one of the first to address the question of whether olfactory stimuli wake from sleep, with an emphasis on the difference between auditory and olfactory processing. The authors presented two odors, four concentration of each, of comparable trigeminal strength but opposite hedonic values: peppermint, which is generally perceived as pleasant and pyridine which is unpleasant and at high concentrations, aversive. Subjects readily detected the odors at wake and were then re-exposed during the night with the instruction to report the detection of odors. Behaviorally, the overall response rate was about 90% in trials conducted in stage 1 sleep, but when tested in stage 2 sleep, REM, and SWS, response rates to pyridine exposure dropped to 45%, 33%, and none, respectively. Trials conducted during the same sleep stages but with the presentation of peppermint did not induce wake at all. In contrast, response rates for the auditory stimuli remained higher than 75% during all sleep stages (Fig. 45.5a). This dissociation was echoed humorously in the article's title – *Why odor alarms will not work for humans* [45.26]. With that in mind, it is further amusing to note that a different research group proceeded to design exactly that, namely an odor-emitting fire alarm designed for the deaf. This, however, is not a contradiction, as the alarm used the odor of Wasabi, a highly trigeminal odorant [45.38].

The notion that olfactory stimuli do not wake was further established in a series of studies. In one, subjects were exposed during sleep to four concentrations of either the odorless trigeminal carbon dioxide (CO_2) or the purely olfactory odorant hydrogen sulfide (H_2S). While exposure to CO_2 resulted in an increase in EEG frequency indicative of brief arousal, the presentation of H_2S did not have such an effect, even at high concentrations (Fig. 45.5d) [45.28]. A second study assessed the latency of arousal following olfactory stimulation. The authors reported a concentration-dependent increase in frequency and latency of arousals only for trigeminal odorants [45.36]. Moreover, whereas administering combinations of trigeminal and olfactory odorants increased arousals from light sleep, no effect was observed for REM or SWS [45.39]. An interim conclusion of the above is that exposure to purely olfactory and mildly trigeminal odorants during deeper stages of sleep does not cause an arousal.

45.4 Odors May Act as Sleep-Promoters: From Aromatherapy to Neuroscience

In the previous paragraph we reviewed evidence implying that odors presented during sleep do not wake. Whereas the neurobiological mechanisms permitting this are being studied, evidence for potential applicative benefits of this phenomenon has been around for millennia.

Aromatherapy is the therapeutic use of essential oils, usually applied through the skin or via the olfactory system. It is an ancient practice claiming significant physiological and psychological effects, particularly on sleep. Although Egyptian, Chinese, and Romans were already practicing aromatherapy in ancient times, the term *aromatherapy* was coined in the late 1920s by the French chemist Rene-Maurice Gattefosse [45.40]. The sleep-promoting effects of specific odorous oils such as lavender or jasmine administered near sleep onset are still widely practiced.

Lavender (*Lavandula spica*) oil is often reported to improve sleep-efficiency parameters (e.g., time awake during the sleeping period and total time asleep) both in healthy individuals and those in need of special care [45.41–43]. A 3 min exposure to lavender oil increased the percentage of time spent in SWS and stage 2 sleep on the account of REM time. This was backed by self-reported increases in vigor the following morning [45.44]. Lavender oil not only enhanced normal sleep but also improved abnormal sleep in a similar magnitude as medication. Total sleep time of insomnia patients who underwent two weeks of medication withdrawal resumed the same levels of sleep as under

medication when ambient lavender was diffused in the room [45.45].

Lavender is not the only aromatic essence known to affect sleep. Cedar extract shortened NREM sleep stage 2 latency in human daytime nap and increased NREM sleep duration in rats [45.46]. Plant-derived odorants such as α-pinene increased paradoxical sleep (PS) duration during a specific night-time zone in adult rats [45.47]. Valerian (*Valeriana officinalis*) inhalation shortened sleep latency in rats and prolonged total sleep time, and both valerian and rose odor prolonged pentobarbital-induced sleeping time [45.48].

Not all aromatic extracts enhance sleep: some exert no influence on sleep while other specific odors may interfere with sleep; Presentation of peppermint essential oil before bedtime did not influence human night sleep when subjective factors were not taken into consideration [45.49], and reduced sleepiness during daytime [45.50]. Similarly, inhalation of lemon odor increased sleep latency in rats [45.48].

Apart from possessing an olfactory percept, many essential oils contain volatile chemical agents capable of exerting a pharmacological effect that cannot be disregarded when discussing their sleep-promoting abilities. These volatiles may readily reach the central nervous system through the blood stream, upon being absorbed by the mucous membranes of the respiratory tracts. For instance, substances found in lavender and valerian oils interact with γ-aminobutyric acid (GABA) receptors, inducing systemic sedative and anxiolytic effects [45.51–53].

Another aspect of the sleep promoting effects of specific odors is cognitive. Peppermint odor enhanced SWS and total sleep time in subjects who perceived it as very intense, in comparison to subjects perceiving peppermint as moderately intense. Furthermore, subjects who found peppermint stimulating had more NREM and less REM sleep while those perceiving it as sedating took longer to reach SWS [45.54]. One may speculate that odors contextually linked with familiar settings or positive experiences can also exert an anxiolytic effect, perhaps through a reduction in sympathetic tone.

Finally, in an international bedroom poll conducted by the USA National Sleep Foundation in 2013, bedroom odors received a central role in sleep hygiene. The majority of respondents answered that they feel more relaxed if their bedroom has a fresh, pleasant scent and reported that they will take steps to make sure their bedroom smells this way. Malodors reported to have a most detrimental effect on sleep quality varied across nationalities but the *usual suspects* were mold, body or pet odor, and stale air [45.55].

The above findings converge to suggest that inhalation of aromatic substances can enhance sleep in terms of reducing sleep latency and affecting sleep architecture. While some of the effects attributed to aromatherapy may be exerted pharmacologically, others are more idiosyncratic as they are modulated by the perceived characteristics of the odor. In turn, the notion that odors may promote sleep follows animal studies as well. For example, high-frequency stimulation of the olfactory tubercle of cats elicited sleep, similar in every respect to physiological sleep, and facilitated SWS [45.56]. Further support for the role of the olfactory tubercle in the physiological mechanism of sleep came from lesion studies which reported decreased sleep time following the lesion of the olfactory tubercle [45.57]. Taken together, these findings imply that beyond not waking, odors may in fact promote sleep.

45.5 Processing of Olfactory Information in Sleep

While basic science and aromatherapy converge to suggest that odors do not wake, odors remain processed by the sleeping brain. For example, rhythmic activity in the rodent olfactory bulb was detected following the sensory stimulation of other modalities [45.58]. Additionally, the biologically relevant odor of fox urine presented during sleep in rats induced increased Fos (an immediate early gene whose expression is an indirect marker for neural activity) expression in the olfactory system [45.59]. In an initial assessment of cortical chemosensory event-related potentials (ERPs) during human sleep, olfactory and trigeminal ERPs were detected in some but not all the participants. Consistent with other sensory modalities, olfactory ERPs latencies were longer, and amplitudes larger, during NREM sleep in comparison to wakefulness [45.60]. In a separate study, fully monitored participants were repeatedly presented with 3 min periods of either clean air or peppermint odor. In a minority of the cases, odors modulated heart rate, as well as EEG and EMG activity [45.37]. Finally, the sniff response is an odorant-specific change in nasal airflow whereby pleasant or mild odors drive stronger sniffs and unpleasant or intense odors drive weaker sniffs [45.62, 63]. The sniff response is therefore a nonverbal implicit measure of olfactory processing. The sniff response remains conserved during sleep [45.27]. For example, the pleasantness of the odorant modified subsequent inhalation

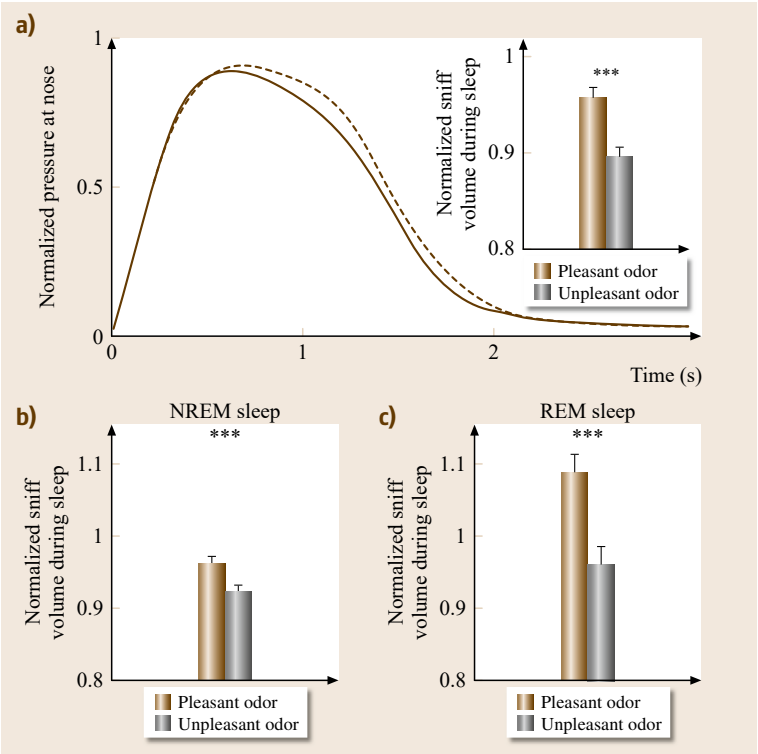

Fig. 45.6 (a) The sniff response, an odorant-specific change in nasal airflow is conserved during sleep, both in REM (b) and NREM (c) stages (after [45.61])

volume in a hedonic valence-dependent fashion, during sleep (see Fig. 45.6) [45.61]. In conclusion, odors do not wake and perhaps even promote sleep, yet they remain processed by the sleeping brain. In this, olfaction differs from other sensory modalities that typically wake from sleep. What is it that allows this special relationship between sleep and smell? Although we do not have a mechanistic answer to this question, we speculate that the answer lies within the unique aspects of olfaction neurophysiology as they relate to sleep neurophysiology. Here, we will detail these unique characteristics of olfaction.

45.5.1 Unique Aspects of Olfaction Neurophysiology May Underlie the Unique Interaction of Sleep and Smell

Olfactory information is projected from the olfactory epithelium to the olfactory bulb, and from bulb directly to olfactory cortex in the ventral temporal lobe [45.64]. This is in contrast to other sensory systems that relay information to primary cortical regions via the thalamus. Synchronized thalamo-cortical oscillatory activity evident during SWS is assumed to aid in the attenuating of sensory processing during sleep [45.65–70]. Given that olfactory information reaches the olfactory cortex directly, it remains independent of this potentially attenuating mechanism (note that olfactory information does traverse a thalamic relay downstream [45.71]). That said, given that olfactory processing during sleep differs from wake, an alternative gating mechanism is likely in effect. Two plausible candidate substrates for such sensory gating are the olfactory bulb and the olfactory cortex. The olfactory bulb and thalamic nuclei share gross structural similarities, and are both subjected to modulation by similar neuronal circuits [45.72]. In addition to the anatomical resemblance, the electrical properties of the olfactory bulb also support its candidacy for a sensory-gating mechanism. The olfactory bulb presents fast electrical activity (30–40 Hz) that decreases during sleep [45.73]. The patterns of electrical activity in the olfactory bulb varied during different states of wakefulness, during physiological sleep, and during unconsciousness produced by central anesthesia or by brain stem lesions [45.58]. The olfactory system of rodents generates slow-wave activity, tightly locked with respiration during sleep and under anesthesia [45.74, 75]. These oscillations between the olfactory bulb and cortex share characteristics with oscillations between the thalamus and cortex during SWS. The periodic respiratory rhythm, specifically the sensory signal resulting from the entry of air into the nasal cavity was speculated to be the driving force of these sleep-

like oscillations in the bulb. Such respiration-induced oscillations are capable of further propagating in the rodent brain, thus contributing to global slow-wave activity [45.76].

An alternative brain region proposed to act as a sensory gate in the olfactory system is the olfactory cortex. Neocortical EEG of anesthetized rats exhibits periodical alternation between a slow wave and a fast-wave state (FWS). Olfactory cortex neurons responded more strongly to odorants during FWS than during SWS. Olfactory bulb neurons however, rarely exhibited such contrast. The authors therefore concluded that during SWS, the olfactory cortex acts similar to thalamic gating mechanisms [45.77].

45.6 The Olfactory System and the Wake–Sleep Regulation System are Highly Connected

An additional distinctive feature of the olfactory system is its widespread connection with the arousal system controlling the sleep-waking cycle. The sleep-waking cycle is regulated by a group of nuclei located in the brainstem through the release of neuropeptides and neurotransmitters. It is established that these nuclei project monosynaptically and polysynaptically to primary and secondary areas of the olfactory system [45.78–80].

A key player in the induction of an alert waking state is the locus coeruleus, a noradrenergic pontine nucleus [45.81]. Approximately, 40% of all locus coeruleus neurons of rats project to the olfactory bulb. This dense connection to the olfactory bulb is almost 10 times greater than to any other part of the cerebral cortex [45.82, 83]. Norepinephrine released by the locus coeruleus disinhibited olfactory bulb neurons [45.84, 85], enhanced detection of relatively weak odors [45.86], and was implicated in olfactory bulb neural responses toward learned odors [86-88].

Orexin, also known as hypocretin, is a hypothalamic neuropeptide implicated in promoting wakefulness. Orexin absence results in difficulty to maintain wakefulness, and REM sleep intrusions. Orexin receptors are present at different levels of the olfactory system, from the olfactory epithelium, through the mitral-tufted cells of the olfactory bulb, and up to the piriform cortex and amygdala nuclei. Although the vast distribution of orexin along the olfactory system suggests a possible modulation of olfactory perception by this neuropeptide [45.87, 88], Orexin's role in olfaction may be more tightly linked to its role in regulating feeding than to its role in sleep [45.89]. Nevertheless, orexin produced hyperpolarization of the olfactory bulb [45.90], and reduced mitral cell firing, without changing the olfactory bulb responsiveness to odors. Other studies report contradicting evidence for the effect of orexin on the olfactory bulb; Orexin significantly increased the firing rate in one study [45.91] and increased olfactory sensitivity in another [45.89]. Even if contradicting, the above findings support a role for orexin in the modulation of olfaction, and provide the grounds for a mechanistic link between olfaction and the wake-sleep cycle.

While it is clear that olfactory function is modulated by brainstem and hypothalamic arousal signals, there is evidence of a reciprocal or complementary effect of olfaction on arousal. The suprachiasmatic nucleus (SCN) located in the anterior hypothalamus is considered the *master* circadian pacemaker, orchestrating the timing of physiological functions and behaviors [45.92] (Fig. 45.4). To adjust the internal clock to outside conditions, the SCN receives input from different systems, including olfactory regions [45.93]. Elimination of free-running circadian rhythms of locomotion by SCN lesions was partially restored using a rhythmic administration of odors [45.94]. Moreover, under constant darkness, cedar oil-induced c-Fos circadian rhythms were observed in the olfactory bulb and in the piriform cortex of mice kept under constant darkness [45.95]. Taken together, these results imply that olfactory stimuli can act as circadian time cues to modulate circadian behavior.

45.7 Olfaction in the Study of Sleep Mentation

So far, we have seen that odors do not wake and possibly promote sleep. Nevertheless, the sleeping brain processes odor information. We speculate that this unique interaction is related to the unique path of odor information in the brain, reaching cortical targets without a thalamic relay. Although a detailed mechanistic explanation for the interplay of sleep and smell remains lacking, this interplay provides a unique window into the study of mentation in both sleep and wake.

45.7.1 Odors as Cues in the Study of Memory and Consolidation

It is largely accepted that sleep plays a central role in facilitating learning. An ever-growing body of evidence demonstrates that memories acquired during prior wake and later reactivated during sleep, were better recalled following the sleeping period. The phenomenon of memory reactivation during sleep, as part of the process of consolidation, is memory type- and sleep stage dependent [45.96–98]. Spontaneous reactivation of neuronal patterns activated during prior wake is widely reported [45.99, 100]. From rodent hippocampal place cells replaying their sequence of activation during a recently learned route [45.101, 102], through juvenile songbirds attempting to master their tutor's song pattern [45.103], to human brains displaying correlation of activation patterns between sleep and preceding wake [45.104–107].

The experimental reactivation of specific memories during sleep, sometimes referred to as *targeted memory activation* (TMR) is a method for the study of the role of sleep in memory processes. Crossing the boundary from scientific research to everyday life, it is a captivating concept that one can achieve such self-augmentation simply by sleeping.

The unique ability of purely olfactory odors to be processed without inducing wake renders them a compelling tool for the study of the role of sleep in the mechanisms of learning, memory, and particularly, memory consolidation. Odors serve as an elegant and covert cue for the reactivation or modification of mental processes experienced or learned prior to the sleep session and even to some extent, to enable learning of new information within the sleep period. For example, task performance was improved by the presentation of an odor cue, as context in prior learning in humans; Volunteers learned a visuospatial object-location task while an odor was present. Next, the odor was presented again during SWS or REM to selectively reactivate the memory linked with it. Re-exposure to the odor during SWS, but not during REM improved the retention time of object location. Functional magnetic resonance imaging (fMRI) revealed hippocampal activation in response to odor presentation during SWS, concurrent with the behavioral findings [45.108]. Another study highlighted the broad state dependence of memory reactivation. Memory reactivation by an odor cue during waking destabilized memories; however, its presentation during SWS immediately stabilized memories. fMRI scans aimed at capturing the differential brain activation responsible for this phenomenon revealed that reactivation during SWS mainly activated hippocampal and posterior cortical regions, whereas reactivation during wakefulness primarily activated prefrontal cortical areas [45.109]. In an ensuing study, odors again served as cues for the reactivation of previously acquired memories, this time within the frame of a short sleep period rather than a full-night sleep. External reactivation by an odor cue during a 40 min sleep period enhanced memory stability to the same extent as 90 min of sleep without odor reactivation, further supporting the role of odor as a potent cue for memory reactivation [45.110]. Employing a similar visuospatial memory task, the authors also looked into the role of odor specificity in the context of memory reactivation, that is, whether odor-induced memory activation requires the same odor during learning and subsequent sleep. Odor re-exposure during sleep significantly improved memory when the same odor was presented both during wake and sleep. This was not the case however, when a new odor or an odorless vehicle was presented (Fig. 45.7) [45.111].

As with the case of consolidation, sleep is suspected to exert a beneficial effect on creativity, and specifically creative insights. While the mechanism responsible for

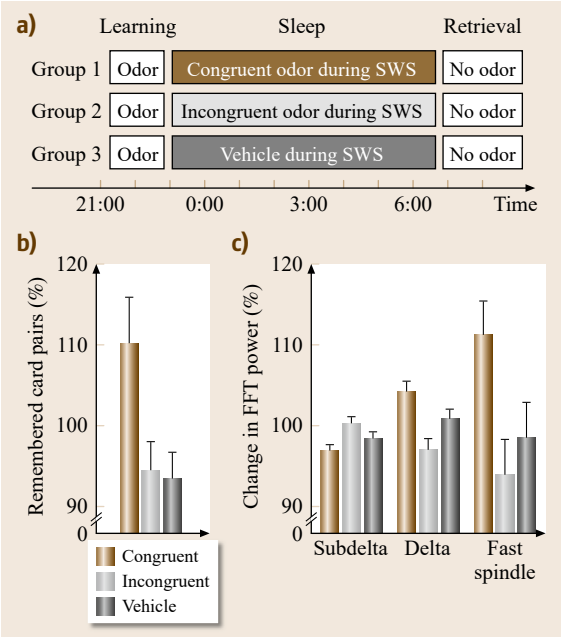

Fig. 45.7 **(a)** Procedure: Participants practiced an object-location task under the presence of a specific odor. Next, the same odor (congruent), a novel odor (incongruent) or an odorless stimulation (vehicle) was presented during SWS. Retrieval took place the morning after sleep without odor. **(b)** Percentage of remembered card pairs after sleep relative to the session before sleep. **(c)** Odor-induced changes in EEG delta power imply consolidation (after [45.111])

producing such *Eureka!* exclamations remains elusive, it has been proposed that reactivations during sleep benefit from the cognitive flexibility of the sleeping state, thus allowing remote associations [45.112, 113]. In a study by *Ritter* et al. subjects were presented with a problem that required a creative solution, while an ambient odor was present. Presenting the same odor in the following sleep session resulted in an increased creativity score when facing the same problem the following morning [45.114]. These results suggest that similar to memory consolidation, the creative process is also reliant on nighttime reactivations, which can be covertly prompted using olfactory cues.

45.7.2 Learning New Olfactory Associations During Sleep

So far, we reviewed the use of olfactory cues promoting the strengthening of memories previously acquired during wake. But can new information be acquired while we are asleep? Studies focused on the subject repeatedly argued that unless an arousal had occurred while information was presented, it became irretrievable upon ensuing wake. A recent study revisited learning of new information during sleep, with the aid of olfactory cues. Partial-reinforcement trace conditioning was used to pair pleasant and unpleasant odorants (shampoo or perfume and rotten fish or carrion, respectively) with two distinct tones during sleep. Respiration was monitored as part of full polysomnography with emphasis on the sniff response following odor presentation (Sect. 45.5). Sniffing following the presentation of tones alone was modulated according to the hedonic value of the odors paired during sleep. Specifically, a tone alone previously paired with a pleasant odor yielded larger sniff volume in comparison to a tone alone previously paired with an unpleasant odor. This implied that a novel association between tones and odors was formed and implemented while asleep. Remarkably, this acquired behavior persisted well into ensuing wake, without any awareness of the learning process. Nighttime effects of tone on sniffing were stronger in REM than in NREM; however, only conditioning which took place during NREM was successfully transferred to wake. This study implies that simple forms of learning such as associative conditioning can be activated during sleep [45.61].

Further support for the concept that new information can be learned during sleep comes from a study which focused on extinction, the process of *forgetting*, or relearning of associations. Subjects underwent contextual fear conditioning during wake in which images of faces were associated with electrical shock while a specific odor was implicitly introduced. Reapplying the odor during a short afternoon nap did not reinforce fear memory. Instead, it reduced fear responses for the face linked with the specific odor, relative to other faces, implying stimulus-specific fear extinction. The physiological fear response was backed up by a parallel reduction of hippocampal activity and reorganization of amygdala activation [45.115]. When an odor was used as a conditioned stimulus in odor-electrical shock fear conditioning, and not as a context, presentation of the odor during sleep resulted in an enhanced fear response when tested during subsequent wake. Injecting the amygdala of mice with a protein synthesis inhibitor following memory reactivation attenuated the previously conditioned fear response in the following wake [45.116]. Despite several methodological differences and possibly contradicting results, both studies illustrate that olfactory stimulation during sleep is a potentially powerful tool for the extinction of painful or maladaptive memories. These methods provide potential treatment for traumatic memories that may lead to phobias or post-traumatic stress disorder (PTSD) [45.116, 117].

45.7.3 The Effects of Odor Stimuli on Dreaming

Dreaming is a profound element of human experience. It is a state of consciousness in which incontrollable internally generated sensory-motor stimuli are experienced. An amalgam of complex, often emotionally charged experiences, woven into intangible plots. Despite its importance, there is little we can confidently say about a phenomenon that takes place every night, in each of our minds (and probably our pet companions' minds too). Our understanding of the function of dreaming is even less clear than that of the functions of sleep in general.

Dreaming was initially associated exclusively with REM sleep because dream recall was more frequently associated with arousals from REM. Nevertheless, it is widely accepted that dreaming takes place during NREM sleep as well [45.118, 119]. As dreams are an altered state of consciousness, a systematic, quantitative study of the dreaming state is a formidable task. The memory of dreams is ethereal, rapidly decaying, and often unintentionally distorted upon being recalled, due to memory-reconstruction mechanisms, verbal description difficulties or self-censorship [45.120].

Most readers will agree that the average dream is rich in visual and auditory experiences, but relatively few olfactory or gustatory experiences. According to a questionnaire-based wide range study only about 1% of all dreams analyzed were reported to contain olfactory or gustatory experiences. Most olfactory reports

came from women, a finding that the authors ascribed to higher olfactory awareness among women. The overall low prevalence of nocturnal olfactory experiences, was linked with the poor ability of humans to generate olfactory imagery in general [45.121]. Despite the fact that the dreaming brain is engaged in internal representations, external stimuli influence dream content. External stimuli may exert an effect on dream *ambiance* and themes, and in some cases even undergo direct incorporation (e.g., experimentally spraying water on skin was reported as being soaked in the dream). In a standard procedure for investigation of such influences the subject is presented with a stimulus in the middle of an REM sleep epoch. Next, the subject is woken up in order to interrogate him or her about their dream content. Tones, words, rocking of the bed, somatosensation, and even pain were demonstrated to be occasionally incorporated into dreams [45.122].

An initial anecdotal report regarding odors in dreams came from a clever self-experimentation session conducted by Hervey de Saint-Denys, a French oneirologist (a specialist in the study of dreams) of the nineteenth century. During a trip that lasted a few weeks, he used a new perfume and hermetically closed the bottle of perfume before traveling back home. A few months later, de Saint-Denys asked his servant to put a few drops of the perfume on his pillow, also instructed his servant not to tell him what night that would be. Ten days later, Hervey de Saint-Denys suddenly dreamed of the same place that he visited during his trip, the very same night the servant had put some of the perfume on his pillow. Such results led him to hypothesize that the stream of dreams is guided by associations, or as he defined them – *psychological affinities* [45.123].

About a century later, a small-scale study examined effects of various olfactory stimuli (both pleasant and unpleasant) on dream content through subjective reports. An incorporation rate of 19% was reported [45.124]. Twenty years later, the question of the effect of olfactory stimuli on dream content was revisited. Considering the direct connectivity between the olfactory pathway and the amygdala, the authors hypothesized that the strongest effect will be on dream emotions rather than direct incorporation of the stimulus. Using an air-dilution olfactometer, they presented two purely olfactory odorants of opposing hedonic values – the pleasant PEA (smell of roses) and the unpleasant H_2S (smell of rotten eggs). The odorants were embedded in constant airflow to prevent additional somatosensory stimulation and odorless stimuli were presented as controls. Reports obtained following odor presentation were scored for realism and dream emotions. Indeed, olfactory stimuli significantly affected the emotional ambiance of dreams, such that the positively toned stimulus yielded more positively toned dreams whereas the negative stimulus led to more negatively toned dreams. Direct incorporations however (the perception of the very object whose odor was presented) were not reported. The authors proposed a clinical application of this finding as a treatment for recurring nightmares, a sleep disorder common among children [45.122].

45.8 Olfaction and Sleep Disorders

Sleep disorders have tremendous physiological, behavioral, and psychological ramifications on life quality. Not only do they affect our sleep they also shape the way we perceive the world when we are awake.

Narcolepsy is a chronic sleep disorder characterized by the inability to regulate the sleep-waking cycle. Narcolepsy patients exhibit persistent daytime sleepiness, abnormal short REM latency, sleep paralysis, and cataplexy, and sudden episodes triggered by strong emotional responses [45.125, 126]. Orexin, a neuropeptide essential for maintaining wakefulness and regulating sleep and wake transitions, is associated with narcolepsy [45.127]. In a recent study, both appetitive and aversive odors presented to orexin-deficient mice, significantly increased the number of narcoleptic attacks. This provided further support for the tight link between odors and the emotional system [45.128]. Furthermore, olfactory dysfunction was observed in narcolepsy patients as reflected from poor scores in olfactory tests as well as a significantly higher olfactory threshold and impaired odor identification in comparison to controls [45.129]. Finally, intranasal orexin administration improved the olfactory threshold for PEA [45.130].

REM sleep behavior disorder (RBD) is characterized by loss of normal skeletal atonia during REM sleep, with prominent motor activity and dreaming. RBD can be linked with neurodegenerative disorders such as Parkinson or dementia with Lewy bodies manifest as an idiopathic disorder (iRBD) which is not linked with any comorbidities [45.131, 132]. Assessment of olfactory tasks revealed higher olfactory threshold, lower discrimination and lower identification scores in individuals with iRBD in comparison to age and sex-matched controls [45.131, 134–137].

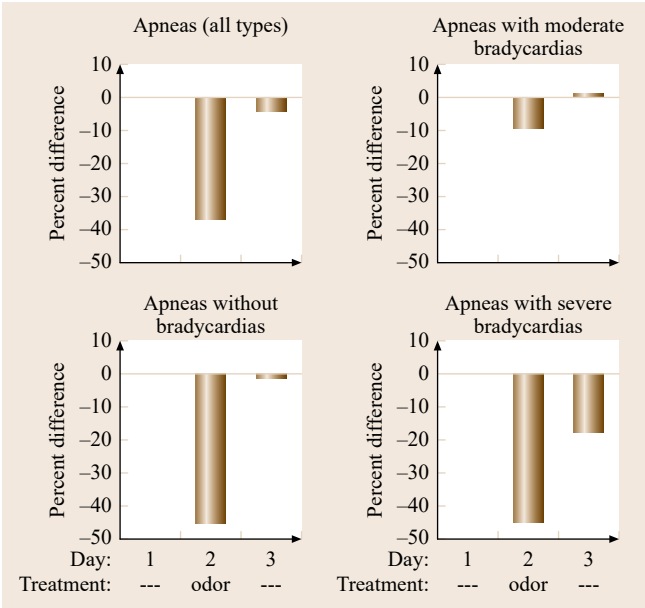

Fig. 45.8 Olfaction stimulation prevented apnea in premature newborn infants. Mean percent difference in the number of apneic spells of different types occurring during (day 2) and after (day 3) exposure to vanillin compared with baseline (day 1) (after [45.133])

45.8.1 Odor Administration During Sleep as a Treatment for Sleep-Disordered Breathing

Sleep apnea is a prevalent sleep-disordered breathing condition, characterized by the repetitive cessation of breathing during sleep leading to brief arousals. The clinical consequences of this disruption of normal sleep architecture cover a wide spectrum of metabolic, neurocognitive and cardiovascular disorders. The majority of sleep apnea is due to obstruction of the soft tissues of the upper airway [45.138, 139]. Sleep apnea is also associated with a deteriorated sense of smell. Sleep apnea diagnosis and severity are assessed by the rate of apneic events per hour named apnea-hypopnea index (AHI) [45.3]. In a recent study in which a comprehensive evaluation of olfactory performance was conducted, a correlation between AHI and score in olfactory tests was reported portraying poorer performance as apnea severity increased. Moreover, the volume of both left and right olfactory bulbs was negatively correlated with AHI [45.140].

Administration of vanillin, a purely olfactory pleasant odorant, to the periphery of a baby's pillow ameliorated bradycardia-associated apnea of prematurity in preterm babies who did not respond to standard pharmacology [45.133] (Fig. 45.8). Recently, the beneficial effects of vanillin on apnea of prematurity were replicated and extended in another study [45.141].

The transfer of this paradigm from prematurity-related apnea (which occurs both in wake and sleep) to adult sleep apnea was attempted as a method to influence respiratory patterns by olfactory stimulation, without inducing arousal or wake. The authors proposed a test of feasibility for treating sleep apnea with olfactory stimulation. Odorants (both pleasant and unpleasant) were introduced during sleep-modified respiration patterns, such that they decreased nasal inhalation and increased nasal exhalation. These findings provided evidence for the manipulation of respiratory patterns during sleep using olfactory stimuli [45.27].

45.9 Final Words

The evidence reviewed in this chapter implies that pure olfactory odors do not wake or arouse during sleep. In turn, the sleeping brain clearly processes these stimuli, and they continue to influence the sensory-motor loops of olfaction, namely sniffing, during sleep. This combination allows the use of odors in the study of sleep mentation. Such use has revealed that odors presented during sleep can strengthen memories of events associated with them during wake. Moreover, pairing of novel odors with novel information during sleep can be learned, and this pairing can later influence behavior during ensuing wake. In conclusion, the nonwaking features of olfaction have already uncovered novel aspects of sleep mentation, and we speculate that they will continue to serve in the study of sleep neurophysiology on one side, and olfactory neurophysiology on the other.

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46. The Intranasal Trigeminal System

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The trigeminal nerve is the fifth and thickest cranial nerve and not only it is responsible for facial sensation and motor functioning, but it is also responsible for chemosensory perception. On top of innervating the skin of the face, the trigeminal nerve also innervates the mucosa of the nose and mouth. Here, chemosensory perception starts with the activation of different receptors, the most important and best known ones being the transient receptor potential (TRP) channels which give rise to sensations such as burning, warmth, coolness, coldness, and pain. From the mucosa, the trigeminal chemosensory information is conveyed through the trigeminal ganglion to the thalamic nuclei in the brain stem; from here fibers project to both, the somatosensory cortex and chemosensory areas of the brain.

Most odors stimulate the trigeminal system, in addition to the olfactory system, especially in higher concentrations. However, overlaps between both sensory systems are not limited to the stimulus level, as they interact with each other on peripheral (mucosa) and central (brain) levels. As a consequence, subjects with a lacking olfactory system show lower trigeminal sensitivity and subjects with a lack of trigeminal sensitivity show lower olfactory activations.

Different techniques are available to assess the state and the functionality of the trigeminal system. Such techniques include behavioral assessment by testing participants lacking a sense of smell – in order to exclude olfactory interference – as well as administrating different types of stimuli (pure odorants, pure trigeminal, or a mix of both). More objective measures include electrophysiological methods that evaluate the peripheral and central activations via the negative mucosal potentials (NMPs) and the trigeminal event-related potentials (ERPs), respectively, as well as functional magnetic resonance imaging, and to a lesser extent, positron emission tomography (PET).

The trigeminal nerve is responsible for facial sensations (touch and pain) and to a considerably lesser degree to motor functioning (masticating). In addition, it also provides chemosensory information from the oral and nasal mucosa, such as the perception of piquancy when eating something spicy. Although a limited amount of

research has focused on the chemosensory part of the trigeminal system, this chapter aims at providing an overview over key knowledge on the trigeminal system. Specifically, the anatomy, the different pathways, the various methods of studying the system, the interactions between the olfactory and the trigeminal system as well as the impact of the loss of one system on the other are discussed.

46.1 General Neuroanatomy of the Trigeminal System

The trigeminal nerve is the fifth cranial nerve (CN V). It is the largest of the 12 cranial nerves; its name stems from the fact that it has three major branches (tri = three; geminus = twin; hence triplets). These three branches are the ophthalmic nerve (CN V1), the maxillary nerve (CN V2), and the mandibulary nerve (CN V3). The trigeminal nerve is primarily a sensory nerve with some motor functions.

46.1.1 Trigeminal Ganglion, Trigeminal Nuclei

Sensory information from the three branches converges on the trigeminal ganglion (Gasserian ganglion, semilunar ganglion); here, the cell bodies of the incoming sensory fibers are located. The trigeminal ganglion has analogous features as the dorsal root ganglia of the spinal cord. Although the cell bodies of the neurons are located in the trigeminal ganglion, there are no synapses; thus the neurons are called pseudo-unipolar. Somatotopic information from the three branches is preserved [46.1], which means that the relative location of the three branches maintains the same location within the trigeminal ganglion. To illustrate, the branches from the ophthalmic nerve will be placed above the branches from the mandibular nerve as their layout on the human face. From here, afferent fibers enter the brain stem in the angle between pons and the middle cerebellar peduncle [46.2]. In the brain stem, they travel to the trigeminal nuclei, which are arranged from the mesencephalon (mesencephalic nucleus) to pons (main sensory trigeminal nucleus) and the rostral portions of the spinal cord (spinal trigeminal nuclei). It is important to note that the fibers are still arranged somatotopically [46.3]. See Fig. 46.1 for an overview over the trigeminal pathway.

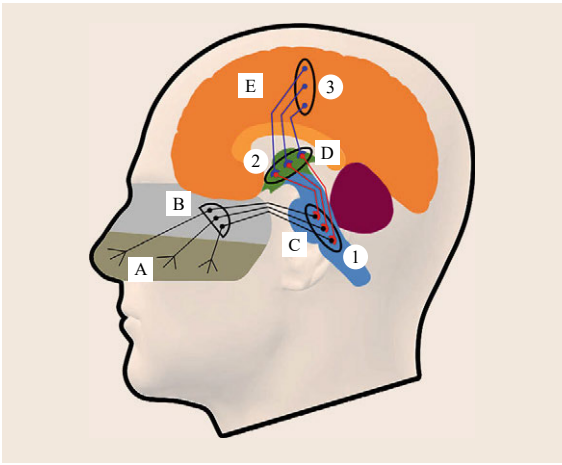

Fig. 46.1 The trigeminal pathway. A: Fibers of the trigeminal nerve (neurons) are located throughout the epithelium of the nasal cavity (*gray*). B: Cell bodies of the trigeminal neuron are located in the trigeminal ganglion. Nevertheless, no information is transferred between neurons at this stage. C: The first (1) relay station is located in the brainstem (*blue*). D: The second (2) relay station is located in the thalamus (*green*). E: Third-order neurons (3) project from the thalamus to the somatosensory regions of the cortex (*orange*). Note that somatotopic information is preserved throughout the pathway. *Purple*: cerebellum; *light orange*: corpus callosum

46.1.2 Central Nervous Processing Structures

The first synaptic relay is found in the trigeminal nucleus, a structure located in the brainstem. Here the information is transmitted to neurons projecting to the second relay station in the thalamus which is a regulator for consciousness and alertness. Specifically, trigeminal information travels to lateral (ventrobasal) and central (centromedial and parafascicular) thalamic nuclei. From here, the third neuronal projection sends the information mainly to the somatosensory cortex which represents the brain area responsible for sensations, such as touch, pain, or heat. In humans, the ophthalmic regions are represented in the inferior portion of the postcentral gyrus, whereas information from the mandibular nerve is processed in more superior regions in the central sulcus [46.4, 5]. Consequently, the relative layout of the three trigeminal branches is preserved in all portions of the trigeminal pathway, from the trigeminal ganglion to the somatosensory cortex.

46.2 Chemosensory Trigeminal Neuroanatomy

In addition to the facial skin, different branches of CN V innervate the mucosa of nose and mouth. Specifically, the ethmoidal nerves from the ophtalmic nerve and the nasal and some alveolar branches from the maxillary nerve innervate the nasal mucosa; further, other branches of the maxillary nerve innervate the oral mucosa together with the lingual nerve, the buccal nerve, and the alveolar nerves from the mandibular nerve. In the mucosa, the nerve endings of the trigeminal nerve are capable to detect chemosensory information in addition to somatosensory information, via specific chemoreceptors.

46.2.1 Peripheral Structures, Receptors

On receptor levels, chemical stimuli activate specific receptors of the trigeminal nerve, most of them being ion channels belonging to the subfamily of transient receptor potential (TRP) receptors, which have been discovered only relatively recently and are discussed in detail in Chap. 34 of this book. One of their main characteristics is that they are activated by both, a certain temperature range and chemical stimuli, such as capsaicin, menthol, camphor, and many more.

In addition to the TRP channels, there is also evidence for non-TRP receptors. These receptors are activated by nicotine (nicotinic acetylcholine receptors) [46.6] and acids (proton-gated ion channels) [46.7–10]. Further, in addition to these receptors on free trigeminal nerve endings solitary chemoreceptor cells have been described in the nasal cavity, although not yet in humans [46.11]. These cells are activated by bitter substances via specific receptors and reach the surface of the nasal epithelium and form synaptic contacts with trigeminal afferent nerve fibers. They may add to the repertoire of compounds that can activate the intranasal trigeminal system.

46.2.2 Central Nervous Processing Structures

Chemosensory information shares the processing units with this classical somatosensory pathway. However, chemosensory trigeminal stimuli activate brain regions in addition to these somatosensory structures such as the insula, the orbitofrontal cortex, and the piriform cortex [46.12–14]. Usually, these additional areas are considered being olfactory and gustatory regions. In order to include trigeminal processing, the term *chemosensory areas* has been put forward to describe the ensemble of these regions. While most trigeminal stimuli also evoke odorous sensations, that is, have a smell [46.15], it is unclear whether the activation of the chemosensory areas occurs via the olfactory pathway – stimulation of olfactory receptors by the trigeminal stimulus leads to the activation of olfactory nerves – or whether there is a direct and chemosensory-specific connection between the somatosensory pathway and the chemosensory processing areas, with a hitherto unknown exact neuroanatomy. The latter hypothesis is supported by the fact that even the odorless trigeminal stimulus carbon dioxide leads to the activation of chemosensory brain areas [46.12]. In fact, there are some collaterals of the trigeminal nerve that end within the olfactory epithelium; some even re-enter the central nervous system and terminate within the olfactory bulb [46.16]. While the exact function of these fibers is unclear, they may provide one of the links by which the trigeminal and the olfactory system interact.

46.3 Trigeminal Perception

Compared to the olfactory nerve, chemoreception within the trigeminal system is relatively unspecific. Still, the sensations arising from trigeminal chemosensation are well beyond simple pain perception as trigeminal stimulation may lead to such diverse perceptions as cooling, burning, stinging, or tingling. The TRP channel receptors play a key role in trigeminal perception.

46.3.1 Receptors and Perception

The first TRP receptor to be discovered and described in detail is the TRPV1 receptor. It is activated by noxious heat above 43 °C [46.17] as well as a variety of chemical stimuli, including capsaicin (the spicy ingredient of hot peppers) [46.17], eugenol (the key compound of essential oil of cloves) [46.36], and acids [46.33]. Stimulation of TRPV1 receptors leads to the perception of a tingling perception, which in higher intensities becomes sharp, burning, and painful [46.44]. There are several other TRPV such as TRPV3. This receptor is activated at lower temperatures starting from 39 °C [46.45, 46]. This receptor is also chemosensitive as different compounds, such as thymol (a main component of spices such as oregano and thyme) [46.24, 26], act as agonists. Activation of TRPV3 is associated with the sensation of warmth [46.26].

Table 46.1 Different stimuli activate different TRP receptors

Substance	Receptor	
Acetylcholine		[46.17]
Adenosine triphosphate		[46.17]
Amyl actetate		[46.18, 19]
Allicin		[46.20–22]
Benzaldehyde		[46.18, 19, 23]
Borneol		[46.24]
Bradykinin		[46.17, 25]
6-tert-Butyl-*m*-cresol		[46.24]
Camphor		[46.24, 26–32]
Cannabinoids		[46.21, 33, 34]
Capsaicin		[46.17, 19, 26, 33]
Capsazepin		[46.17]
Carvacrol		[46.24, 26]
Carveol		[46.24]
L-Carvone		[46.25]
Cinnamaldehyde		[46.25, 28]
Citral		[46.35]
Diallylsulfide		[46.20]
Dihydrocarveol		[46.24]
Eucalyptol		[46.27, 31]
Eugenol		[46.23, 25, 26, 36]
Formalin		[46.21, 37, 38]
Geranial		[46.35]
Geraniol		[46.35, 39]
Gingerol		[46.25]
Glutamate		[46.17]
HC-030031		[46.37]
Helional		[46.39]
Heliotropyl acetone		[46.39]
cyclo-Hexanol		[46.27]
cyclo-Hexanone		[46.19, 23]

Table 46.1 (continued)

Substance	Receptor	
Histamine		[46.17]
4-Hydroxynonenal		[46.21, 38, 40]
Hypertonic saline		[46.17]
Icilin		[46.27]
Incensol acetate		[46.41]
Isopulegol		[46.25]
Javanol		[46.39]
Limonene		[46.19]
cis-p-Menthane-3,8-diol		[46.25]
trans-p-Menthane-3,8-diol		[46.25]
Menthol		[46.25, 27, 28, 42]
Menthone		[46.27]
Menthyl lactate		[46.25]
Methyl salicylate		[46.25]
Mustard oil		[46.25, 33, 37]
Neral		[46.35]
Nerol		[46.35]
(−)-Nicotine		[46.19]
Phenyl ethanol		[46.39]
Putrescine		[46.43]
Protons		[46.17]
Resiniferatoxin		[46.17]
Sandalore		[46.39]
Sandranol		[46.39]
Serotonin		[46.17]
Substance P		[46.17]
Spermidine		[46.43]
Spermine		[46.43]
Terpineol		[46.18]
Toluene		[46.18, 19]
Thymol		[46.24, 26]

Table 46.1 (continued)

Substance	Receptor	
Vanillin		[46.39]
WS23		[46.25]
WS3		[46.25]

Dark blue: TRPA1; *light blue*: TRPM8; *orange*: TRPV3; *red*: TRPV1; *full square*: agonist; *black diagonal stripes*: antagonist; *white diagonal stripes*: partial agonist; *empty square*: no effect; *empty space*: not tested

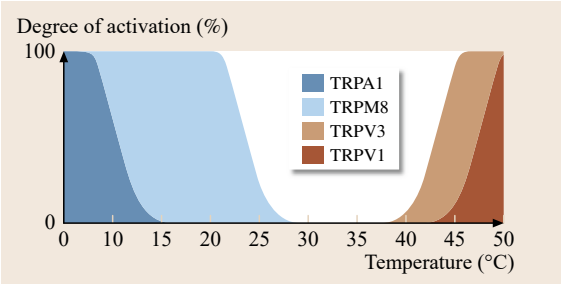

Fig. 46.2 Receptive range of four intranasal TRP receptors. The *green area* indicates the physiological temperature in the nasopharynx, that is, the temperature to which the nose conditions the inhaled air (after [46.49])

On the other side of the physiological temperature range, we find the TRPM8 receptor. This particular receptor is activated by cool temperatures starting from 39 °C [46.27], as well as chemical substances such as menthol (main component of peppermint) [46.27], and eucalyptol (eucalyptus) [46.47]. Stimulation of this receptor provides the sensation of cooling without being particularly painful. Further toward the range of noxious cold is the TRPA1 channel, which is activated by cold temperatures below 17 °C [46.25, 33]. Again, this receptor is chemosensitive as compounds such as allyl thioisocyanate (mustard oil) [46.25, 33] serve as agonists. TRPA1 stimulation gives rise to a dull, painful sensation. See Fig. 46.2 for an overview over the temperature ranges of these four receptors. It is interesting to note that many of the stimuli are ingredients of spices and herbs, such as hot pepper, cloves, oregano, thyme, eucalyptus, peppermint, mustard, etc. See Table 46.1 for an overview of the different stimuli that activate the four TRP receptors mentioned in this chapter.

When humans breathe air at room temperature normally, inhaled air is heated within the nasal cavity to an average of 28–32 °C [46.48, 49]. This temperature lies between the temperature ranges of TRPM8/TRPA1 on one hand and TRPV3/TRPV1 on the other hand. Thus, none of these receptors should be activated when we breathe clean air at room temperature. Upon stronger inhalation of air at room temperature, however, the TRPM8 receptor may be activated, since intranasal temperature drops [46.49]. Further, changes of the air temperature induce receptor potentials and, as a consequence, the perception of cold, cool, warm, or hot. Similarly, the presence of one of the multiple trigeminal stimuli may induce receptor potentials evoking according sensations even in the absence of a real temperature change. In fact, the inhalation of menthol does lead to the perception of coolness and, by analogy, the sensation of an increased nasal patency. Objective measures of nasal air temperature and nasal patency however show that menthol inhalations do not affect any of both measures [46.48].

We do not yet know how different trigeminal receptors are distributed within the nasal epithelium. In this context, it is interesting to note that three different trigeminal stimuli, which most likely bind to different receptors, evoked comparable activation patterns at different sites within the nasal epithelium [46.50]. Therefore, different areas of the nasal cavity seem to exhibit distinct concentrations of receptors, but the ratios between different receptors seem to be stable throughout the nasal cavity.

46.4 Assessment of the Trigeminal System

Different methods are available to assess the sensitivity of trigeminal system in humans in vivo. One challenge a researcher faces is the fact that olfactory and trigeminal system are intimately related. Specifically, most trigeminal stimuli activate the olfactory system in addition to the trigeminal system, and they do so at lower concentrations [46.51]. Therefore, although the intensity of the trigeminal sensation is usually stronger than the olfactory one, there usually is some form of interference from the olfactory input. Several methods have been used to overcome this problem, each of which has its own advantages and disadvantages.

46.4.1 Behavioral Methods

Different methods are available to assess sensitivity in the trigeminal system with behavioral measures. In order to avoid the effects of olfactory interference, one could either test participants with anosmia, i. e., lack of

olfactory perceptions, or focus on tests which rely more or less exclusively on trigeminal sensations.

Behavioral Testing in Anosmia

Anosmia, acquired or congenital lack of olfactory function is a relatively common finding as 1 in 20 is affected in the general population [46.52, 53]. Of course, persons with anosmia do not perceive the olfactory component of mixed olfactory-trigeminal stimuli; thus minimizing olfactory interference. In fact, persons with anosmia can only detect mixed olfactory-trigeminal stimuli by virtue of the test compound's trigeminal component. This can be used to assess the trigeminal impact of a substance, since the percentage of subjects with anosmia who detect a stimulus is correlated with the stimulus' trigeminal impact [46.15, 54]. The typical method to assess olfactory thresholds in persons with normal olfactory function is to ask participants to detect an odorous substance in different concentrations amongst several odorless blanks. If the same stimulus with the same paradigm is used in subjects with anosmia the resulting threshold will be based on the trigeminal sensation [46.55, 56], thus providing a method to assess trigeminal thresholds.

However, one has to be careful when generalizing the findings from persons with anosmia to the general population. We know that anosmia is associated with a reduced trigeminal sensitivity [46.57–60]. The difference in trigeminal sensitivity between subjects with anosmia and participants with a normal sense of smell may be small, and therefore not be detected when investigating only a small number of subjects [46.55, 56, 61], but is a clear limiting factor of this approach.

Tests Depending on Trigeminal Stimulation

In addition to testing subjects who cannot perceive olfactory information, olfactory interference can be excluded in those tests, which depend solely on trigeminal perception. One exemplary method is the lateralization task, in which subjects are asked to identify the nostril to which a stimulus has been delivered (lateralization task). This task is based on the fact that humans are unable to localize pure odorants [46.62, 63], but can do so for stimuli which activate the trigeminal system, mixed olfactory/trigeminal stimuli. Depending on the trigeminal impact [46.44], such stimuli can be localized with high accuracy [46.51, 62, 64–67]. Using this fact allows for two different tasks: first, one can determine the sensitivity of the trigeminal system in a given population or compare sensitivity between two groups of subjects [46.66, 68]; second, one can determine the trigeminal potency of different chemical compounds [46.44, 56]. Thus, by using the lateralization test, one can determine the threshold for a given substance, by using a staircase procedure [46.51]. Such a procedure is often used as an experimental design to assess the thresholds by lowering or elevating the task difficulty until we find the minimally intense stimuli to which the participant would respond accurately. Alternatively, a single concentration of a test compound with strong trigeminal impact may be used in a semiquantitative screening protocol. Here, the sum of correct identifications is used for further statistical analyses. The latter is mostly used in a clinical setting. The lateralization test can be cumbersome, since it is important to keep a relatively long inter stimulus interval of at least 40 s in order to avoid habituation [46.69]. An alternative method is to register response times in a lateralization task [46.70], which has the advantage to provide parametric data with fewer trials and thus shorter test times.

As an alternative, some groups take advantage of the fact that the cornea and conjunctiva of the eye are also innervated by the trigeminal nerve. These epithelia, in fact, allow for the perception of painful sensations, but do not respond to pure olfactory stimuli. Thus, vapor-phase chemical stimuli which evoke the sensations of burning or stinging in the cornea can be used to assess trigeminal irritation thresholds in the eye. However, one has to be careful to avoid co-stimulation of the nose and thus the olfactory system. Corneal thresholds can be used as estimates of trigeminal sensitivity since irritation thresholds obtained in the eye and nose are significantly correlated [46.71].

Another approach is to instruct subjects with a normal olfactory function to distinguish between olfactory and trigeminal sensations. Therefore, after being trained to focus on trigeminal sensations subjects learn to disregard the simultaneous olfactory sensation. Typically, subjects receive instructions such as *have you felt any sensation like burning, stinging, cooling, or tickling?* By using this method, one can assess trigeminal perception thresholds [46.58, 72–74]. However, one has to keep in mind that olfactory interference will occur and can unconsciously affect subjects' responses, which limits the application of this method.

Another method rules out olfactory interference, by using *pure* trigeminal stimuli, stimuli which exclusively activate the trigeminal system. However, a possible concomitant olfactory stimulation is very difficult to exclude. Only few stimuli are available in this category; they include CO_2 or capsaicin. Here it is important to note that CO_2 acts as a trigeminal stimulus only in very high concentrations ($> 100\,000$ ppm) [46.75], which may be dangerous for participants' safety. Thus, CO_2 as a nasal trigeminal stimulus can safely be employed only as brief (< 3 s) stimulus, or alternatively with mouth breathing and velopalatine closure (isolation of the nasal cavity).

46.4.2 Electrophysiological Methods

In contrast to behavioral techniques, electrophysiological measures rely less on participants' response and collaboration. They therefore provide a more objective assessment of trigeminal sensitivity. Still, the problem of olfactory interference is difficult to avoid.

Event-Related Potentials

Trigeminal ERP are electroencephalography (EEG)-derived polyphasic signals obtained at the surface of the scalp [46.76] due to the activation of cortical neurons that generate electromagnetic fields. In other words, trigeminal ERP are a central nervous representation of the processing of trigeminally mediated sensations. The EEG is a noisy signal containing activity from many cortical neurons; therefore ERP have to be extracted from the background activity; this can be done by averaging responses to single stimuli reducing the random background noise [46.77]. Again, single stimulations have to be separated by a relatively long inter-stimulus interval of at least 30–40 s to avoid effects of habituation [46.69]. Further, in order to obtain meaningful averages with an acceptable signal-to-noise ratio, at least 10 single responses have to be recorded resulting in ERP sessions of 45 min to 2 h. This requires subjects' vigilance to be stabilized by simple tasks, such as a tracking task on a computer screen [46.78]. The main advantage of ERP is the very high temporal resolution in the range of milliseconds. However, this comes with a relatively poor spatial resolution. Further, ERP are prone to artifacts; therefore successful ERP recording requires an olfactometer, a device which allows for the delivery of stimuli with:

1. A sharp onset
2. Exactly defined duration
3. Without concomitant mechanical co-stimulation
4. Without concomitant thermal co-stimulation [46.76].

The nomenclature of the trigeminal ERP responses follows that of other sensory domains; a small first positive peak (P1) typically occurs at latencies later than 200 ms, followed by a first major negative peak (N1; approximately 400 ms), and the late positive complex (P2 or P2/P3; approximately 650 ms) [46.77, 78] (Fig. 46.3 for an overview). Largest responses are obtained from central and parietal electrodes; measures of interest are usually amplitudes and latencies of the major peaks. ERP are mostly used in a research setting, since they require a relatively high effort in terms of cost and time. Further, the discussion on olfactory interference also applies to trigeminal ERP: since most trigeminal stimuli also activate the olfactory system, trigeminal ERP will in most cases also contain signals from olfactory processing areas.

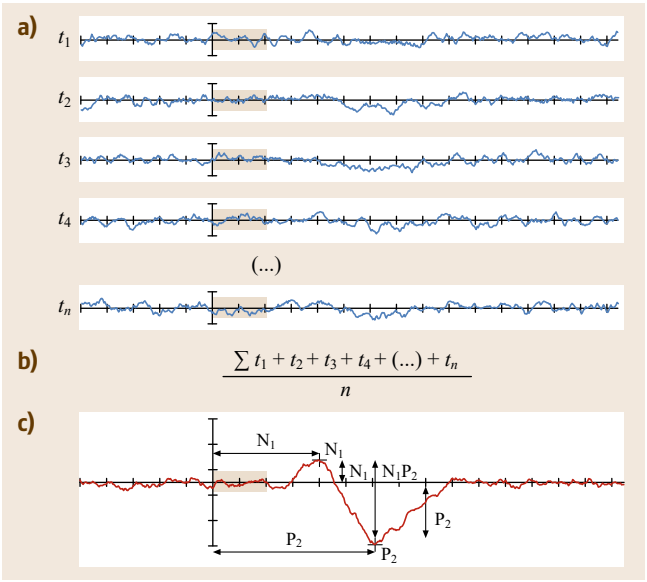

Fig. 46.3a–c Procedure of trigeminal ERP recording: **(a)** Single EEG recordings in response to a 200 ms stimulus (*shaded area* indicates stimulus). Note that response to the stimulus is overlapped by random brain activity. In order to extract specific response, several trials are recorded (t_1 to t_n). **(b)** EEG response are averaged. **(c)** Event-related potential is visible after averaging. The most important peaks, N1 and P2 are indicated by *crosshair*. For each peak one can analyze latency (*blue*; N1 and P2), and amplitude (*red*; baseline to peak amplitude: N1 and P2; peak-to-peak amplitude: N1P2)

Negative Mucosal Potential

Another electrophysiological procedure is relatively free from olfactory interference: the NMP is measured on the level of the nasal mucosa and thus the periphery of the trigeminal system [46.79–83]. The NMP is the summating receptor potentials of chemoreceptors of the trigeminal nerve [46.84] and thus an electrophysiological correlate of trigeminal activation of the nasal respiratory epithelium [46.79–83]. As such it is independent from olfactory stimulation. NMPs are recorded by means of an electrode placed on the respiratory mucosa [46.85]; electrode placement should be done under endoscopical control [46.86]. Again, the signal is averaged, but due to the lower background noise, fewer recordings are needed to obtain a meaningful NMP. In fact, one single recording may be enough for interpretation. Similar to ERP, NMP recording requires an olfactometer for stimulus presentation to assure a sharp stimulus onset and exactly defined duration and to avoid concomitant mechanical and thermal co-stimulation.

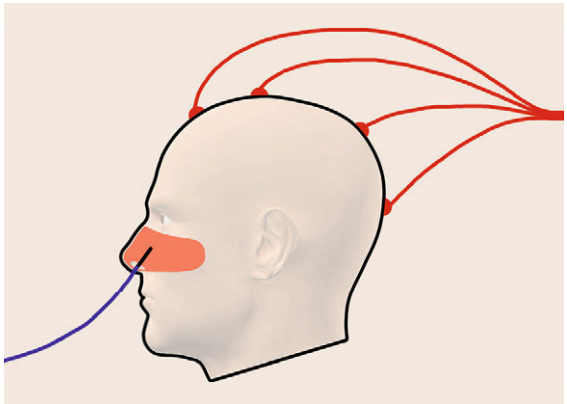

Fig. 46.4 Electrophysiological recording sites: event-related potentials are EEG derived signals and recorded from the surface of the skull (*red*), central responses; negative mucosal potentials are mucosa-generated responses and recorded from the epithelium (*blue*), peripheral responses

NMP consist of a slow negative wave with a latency of approximately 1000 ms [46.87]. The largest NMP indicating the highest sensitivity is observed at the nasal septum, lowest on the nasal floor and the olfactory cleft [46.50, 88]. See Fig. 46.4 for an overview over electrophysiological recording sites.

46.4.3 Brain Imaging

Over the last three decades, several techniques have been made available to neuroscientists, which allow for the investigation of brain structure and brain function in vivo. The most commonly used technique is functional magnetic resonance imaging (MRI), which measures the ratio of oxygenated hemoglobin/deoxygenated hemoglobin in the brain. From this one can infer the activation level of different brain areas (neurovascular coupling). Specifically, researchers can observe which areas of the brain are activated by different tasks. Functional MRI permits the investigation of brain activation with a relatively high spatial resolution. Still, a voxel – the 3D analog of a pixel – typically has the size of 27 mm^3 (3 mm × 3 mm × 3 mm); thus each voxel contains the information of the average activation of ten thousands of neurons. Functional MRI has been used to explore sensory processing; in some studies the trigeminal system has been examined with this technique. Functional MRI has shown that chemosensory trigeminal stimulation leads to the activation patterns which only partly overlap with somatosensory stimuli (brainstem [46.14], thalamus [46.13], SI/SII [46.89], anterior cingulate [46.14]). However, chemosensory trigeminal stimuli also activate olfactory regions, piriform, orbitofrontal, and insular cortex [46.12, 14, 90]. It is worth mentioning that especially orbitofrontal areas are susceptible to image distortion in MRI due to the proximity of the nasal sinuses making the examination of this chemosensory area somewhat difficult.

Other brain imaging techniques can be used to investigate the trigeminal system, but have so far only been used in a few studies. One of these techniques, PET assesses the concentration of radioactive markers. Importantly, it allows for imaging of the orbitofrontal areas of the brain free of distortion; however, this comes with a weaker spatial and temporal resolution compared to functional MRI. PET-based experiments reported additional activation in the amygdala, claustrum, and lateral hypothalamus due to trigeminal chemosensory stimulation [46.91].

46.5 Interactions Between Olfaction and Trigeminal System

As mentioned earlier, most stimuli activate the trigeminal system as well as the olfactory system [46.51, 92, 93]. Additionally, psychophysical and electrophysiological findings report that these two systems interact [46.93], by suppression and enhancing each other [46.92, 94–98]. It is hypothesized that this interaction occurs at three levels:

1. On the stimulus level
2. In the periphery (mucosa)
3. On central levels (brain; Fig. 46.5) [46.92].

In order to understand this interaction, it is necessary to evaluate both sensory systems separately. However the fact that they are usually stimulated together raises a serious methodological challenge. One possibility to overcome this problem is to investigate the trigeminal function in subjects without a working sense of smell [46.93].

46.5.1 Stimuli

Subjects with anosmia are able to detect a large number of volatile chemicals despite the lack of functionality of the olfactory nerve, suggesting that stimuli perception was processed by the trigeminal nerve [46.15]. Moreover, patients with olfactory dysfunction also demonstrate lower trigeminal sensitivity which highlights the interdependence of the two systems [46.99, 100]. Additionally, healthy subjects have higher trigeminal thresholds compared to olfactory ones suggesting

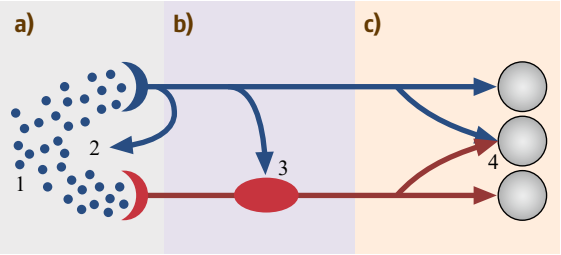

Fig. 46.5 Sites of interaction between the trigeminal system (*blue*) and the olfactory system (*red*). (a) Mucosal level: 1. The same molecules can stimulate both, the olfactory system and the trigeminal system; 2. Trigeminal reflexes may alter nasal patency. (b) Olfactory bulb: 3. Some trigeminal nerve endings terminate in the olfactory bulb. (c) Central nervous system: 4. The trigeminal system and the olfactory system share central processing units

that it takes a higher concentration of an odorant to stimulate the trigeminal nerve as compare to the olfactory nerve [46.51]. Thus, most odorants also activate the trigeminal system. It should be noted however that although most odorants activate both, the trigeminal and the olfactory nerves, there are a few exceptions. For instance, CO_2 activates the trigeminal system with little or no concomitant olfactory stimulation [46.101–103]. On the other hand only a few odorants activate the olfactory nerve selectively with little activation of the trigeminal nerve [46.15] such as vanillin [46.104] decanoic acid [46.15], hydrogen sulfide (H_2S) [46.104] and phenylethyl alcohol (PEA) [46.51, 63].

Further, most irritants that stimulate the trigeminal system can activate the olfactory system as well. This has been shown indirectly, as trigeminal stimulation activates regions typically involved in the olfactory processing such as piriform cortex [46.12, 105], the anterior orbitofrontal cortex [46.12, 106–108], rostral insula [46.12, 109], and the superior temporal gyrus [46.12, 110]. In addition, CO_2, which is usually considered to be a selective trigeminal stimulus, recruited the olfactory pathways in low concentrations [46.111]. Moreover, roughly 10% of piriform cortex neurons responded to both, olfactory and trigeminal stimuli, further highlighting the overlap between the two systems. Altogether, these studies show that both sensory systems exhibit a large extent of promiscuity already on the level of the stimuli.

46.5.2 Indirect Interaction

On top of an interaction at the level of the stimuli, there is also an indirect interaction between the trigeminal and the olfactory systems. For instance, trigeminal reflexes trigger alteration of nasal patency and respiration as well as changes in the mucus covering the epithelium as a result of the stimulation of glands and secretory cells [46.112]. Additionally, electrophysiological studies indicate that odors can modify, via local axon reflex, the spontaneous activity of olfactory receptor cells which triggers the release of different peptides as well as analgesic effects from trigeminal fibers innervating the olfactory epithelium resulting in a modification of odor perception [46.113–115]. Putting together, these studies indicate that other substances can influence the olfactory and the trigeminal systems [46.93].

46.5.3 Peripheral Interaction

In addition to a complete loss of olfaction, subjects with anosmia also have a lower trigeminal function, when assessed with behavioral measures [46.93, 100, 103, 116, 117]. On peripheral levels however, subjects with anosmia exhibit larger NMP, which indicated increased peripheral susceptibility [46.68, 87]. Thus, a model with mixed sensory adaptation/compensation was put forward to explain the interaction between the two systems at a peripheral level. Some neuroanatomical studies provide underpinnings for these interactions. For example, axons of the trigeminal nerve re-enter the central nervous system (CNS) and terminate in the glomerular layer of the olfactory bulb [46.16, 118]. They may be activated by the lateral excitatory network within the olfactory glomeruli [46.119] of a working olfactory system, which is associated with a reduced peripheral responsiveness [46.74]. In the case of olfactory loss, however, this excitatory network is no longer activated; as a consequence, the intrabulbar trigeminal collaterals become disinhibited, resulting in an increased activation of the trigeminal system at peripheral levels [46.93]. In other words, the lack of a functioning olfactory system leads to the disinhibition of the intrabulbar trigeminal collaterals which results in higher NMP activations. This type of mechanism is also found in the gustatory system in which input from the chorda tympani inhibits that of the glossopharyngeal nerve. Damage to the chorda tympani abolishes this inhibition which increases the input from areas innervated by the glossopharyngeal nerve [46.120].

46.5.4 Central Interaction

Studies on patients with anosmia also revealed interactions of the two systems at central levels. When evaluating such interaction at central levels, electrophysiological measures are particularly useful. Trigeminal event-related potentials (tERPs) are electrophysiological responses generated by the cortex. Compared to controls, subjects with acquired anos-

mia exhibited smaller tERPs [46.87], whereas those with congenital anosmia presented similar activations as controls [46.68]. Moreover, low concentrations of isopentyl acetate (an odor with trigeminal components), triggered odor-like responses in the piriform cortex of mice whereas at high concentrations triggered trigeminal-like responses providing support that some neurons of the piriform cortex can be modulated to respond to both types of stimuli [46.121]. Additionally, the duration of olfactory dysfunction and tERP amplitudes are positively correlated [46.59] suggesting that anosmia-triggered reduction of trigeminal sensitivity could improve over time. Therefore, these studies advocate that on one hand, a fully functional trigeminal system relies on the functionality of the olfactory system [46.58–60, 68, 122, 123], and on the other hand, longer the period of time of olfactory loss, better the adaptive mechanisms. FMRI studies provide further evidence as to the key areas of central interaction between olfactory and trigeminal stimulation [46.12, 90]. While each sensory system has unique central processing areas, they share a considerable amount of central processing areas. For example, activation of brainstem, ventrolateral posterior thalamus, anterior cingulate, precentral gyrus, and somatosensory areas are commonly observed following trigeminal stimulation, but not after exposure to odors [46.14]. In turn, activation of amygdala and the ventral putamen is a typical feature of olfactory but not of nonpainful trigeminal stimulation [46.124]. However, both trigeminal and olfactory stimulation activate piriform cortex, the orbitofrontal cortex and the rostral insula [46.14, 124]. Thus, these are the prime candidates on the quest for the areas of interaction between the trigeminal and the olfactory system. In fact, patients with anosmia exhibited, when compared to controls, less activations in, amongst other regions, the orbitofrontal cortex and the insula, but also primary somatosensory cortex [46.89]. This may help explaining smaller tERP amplitudes and higher trigeminal thresholds in this patient group [46.93]. In healthy subjects a mixture of CO_2 (trigeminal stimuli) and PEA (pure odorant) led to higher activations than the sum of activation of CO_2 and PEA presented independently, in both, chemosensory areas (orbitofrontal cortex) and multisensory integration centers (intraparietal sulcus) [46.125].

Putting together, these imaging studies suggest a great overlap between the two chemical senses highlighting the intimate connections between the two systems.

46.6 Clinical Aspects of the Trigeminal System

Different factors may affect trigeminal sensitivity. The most prominent amongst them is aging as older people consistently exhibit lower trigeminal sensitivity [46.83, 123]. Additionally, different diseases and olfactory dysfunction also impact the trigeminal function [46.59]. As previously mentioned, the olfactory and trigeminal systems interact with one another at different levels and therefore examining a clinical population with olfactory and trigeminal dysfunction could shed some light on the co-dependence of the two systems.

46.6.1 Olfactory Dysfunction and Trigeminal Perception

Hyposmia (a reduced sense of smell) and anosmia are relatively common in the normal population. It is estimated that around 15% of the population exhibit hyposmia and 5% suffer from anosmia [46.52, 53]. Generally, typical causes of hyposmia and anosmia include sinunasal disease, head trauma, upper respiratory tract infections, or neurodegenerative diseases whereas congenital anosmia occurs only in around 2% of those with anosmia [46.126–128]. Anosmia and hyposmia are associated with lower trigeminal sensitivity when tested with several different techniques. Specifically, subjects with anosmia exhibit less changes in their breathing pattern when inhaling airborne chemicals [46.122, 129], have lower intensity ratings of trigeminal stimuli [46.60], exhibit higher thresholds to irritants [46.58, 130], and have fewer correct answers on lateralization tests [46.62, 123]. Lower trigeminal chemosensitivity seems to be a general feature of acquired anosmia and independent of its etiology [46.123], while congenital anosmia is somewhat different from acquired anosmia. As a matter of fact, congenital anosmia is associated with similar behavioral measures of trigeminal sensitivity as controls [46.68]. Interestingly, somatosensory measures (such as responsiveness to touch) are not affected by olfactory dysfunction [46.57]. Further, peripheral susceptibility seems to be larger in patients with anosmia (congenital or acquired), in sharp contrast to the findings from central and behavioral measures [46.93].

The interactions between anosmia and the trigeminal system seem not be stable over time: the duration of the olfactory loss and the functionality of the trigeminal system are correlated as the latter gets better over time [46.87]. Further, those 10–30% [46.131] of cases

of anosmia which show recovery of olfactory function actually exhibit even larger peripheral responses than those without recovery [46.87] suggesting additional complex mechanisms taking place. Puttting together, these studies show that acquired anosmia is associated with lower trigeminal sensitivity, while anosmia in general is associated with increased peripheral responsiveness in the trigeminal system.

46.6.2 Trigeminal Dysfunction and Olfactory Perception

As previously mentioned, the trigeminal nerve is the thickest of the cranial nerve which makes it more resistant to trauma compared to the olfactory nerve [46.87]. Consequently, cases in which the trigeminal nerve is damaged are very scarce. In one experiment, local anaesthesia of the middle nasal meatus resulted in an elevation in detection thresholds of *n*-butanol suggesting that blocking the trigeminal system results in a reduced olfactory function [46.132]. In another report, two subjects with a total unilateral destruction of the trigeminal nerve due to the removal of an acoustic neuroma, reported lower odor intensity ratings compared to controls with normal trigeminal function [46.133]. In another case study, a women who, as a result of a meningioma, had a loss of trigeminal function on her left side, exhibited lower olfactory function on the affected side [46.103]. Specifically, NMPs responses, tERPs responses to H_2S (pure odorant) as well as to CO_2 (trigeminal irritant) were reduced on the left side compared to the right side. Additionally, pure odorant detection threshold was 64 times higher on the left side compare to the right side. Puttting together, a damaged trigeminal system may have an impact on olfactory perception highlighting once more the interdependence of the two systems at different levels of odor processing.

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47. Cross-Modal Integration in Olfactory Perception

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In everyday life, odorous sensations are a multisensory experience. In other words, odors are perceived before, during, or after inputs of other sensory systems like the visual, gustatory, auditory and tactile system. Thus, multisensory processing of olfactory cues should be assumed as a realistic stimulation for the perception of odors.

Odors are perceived through two main pathways: orthonasal and retronasal routes and odors are processed in a different manner depending on the pathway. Therefore, before reviewing effects of other sensory cues on olfactory perception, both orthonasal and retronasal olfactory systems are discussed at psychophysical, cortical electrophysiological and neuroanatomical levels.

This chapter introduces cross-modal correspondence between olfactory and other sensory cues; it also features the effects of other sensory inputs, such as visual, gustatory, auditory, trigeminal and tactile cues, on olfactory perception, with a focus on key modulators in cross-modal integration.

Overall, most of the cross-modal integration between olfactory and other sensory cues appears to occur at a central nervous level. Many studies have emphasized the role of congruency between bimodal cues in cross-modal integration. The modulatory effect of congruency was found to be influenced by many factors such as odor delivery route (orthonasal and retronasal pathways), selective attention, experience (associative learning), cultural background, type of given task (analytical versus synthetic) and characteristics of a given stimulus.

Odors are rarely experienced without accompanying other sensory cues. For instance, while consuming potato chips, we experience potato chip odor with its visual (shape and color), gustatory (taste), tactile (texture) and auditory (the sound of biting chips) cues. This example shows that olfactory perception is subject to the multifaceted combination of diverse sensory inputs. That is, perceived intensity or pleasantness for a certain odor (e.g., coffee odor) may be altered by different sensory input. For instance, let us assume that someone is asked to describe odor quality of a white wine artificially colored red. Can the person identify it as a white wine? Interestingly, *Morrot* et al. [47.1] showed white wine-colored red was identified as red wine by tasters, indicating that odor perception can be altered by visual input.

This chapter is composed of three main sections:

1. Orthonasal and retronasal olfaction
2. Cross-modal correspondence between olfaction and other sensory cues, and
3. Influences of different sensory inputs on olfactory perception.

There exists a difference in the perception of aromatic volatile compounds depending on whether they are perceived through the nose or the mouth. In this way, as a result of eating and drinking, odors which are perceived via the mouth will be more frequently encountered with other specific sensory cues such as gustatory, somatosensory and tactile stimulations, compared to those perceived via the nose. This point suggests that cross-modal integration of olfactory cues can be different depending on whether the odor is perceived via the nose or via the mouth. Therefore, the concept of a *dual olfactory system* [47.2] will be addressed at psychophysical, cortical electrophysiological and neuroanatomical levels. Next, earlier studies regarding the concept of *cross-modal correspondence* will be reviewed. Finally, this chapter will emphasize influences of different sensory cues on olfactory perception.

47.1 Orthonasal and Retronasal Olfaction

There are two main pathways through which aromatic volatile compounds reach the olfactory epithelium: via the nose and via the mouth (*dual olfactory system*, [47.2]).

47.1.1 Conceptual Definitions

By inhaling or sniffing, airborne volatile compounds arrive at the olfactory epithelium (e.g., sniffing roasted coffee beans), which refers to as *orthonasal olfaction* [47.2]. Orthonasally perceived odors provide the information about the external world associated with edibility, acceptability, toxicity and danger/threat [47.2–5]. It is also well known that body odors carry information about emotional states [47.5, 6], age-related information [47.7], individual identity [47.8], or social interaction [47.9]. In this way, people who have lost their sense of smell tend to be more exposed not only to the risks of poor food intake [47.10], food poisoning [47.11, 12], gas leaks and smoke [47.11, 12], but also to a decrease of social relationships [47.13], compared to people with normal olfactory function.

When people consume foods and drinks, aromatic volatile compounds are released from the food matrix by masticating and swallowing [47.14]. The released volatiles are then pumped from the back of the oral cavity up though the nasopharynx to the olfactory epithelium [47.15, 16]. This pathway of smelling is called *retronasal olfaction* [47.2].

Are humans able to differentiate the location where aromatic volatiles come from? Yes, they are. There is empirical evidence that human subjects can localize whether the odors came from either the front of the nasal cavity or the back of nasal/oral cavity [47.17, 18]. An interesting point to note is that since retronasal olfaction occurs in the mouth, people often refer to the retronasally perceived odors as taste; this is known as *smell-taste confusion* [47.2, 19, 20]. For example, when food being eaten is delicious, people are used to describing it as *It tastes good* rather than *It smells good*. Furthermore, certain languages, such as Swiss German, have adequate vocabulary of expressing *tasting*, but not *smelling* [47.18].

47.1.2 Comparison Between Orthonasal and Retronasal Olfaction

As summarized in Table 47.1, orthonasal and retronasal olfaction are different in terms of odor pathway, odor sensitivity and identification, odor-associated information and odor-activated brain regions.

Psychophysical Level

Generally, humans are more sensitive (lower detection/recognition threshold) when they smell the odors

Table 47.1 Comparison between orthonasal and retronasal olfaction

	Orthonasal olfaction	Retronasal olfaction
Delivery route	Through the external nares	From the back of the oral cavity through the nasopharynx
Information	External worlds	Ingested substances (e.g., foods)
Odor sensitivity	Orthonasal perception > retronasal perception At suprathreshold level, orthonasally perceived odors are more intense	
Odor identification	At low concentration, orthonasal perception is more accurate At high concentration, there is little or no difference	
Differences in neural activation	Anticipatory phase (e.g., food availability)	Consummatory/reward phase (e.g., foods being eaten)

via the nose (orthonasal route) than via the mouth (retronasal route) [47.21, 22]. At suprathreshold levels, humans perceive odors as being more intense when the odors are delivered through the orthonasal route than the retronasal one [47.17, 22–25]. A plausible explanation for the relatively lower sensitivity for retronasal odors can be found in the fact that airflow from the pharynx to the olfactory epithelium may differ from airflow pattern seen for orthonasal stimulation, resulting in a lower concentration of odors reaching the olfactory cleft [47.18]. Furthermore, since humans experience retronasal odors together with other sensory attributes (taste and tactile components), mechanic movements (mastication and tongue movement) and salivation in the mouth ([47.14, 15]; for a review see [47.27]), humans' attention to the retronasal odors may be decreased [47.28, 29], thus leading to a lower sensitivity for retronasal odors (Table 47.1).

During normal breathing, humans identify aromatic volatiles more correctly when they are perceived via an orthonasal route than via a retronasal one [47.30–32]. However, the effect of odor delivery route on odor identification is not observed when subjects receive relatively highly concentrated odorants or when they perform deep breathing [47.32].

Cortical Electrophysiological Level

Clinical studies using olfactory event-related potentials (OERPs) demonstrate that orthonasal and retronasal stimuli produce different cortical electrophysiological responses [47.26]. *Landis* et al. [47.26] observed patients who exhibited olfactory loss when stimuli were presented orthonasally but not retronasally. Figure 47.1 shows recordings from a 54-year-old woman who lost her sense of smell for more than 10 years following an infection of the upper respiratory tract. While there were no detectable OERPs in response to orthonasal stimulation (A), retronasal stimulation with hydrogen sulphide (H_2S) produced noticeable activation (B). This result is in line with her result of psychophysical tests measuring orthonasal or retronasal olfactory function. That is, corresponding to the difference in OERPs, this patient correctly identified 35% of the retronasally perceived odors, whereas she correctly identified only

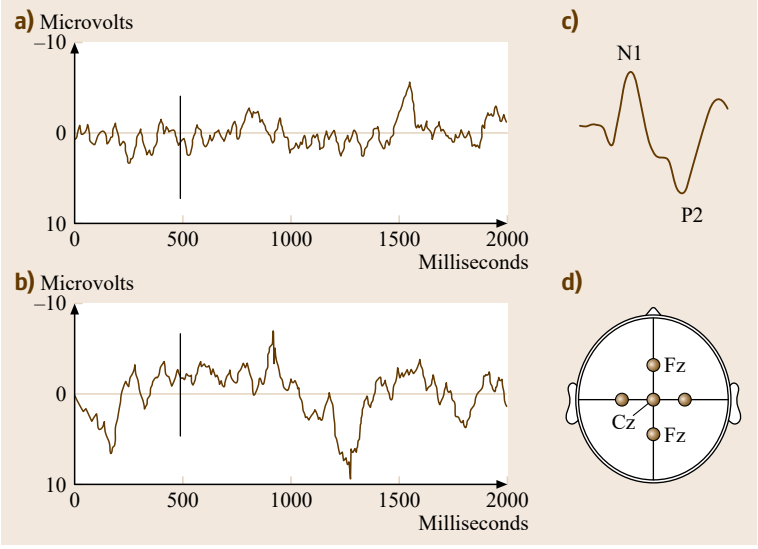

Fig. 47.1a–d Olfactory event-related potentials (OERPs) from a female patient who lost her orthonasal sense of smell following an infection of the upper respiratory tract. OERPs (recording position, Cz; for a schematic drawing of the recording sites, see **(d)**) were measured in response to orthonasal **(a)** and retronasal **(b)** stimulation with hydrogen sulfide (H_2S, 4 ppm). A schematic drawing of an OERP **(c)** and the recording sites **(d)** of electrodes are shown (after [47.26])

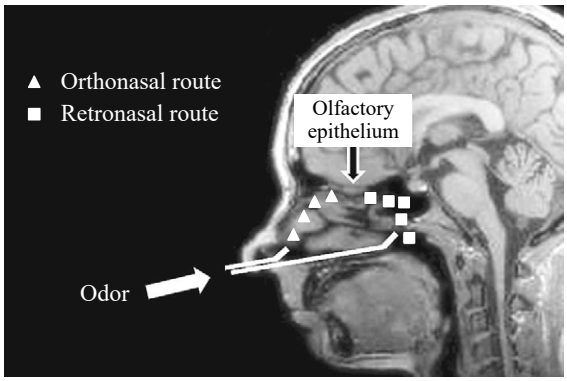

Fig. 47.2 A model for providing retronasal odor stimulation designed by [47.22]. This model allows odor stimuli to reach the olfactory epithelium through either orthonasal or retronasal route at approximately the same concentration and time course

12% of the orthonasally perceived odors. These clinical observations suggest that retronasal function may constitute a separate chemosensory entity [47.26].

In healthy people, when compared to retronasal stimulation, orthonasal stimulation appears to produce greater peak amplitudes and shorter latencies in the olfactory event-related potentials [47.22, 33]. However, when a nonfood odor, which is typically sniffed, is presented retronasally, subjects may be surprised due to this unfamiliar stimulation, leading to an increase in OERP peak amplitudes (especially, in the late positivity, P2) [47.25]. Generally, while the N1 peak, as shown in Fig. 47.1c, is associated with stimulus characteristics such as intensity and quality, the late positivity P2 is strongly related to cognitive processing of olfactory information [47.34, 35].

Neuroanatomical Level

Brain imaging studies demonstrate similar, yet different patterns of brain activation in response to orthonasal and retronasal odor stimulations [47.17, 36, 37]. *Cerf-Ducastel* and *Murphy* [47.36] demonstrated brain regions in response to retronasal stimulation by odorants delivered in aqueous solution. The brain regions activated by retronasal stimulation, such as piriform cortex, orbitofrontal cortex (OFC), hippocampus, amygdala and insula, are known as the regions activated by orthonasal stimulation. In other words, some brain regions are associated with both orthonasal and retronasal odor stimulation [47.36]. *De Araujo* et al. [47.37] also showed that anterior ventral insula was activated by both orthonasal and retronasal odor stimuli.

In a subsequent study, *Small* and colleagues [47.17] provided four different odors (food and nonfood odors) via the orthonasal and retronasal routes and measured brain responses using the functional magnetic resonance imaging (fMRI) technique. In earlier studies [47.36, 37], retronasal odors were presented via the oral cavity, but this may also activate other sensory attributes such as thermal, mechanical and/or gustatory stimulation [47.18]. To examine the sole effect of retronasal odor stimulation without other sensory influences, *Small* et al. [47.17] used a technique of odor administration which allows the presentation of odor stimuli directly to the epipharynx above the soft palate [47.22], as illustrated in Fig. 47.2. The identical odors produced more neural activation in the brain regions (e.g., a region of the Rolandic operculum at the base of the central sulcus) that typically respond to the stimulation of oral cavity when they were presented via retronasal route than via orthonasal route [47.17]. This result suggests that subjects perceive retronasally administered odors as originating from the oral cavity [47.17, 19]. Furthermore, in their study [47.17], while orthonasally perceived chocolate odor showed greater neural activation in the insular, opercula, thalamus, hippocampus, amygdala/piriform and caudolateral OFC, retronasally perceived chocolate odor demonstrated larger activation in the perigenual cingulate, posterior cingulate, medial OFC and superior temporal gyrus. However, these differences were not obtained for nonfood odors. The different pattern of neural responses between orthonasal and retronasal odor stimuli seems to be related to associative learning (experience) and/or reward context (anticipatory phase versus consummatory phase) [47.17]. For example, since food odors, but not nonfood odors, are experienced in the retronasal route (oral cavity), only the food odor (i. e., chocolate odor) led to neural response difference between orthonasal and retronasal odor stimulations. In addition, brain regions activated by orthonasally perceived odors were associated with the anticipatory rewards (i. e., food availability), whereas those preferentially responding to retronasal odors were related to the consummatory rewards (i. e., foods being eaten) [47.17]. In that orthonasal and retronasal odors are mainly perceived before and during eating, those results are understandable.

47.2 Cross-Modal Correspondences Between Olfactory and Other Sensory Cues

The terms *synaesthesia*, *synaesthetic correspondence*,or *synaesthetic association*are characterized by [47.38, p. 36], [47.39–41]:

> *a conscious experience of systematically induced sensory attributes that are not experienced by most people under comparable conditions.*

That is, synaesthesia refers to a phenomenon that one sensory cue uniquely induces conscious experience of another sensory cue in certain individuals; for example, seeing colors in response to pain [47.42]. *Martino* and *Marks* [47.42] called this form of synaesthesia *strong synaesthesia*, which is rarely observed (approximately 1 out of 2000 people) [47.43].

By contrast, *cross-modal correspondence* or *cross-modal association* does not mean that one sensory cue necessarily induces conscious experiences of another sensory cue [47.44]. The terms cross-modal correspondence or cross-modal association refer to [47.45, p. 973], [47.42, 46]:

> *a compatibility effect between attributes or dimensions of a stimulus (i. e., an object or event) in different sensory modalities (be they redundant or not).*

The cross-modal correspondences appear to be shared by many individuals (be often universal) and exist between all possible pairs of sensory modalities [47.45]. Cross-modal correspondences are innate and they are developed by perceptual learning [47.47]. Cross-modal correspondences seem to be considered as a form of *weak synaesthesia*, being characterized by [47.42, p. 61]:

> *cross-sensory correspondences expressed through language, perceptual similarity and perceptual interactions during information processing,*

but this idea remains controversial [47.44]; thus, further clarification is needed.

Cross-modal correspondences of olfactory cues with other sensory cues are reported below.

47.2.1 Cross-Modal Correspondences Between Olfactory and Visual Cues

Table 47.2 demonstrates examples of the cross-modal correspondences between olfactory and visual cues. It has been reported that certain odors can be matched with specific visual cues such as color brightness [47.48–51], color hue [47.46, 52] and symbol/shape [47.53, 54].

Color Brightness

Von Hornbostel [47.48] provided empirical evidence of the cross-modal correspondences among colors, tones and odors based on their brightness. High pitch tone and light odor (e.g., lemon odor) might be paired according to their similar characteristics of brightness [47.48, 49]. Furthermore, odor intensity was found to be inversely matched with color brightness. For example, stronger odors appear to be matched with darker colors [47.51].

Color Hue

Gilbert and colleagues [47.46] asked subjects to select a color chip that best represented each odor following sniffing 20 fragrance odors one after another. Subjects could match certain odors with specific color chips. For example, cinnamon aldehyde odor was paired with red color; this relationship seems to be mediated by a familiar name of popular cinnamon-flavored candy, *red hots* [47.46]. Similar results were also observed in other studies [47.52]: for example, strawberry odor and pink color; spearmint odor and turquoise color. Using the implicit association test (IAT), the color-odor associations were found to be strong enough for the subjects to implicitly respond to the associations, without relying on explicit cross-modal matching [47.52]. Subjects implicitly responded more accurately and quickly to compatible matching (e.g., strawberry odor and pink color) than to incompatible matching (e.g., spearmint odor and pink color). Furthermore, the color-odor associations were shown to have a high test-retest correlation of 0.53 after a 2-year interval [47.46], indicating that the cross-modal correspondences of colors and odors are robust and replicable.

Symbol/Shape

Several studies have demonstrated empirical examples of cross-modal correspondences between odors and abstract symbols/shape [47.53, 54]. *Seo* et al. [47.53] asked subjects to match 8 odors with 19 different abstract symbols. As illustrated in Fig. 47.3, pleasant odors (e.g., banana, honey melon, mint, vanilla and violet odors) were matched with circle or curve shaped symbols, while unpleasant odors (e.g., parmesan cheese, pepper and truffle odors) were matched with angular or square shaped ones, reflecting that emotional similarity between bimodal cues may mediate the cross-modal correspondence of symbols/shapes and odors. In

Table 47.2 A list of studies showing cross-modal correspondences between olfactory and visual cues

Olfactory cues	Visual cues	References
Orthonasal odors		
Fragrances/food odors	Color brightness	[47.48]
Fragrances	Color brightness	[47.50]
Fragrances/food odors	Color brightness	[47.51]
Fragrances	Color brightness	[47.47]
Fragrances/food odors	Color hue (Munsell color system)	[47.46]
Fragrances/food odors	Color hue (color patches)	[47.52]
Masculine/feminine fragrances	Color hue	[47.55]
Fragrances	Color hue	[47.56]
Fragrances/food odors	Symbols/shapes	[47.53]
Fragrances/food odors (wine)	Angular and rounded shapes	[47.54]

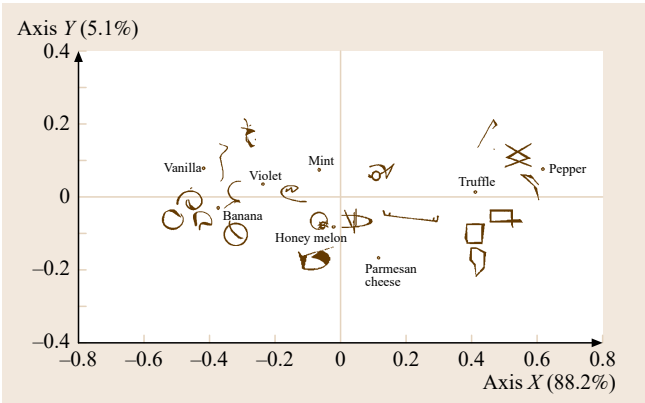

Fig. 47.3 Cross-modal association between abstract symbols and odors. The correspondence analysis shows that certain odors can be matched with specific odors. Overall, pleasant odors are paired with circle or curve shapes, whereas unpleasant odors are paired with angular or square shapes (after [47.53])

a more recent study [47.54], subjects were asked to rate how much 20 individual odors are associated with an angular or rounded shape. The angular shape was matched with more intense, sour and unpleasant odors (e.g., lemon and pepper odors), while the rounded shape was found to be best matched with more subtle, sweet and pleasant odors (e.g., raspberry and vanilla odors).

47.2.2 Cross-Modal Correspondences Between Olfactory and Gustatory Cues

As a result of eating and drinking, gustatory cues are often perceived with olfactory cues (especially retronasal odors). Thus, people tend to confuse the co-occurrences of olfactory and gustatory cues in a mouth as a unitary sensation [47.2, 57]. In this way, when people sniff odors, they often describe the stimulation as *taste-like sensation* [47.58, 59], and they are used to matching certain aromas/flavors with specific tastes. For instance, caramel or vanilla aromas/flavors are commonly paired with sweetness, while lemon aromas/flavors are frequently paired with sourness.

47.2.3 Cross-Modal Correspondences Between Olfactory and Auditory Cues

Previous psychophysical studies demonstrated examples of cross-modal correspondences between olfactory and auditory cues [47.60–65]. As shown in Table 47.3, people match certain odors perceived via orthonasal route (odor, aroma, or scent) with specific pitches [47.60, 66]. For instance, in an earlier study, subjects could consistently match certain auditory pitches (e.g., 200 and 1000 Hz) with specific fragrant odors based on odor quality cues rather than odor intensity cues [47.60].

A recent study by *Crisinel* and *Spence* [47.63] demonstrated that certain odors, often found in wine, could be matched not only with specific pitches, but also with timbres of musical notes. For example, fruit odors were consistently matched with high-pitched sounds. Also, pleasant odors were paired with the tone of a piano instrument more than tones of other instruments. More recently, *Crisinel* et al. [47.65] reported similar findings showing the cross-modal correspondences between odors and pitches. As odors judged as *happier*, *sweeter*, *brighter* and *more pleasant* (e.g., iris flower and candied orange odors), they were well matched with the instrumental sounds of higher pitch (e.g., tone of piano).

Table 47.3 presents that people can match retronasally perceived odors (flavors) with specific timbres of musical notes [47.61, 62, 64]. Flavors were found to be also matched with specific pitches of musical notes [47.61, 62]. However, the cross-modal association of flavors and pitches appears to be relatively weaker compared to the association with timbres [47.61, 64]. That is, the association of flavors

Table 47.3 A list of studies showing cross-modal correspondences between olfactory and auditory cues

Olfactory cues	Auditory cues	References
Orthonasal odors		
Essential oils	Pitch	[47.66]
Fragrances and essential oils	Pitch (200 and 1000 Hz)	[47.60]
Wine aroma kit	Pitch	[47.63]
Aroma kit	Pitch	[47.65]
Wine aroma kit	Timbre (instrumental note)	[47.63]
Aroma kit	Timbre (instrumental note)	[47.65]
Retronasal odors (flavors)		
Flavors (foods and flowers)	Pitch	[47.61]
Flavors (milk)	Pitch	[47.62]
Flavors (foods and flowers)	Timbre (instrumental note)	[47.61]
Flavors (milk)	Timbre (instrumental note)	[47.62]
Flavors (chocolate)	Timbre (instrumental note)	[47.64]

(retronasal odors) and pitches has not been consistently observed in previous studies [47.61, 64]. While a cross-modal correspondence of flavors with timbres appears to be mediated by subjective hedonic valence (e.g., pleasantness versus unpleasantness), the correspondence with musical pitches seems to be driven by other factors, such as *potency* (strong versus weak) and *activity* (active versus passive), of bi-modal cues [47.61, 64].

Deroy et al. [47.67] proposed three hypotheses that explain the cross-modal correspondences between olfactory and auditory cues. Firstly, auditory and olfactory perceptions may share a common amodal dimension such as space (*the amodal hypothesis*). Secondly, another independent dimension, such as emotional similarity of bimodal cues (e.g., pleasantness or unpleasantness), may mediate the olfactory and auditory association (*the indirect hypothesis*). For example, olfactory and auditory cues can be paired because both of them are pleasant. Finally, a network of cross-modal correspondences between sensory cues (or dimensions) may be formed based on statistical (or nonstatistical) co-occurrences (*the transitivity hypothesis*). Furthermore, these hypotheses would be applied to cross-modal correspondences of olfactory cues with other senses (visual or gustatory cues).

47.3 Influences of Visual Cues on Olfactory Perception

Olfactory perception is found to be affected by other sensory cues such as visual, gustatory, somatosensory, auditory and tactile cues as described below. The cross-modal integration between olfactory and visual cues will be introduced with a focus on key modulators in individual integration.

47.3.1 Congruency Between Bimodal Cues

A *congruency* between bimodal cues is the concept that has been most often used in many studies investigating the cross-modal integration involving the sense of smell. Thus, prior to introducing the effect of congruent visual cues on olfactory perception, a concept of the congruency will be addressed.

In the cross-modal study using olfactory and gustatory cues, *Schifferstein* and *Verlegh* [47.68] defined the congruency as *the extent to which two stimuli are appropriate for combination in a food product*. However, since cross-modal integrations of olfactory cues with other sensory cues are not limited to food products, in this chapter the congruency is characterized as *the extent to which bimodal stimuli are appropriate for combination in everyday life* as modified by [47.69, p. 6]. Three types of congruency are proposed: *synaesthetic congruency*,*spatiotemporal congruency*, and *semantic congruency* [47.45, 70]. Synaesthetic congruency refers to *the correspondences between more basic stimulus features (e.g., pitch, lightness, brightness, size) in different modalities* [47.45, p. 972]. Aforementioned examples of *cross-modal correspondence* are close to synaesthetic congruency. Spatiotemporal congruency refers to *the proximity between two unisensory events in time and space* [47.70, p. 992], which has been a main theme of multisensory integration of visual and auditory cues. Finally, semantic congruency refers to *a cross-modal match (versus mismatch) in terms of the identity or meaning of the unisensory component stimuli* [47.70, p. 992]. While synaesthetic congruency (or cross-modal correspondence) occurs mainly between

the more basic (low-level) cues, semantic congruency exists between more complex (high-level) cues [47.45]. For example, a high pitch note can be synaesthetically congruent with a bright color (versus a dark color), whereas it can be semantically congruent with a violin (versus a cello).

Effects of Congruent Visual Cues on Odor Detection, Discrimination and Identification

People have a difficulty in identifying odors without verbal descriptors/labels even when they are everyday odors [47.71]. However, most people are unaware of their poor ability to identify odors because they usually smell the odors with visual cues which assist in identifying the odors [47.72]. For instance, people usually smell coffee aroma while looking at the coffee placed in their mugs. Another example is that people tend to expect a specific flavor of a beverage from the color of the beverage [47.73, 74]. According to *Shankar* and her colleagues' study [47.74], more than half of British subjects could expect lemon flavor when they looked at yellow-colored beverage. Likewise, visual cues may lead people to expect specific odors/flavors associated with the visual inputs based on their experience and associative learning.

Congruent visual cues, such as colors and pictures, facilitate odor detection [47.53, 75], discrimination [47.76] and identification [47.75, 77–80]. For example, people detect orthonasal odors more quickly and correctly when they are presented with semantically congruent visual cues (colors or images) compared to incongruent visual cues [47.53, 75, 77, 78]. On the other hand, when the color of the odorant is not seen (clear odorant) or is inappropriate (e.g., red color for banana odorant), people tend to be slower and less accurate in identifying the odor [47.72].

The enhancement effect of congruent visual cues on the accuracy of odor identification has also been found in the cross-modal integration between visual cues and retronasal odors [47.73, 81–83]. For example, *Blackwell* [47.83] found that 79 and 92% of subjects correctly identified the flavors of orange and blackcurrant solutions when the solutions were colored appropriately. However, the correction rates of identifying the orange and blackcurrant solutions were reduced to 31 and 29% when they were colored inappropriately.

The facilitation effect of congruent visual cues (particularly colors) on odor identification is more obvious when the olfactory cue is more familiar [47.77]. For example, if individuals consume bananas every day but they consume a lemon once a year, they are expected to have a stronger association of yellow color with banana odor rather than with lemon odor. As a result, when the individuals are exposed to yellow color, they are more likely to retrieve the image of banana than of lemon. In this way, yellow color assists the individuals in detecting, discriminating and identifying the banana odor. Likewise, congruent visual cues increase the speed of identifying their corresponding odors, as well as the correction rate of identifying them [47.72].

Herein, one can raise a question. Do red-colored solutions increase the ability to identify the flavors of all reddish foods and beverages, such as strawberry, apple, tomato? The answer would be *yes* or *no*. Aforementioned, the effect of congruent visual cues appears to be dependent on the strength of color-flavor association. *Zampini* et al. [47.73] reported that inappropriate colors deteriorate the ability to identify flavors only when the connection of color and flavor is strong enough. In addition, the congruency effect is modulated by the degree of discrepancy between individuals' expectation-based flavor and the actual perception of the flavor. Based on the *assimilation-contrast* theory [47.84–86], as long as the degree of discrepancy is so small that the individuals can admit that the combination of color and flavor is congruent, the color cue helps in identifying the flavor. By contrast, if the discrepancy is so big that individuals cannot allow that the combination of color and flavor is congruent, the color cue may reduce the individuals' ability to identify the flavor [47.74, 80, 87]. This idea reflects that the effect of congruent visual cues on odor perception is mediated by a *top-down* process at the cognitive level, and this point has been supported by numerous empirical evidence at psychophysical [47.1, 74, 88, 89] and neuroanatomical [47.75, 90] levels. As previously addressed, when a white wine was artificially colored with a red dye, wine tasters described its sensory attributes by using descriptors related to red wine [47.1]. That is, visual inputs alter not only olfactory expectation, but also olfactory perception.

Effects of Congruent Visual Cues on Odor Pleasantness

Likewise, congruent visual cues assist people in identifying the served odors and in being familiar with them, which may result in increasing odor pleasantness. It is generally understood that people like odors significantly more when they are presented with congruent (or appropriate) visual cues compared to when presented with incongruent (or inappropriate) visual cues and/or no additional visual cues [47.78, 91]. However, it should be noted that congruent visual cues do not always enhance odor pleasantness [47.53, 87]. When the hedonic tone of odor is unpleasant, congruent visual cues can increase the unpleasantness of the odor [47.53, 87]. *Seo* et al. [47.53] have demonstrated that congruent symbols increased not only pleasantness for pleasant

odors (2-phenylethanol), but also unpleasantness for unpleasant odors (1-butanol). That is, congruent visual cues appear to strengthen their own hedonic valence of the odors.

Furthermore, incongruent (or inappropriate) visual cues decrease odor pleasantness, which may result from the reduced ability to correctly identify the odors paired with incongruent (or inappropriate) visual cues [47.72, 78]. Notably, in the study conducted by *Zellner* and colleagues [47.78], correctly identified odors were always more pleasant than incorrectly identified odors even when the odors were inappropriately colored. This finding indicates that odor identification plays a critical role in modulating the effect of visual cues on odor pleasantness. Furthermore, these results support the above notion that congruent visual cues facilitate odor identification, thereby enhancing odor pleasantness.

Effects of Congruent Visual Cues on Odor Intensity

An effect of congruent visual cues (especially colors) on perceived intensity of orthonasal and retronasal odors remains unclear because previous results regarding the color effect have not shown a consistent pattern ([47.53, 73, 91–93]; for a review, [47.94]).

It appears that colors affect perceived intensity of odors in a different manner as a function of the route of administration of the odor (orthonasal route versus retronasal route). Overall, when odorants are sniffed through the nostrils, color cues increase perceived intensity of odors [47.23, 92, 93, 95]. *Christensen* [47.95] found that when subjects sniffed appropriately colored processed cheese, they perceived its odor more intense compared to when they sniffed inappropriately (e.g., bright blue) colored cheese. *Zellner* and *Kautz* [47.93] also found that orthonasal odors of flavor solutions (e.g., strawberry flavor) were rated more intense when the solutions were appropriately colored (e.g., red) than when colorless; however, the color-induced odor enhancement did not result from certain color-odor association. That is, the effect of color cue on perceived intensity of the orthonasal odor was not modulated by the congruency between bimodal cues, but by the inherent presence of the color cue. Orthonasal odor intensity seems to be influenced by color intensity whether the color is appropriate for the odor or not. The perceived intensity of orthonasal odor is usually more intense in dark-colored solutions than in light-colored solutions. Furthermore, the orthonasal intensity is often rated weakest in colorless solutions. However, the relationship between the intensities of color and odor has not always been found [47.92, 93].

In contrast to the color-induced orthonasal odor intensity, color cues tend to show no influence or even reduce the intensity of odors when the odorants are smelled through the mouth [47.23, 73, 95]. What makes the difference between orthonasal and retronasal odors in the effect of color on perceived odor intensity? *Zellner* [47.72] argues that the color-enhanced orthonasal odor intensity might be due to a combination of 1) the actual odor experience and 2) the color-induced conditioned percept elicited by the previous experience associated with the pair of color and odor. For example, the odor percept caused by looking at a red solution might be an odor blend of red-colored foods and drinks that the individual has previously experienced [47.72]. In addition, *Zellner* [47.72] proposes that the color-reduced retronasal odor intensity might be due to 1) relatively less visible color cues and 2) intensity contrast between orthonasal and retronasal odors. For example, when subjects try tasting a colored odorous solution, they first see the colored solution, eliciting the color-enhanced orthonasal odor intensity of the solution. Subsequently, when the colored solution is placed in the mouth for judging its retronasal odor intensity, the color cue is no longer available. Thus, the subjects perceive the retronasal odor without the color cue and therefore the retronasal odor might be perceived weaker than the orthonasal odor, resulting in the intensity contrast between the orthonasal and retronasal odors. In this way, color cues may affect odors differently depending on the odor delivery route [47.72].

Compared to the effect of color, relatively little attention has been paid to an influence of other visual cues such as images on odor intensity. It has been reported that visual images such as pictures and symbols also modulate odor intensity [47.53, 91]. *Sakai* et al. [47.91] found that orthonasal odor intensity was increased when the odor was presented with an appropriate picture than when presented with an inappropriate picture. Using olfactory event-related potentials (OERPs), *Seo* et al. [47.53] showed that for phenyl ethyl alcohol (PEA) odor, in comparison to the incongruent symbol, a congruent symbol significantly enhanced the amplitude of the N1 peak, which is associated with stimulus intensity. Based on previous findings, the effect of congruent visual cues on odor intensity appears to be stronger in an image-odor association than in a color-odor association. Compared to the color-odor association, visual images may have a stronger connection with the odors. Due to this point, the visual image-elicited conditioned percept, in comparison to a color-induced conditioned percept, can be more profound and thus the visual image cues might show greater impacts on odor intensity than color cues.

Neuroimaging Evidence for the Effects of Congruent Visual Cues on Odor Perception

Previous neuroimaging studies have found that the left orbitofrontal cortex (OFC) is predominantly involved in the cross-modal integration between visual and olfactory cues [47.75, 96], while the right OFC is more engaged in an odor perception [47.97]. Using fMRI, *Österbauer* et al. [47.96] showed the super-additive neural activation in the OFC in response to congruent color-odor pairs (congruent color-odor pairs > (odors + colors)). Also, the left OFC was more activated in response to a congruent color-odor combination than incongruent combination or odor alone. Furthermore, *Gottfried* and *Dolan* [47.75] found that subjects detected odors more quickly and accurately when the odors were provided with semantically congruent pictures than when provided with incongruent pictures. Further, neuroimaging revealed that the congruent image-induced odor detection and identification was mainly associated with the anterior hippocampus and rostromedial OFC.

47.3.2 Cultural Background

As shown in numerous studies, cross-modal interactions between olfactory and other sensory cues are dependent on experience and associative learning [47.98, 99]. This idea has been supported by studies demonstrating the effect of cultural background on the cross-modal integration between olfactory and visual cues [47.74, 100]. *Shankar* et al. [47.74] sequentially displayed each of the beverages of seven different colors to British and Taiwanese participants and asked them to write down the first flavor or beverage that came to their minds based solely on the color of beverages. Interestingly, a large portion (70%) of British subjects associated the brown beverage with a *cola* flavor (none expected it would be a grape flavor), while almost half (49%) of the Taiwanese subjects associated the same color beverage with a *grape* flavor (none expected it would be a cola flavor). This result appears to be due to a cultural difference between the experience of British and Taiwanese subjects with the brown beverage in their home countries. For example, cola is the most common brown beverage in Great Britain, while grape juice or drink is a common brown beverage in Taiwan [47.72]. *Wan* et al. [47.100] also showed such a cultural difference in the color-odor association between US and Chinese adults. For instance, many American subjects paired the red drink with a *cherry* flavor, whereas Chinese subjects paired it with a *watermelon* flavor.

These findings suggest that the effect of congruent colors on olfactory perception can be different as a function of cultural background. Let us assume that British and Taiwanese subjects are asked to identify a flavor of the brown-colored solution including the mixture of cola and grape flavors. Based on the above finding [47.74], it can be expected that British subjects, in comparison to Taiwanese subjects, may detect and identify the cola flavor more quickly and accurately from the solution because they are apt to judge brown color to be congruent with cola flavor since brown color and cola flavor are strongly connected by their experiences; however, further study is needed to validate this assumption.

47.4 Influences of Gustatory Cues on Olfactory Perception

Odors are perceived with concurrent gustatory stimulation in the mouth while consuming foods and drinks. As a result, it is frequently observed that patients who have lost their sense of smell complain of their taste-loss in the clinic, although they have a normal sense of taste (referred to as *flavor disturbance*) [47.101, 102]. So far, most of the studies regarding the cross-modal integration between olfactory and gustatory cues focused on effects of olfactory stimulation on gustatory perception ([47.68, 103–109], for a review, see [47.29, 99, 110–112]). For example, it has been found that congruent odors increase the perceived intensity of taste stimuli although the enhancement effect was not consistently obtained in earlier studies [47.113, 114].

This section will focus more on the effect of gustatory cues on olfactory perception.

47.4.1 Congruency Between Bimodal Cues

Congruency between olfactory and gustatory cues is a key modulator in their interaction. As seen below, it has been found that congruent gustatory cues affect odor localization, odor intensity and pleasantness.

Effects of Congruent Gustatory Cues on Odor Localization

Using the aforementioned technique (Fig. 47.2), several studies demonstrated that subjects were able to differentiate whether an odorous stimulus was coming from the tip of the nose or from the back of the mouth [47.17, 22]. These findings indicate that nasal airflow direction (anterior delivery versus posterior delivery) across the olfactory epithelium may be adequate to generate

oral localization of the odorous stimulus [47.17, 22, 115].

Von Békésy [47.116] asked subjects to localize the place of odor stimulation when a taste stimulus was presented to the mouth before, during, or after delivering odors through the nose. When the subjects received the odor and taste stimuli (e.g., clove odor and acid solution) simultaneously, they perceived them as a single sensation occurring in the mouth. Based on the result of *von Békésy* [47.116], it seems that the nasal airflow direction is not a necessary condition because subjects could experience the oral localization although the odor was delivered via the orthonasal route [47.117]. *Murphy* and *Cain* [47.20] suggested that the trigeminal system may integrate olfactory and gustatory systems into a single perceptual system during eating. That is, tactile (somatosensory) or trigeminal stimulation in the mouth is expected to capture taste [47.20, 118], thereby inducing the odor localization to the mouth. In a recent study, *Stevenson* et al. [47.117] demonstrated that when subjects sniffed an odor stimulus in the absence of a somatosensory or taste stimulus in the mouth, they accurately localized the location of the odor (at nose). However, in the presence of a taste stimulus in the mouth, subjects localized the orthonasally presented odor to the mouth. *Stevenson* et al. [47.117] proposed that taste stimulation may induce subjects' attention toward the taste stimulus, resulting in an impaired ability to selectively attend to an odor stimulus. Opposite to *Murphy* and *Cain* [47.20] and *Green's* [47.118] suggestion, somatosensory stimulation alone was not a sufficient condition to generate the oral localization when an odor stimulus was sniffed [47.117]. Contrary to taste stimulation, during involuntary swallowing, breathing and talking, oral somatosensory stimulation is constantly present in the mouth [47.117]. Due to such a frequent occurrence, oral somatosensory stimulation seems to be unable to capture selective attention toward it [47.117]. In this way, while a minimum level of somatosensory stimulation may be a necessary condition, somatosensory stimulation alone is relatively ineffective at eliciting oral localization [47.117].

Lim and *Johnson* [47.119, 120] and *Lim* et al. [47.121] demonstrated an oral localization of retronasal odor (retronasal odor referral). Specifically, *Lim* and *Johnson* [47.119] asked subjects to localize an odorous stimulus when food odors were inhaled through a straw and exhaled through the nose in the presence or absence of taste stimuli (water or various taste solutions) in the mouth. When vanilla and soy sauce odors were simultaneously presented with sucrose and sodium chloride solutions, subjects reported that those odors were present on their tongue. This finding indicates that the referral of retronasal odor to the tongue depends on the presence of its congruent taste [47.119]. This effect (i. e., congruent taste-induced odor localization to the tongue/mouth) was also obtained in a more natural food condition (e.g., flavored gelatin samples) [47.120]. However, a presence of tactile stimulation alone was not a sufficient condition to enhance the retronasal odor referral [47.120].

Effects of Congruent Gustatory Cues on Odor Sensitivity and Intensity

As previously mentioned, relatively few studies have been conducted to answer the question as to whether congruent taste increases olfactory perception. *Dalton* and her colleagues [47.122] found that taste stimulus enhances sensitivity to orthonasal odor. For example, following ingestion of 10 mL of the respective solutions (saccharin, monosodium glutamate (MSG), or deionized water), subjects sniffed the odor of benzaldehyde (cherry/almond odor) at subthreshold level. Subjects' sensitivity to the odor was increased in the presence of congruent taste (i. e., saccharin) in the mouth, while their odor sensitivity was decreased in the presence of incongruent taste (i. e., monosodium glutamate) or of deionized water.

Recent studies demonstrated that tasting substances (particularly sucrose solution) can enhance perceived intensities of retronasal odors [47.121, 123, 124]. In a recent study by *Lim* and her colleagues [47.121], subjects rated perceived intensities of three taste substances (sucrose, citric acid and caffeine), as well as of two retronasal odors (citral and coffee odor), both alone and in taste-odor mixtures. The subjects also rated the degree of congruency of all combinations of taste and odor stimuli. The pair of citral odor and sucrose was rated most congruent among the three taste-odor pairs. Also, coffee odor was rated most congruent when it was paired with sucrose or caffeine solutions. That is, sucrose solution was judged congruent with both citral and coffee odors. Sucrose solution significantly increased perceived intensity of its congruent odors (i. e., citrus and coffee flavors) than did either other taste solutions or retronasal odor alone. Furthermore, using olfactory ERPs, *Welge-Lüssen* et al. [47.33] demonstrated that orthonasal or retronasal stimulation of vanillin odor produced shortened latency of P2 peak in the presence of its congruent taste stimulus (sweet taste solution) compared to in the presence of incongruent taste stimulus (sour taste solution), suggesting that congruent taste facilitates retronasal olfactory processing. Similarly, in comparison to incongruent taste stimulus (sour taste solution), congruent taste stimulus (sweet taste solution) produced shorter latencies of P1 and N1 peaks for orthonasally presented vanilla odorant [47.125].

47.5 Influences of Auditory Cues on Olfactory Perception

Recent animal studies have found that the olfactory and auditory sensory inputs converge in the mammalian cerebral cortex [47.126–130]. *Wesson* and *Wilson* [47.128] demonstrated that single units of the olfactory tubercle were activated by tones, as well as by odors [47.128]. Furthermore, 29% of the single units in the olfactory tubercle showed either enhanced or suppressed activations in response to the simultaneous stimulation of odors and tones, suggesting that the olfactory tubercle may play a major role in influencing the cross-modal interaction between olfactory and auditory cues. In addition, *Plailly* et al. [47.131] showed that human brain regions associated with a familiarity to music pieces or odors were overlapped in the left hemisphere: specifically, the superior and inferior frontal gyri, the angular gyrus, the precuneus, the hippocampus and the para-hippocampal gyrus. Building on these findings, this section will address effects of auditory cues on olfactory perception with a focus on congruency between bimodal cue and background noise.

47.5.1 Congruency Between Bimodal Cues

Several studies have demonstrated that congruency between olfactory and auditory cues plays an important role in their interaction, especially for modulating odor pleasantness [47.132, 133]. That is, congruent sounds, in comparison to incongruent sounds, increase odor pleasantness. For example, food odors (e.g., potato chip odor) were rated more pleasant when they were presented with their matching sounds (e.g., the sound of eating potato chips) than when presented with un-matching sounds (e.g., the sound of drinking coffee) [47.132]. Further, as subjects rate the combination of odor and sound stimuli to be more congruent, they rated the odor as more pleasant. More recently, *Seo* et al. [47.133] corroborated the congruent sound-enhanced odor pleasantness. For example, cinnamon odor was rated as congruent with a Christmas carol (Jingle bells), which is based on previous experience that cinnamon-based foods/drinks and ornaments are popular during the Christmas season. The cinnamon odor was more appreciated in the presence of a congruent sound (the Christmas carol) compared to in the presence of an incongruent sound (pop music). In other cross-modal integrations of olfactory cue, congruent visual or gustatory stimuli assisted subjects in identifying the presented odors and in being familiar with them, thereby enhancing odor pleasantness [47.75, 78, 87, 134, 135]. In the same manner, the congruent sound-enhanced pleasantness for cinnamon odor was mediated by an increase in odor identification, as shown in Fig. 47.4 [47.133].

Unlike odor pleasantness, odor intensity was not modulated by congruent (or incongruent) sounds [47.132, 133]. Furthermore, no significant correlation has been reported between the degree of congruency and perceived odor intensity [47.132, 133]. The lack of significant effect on the odor intensity can be explained by the *analytic* characteristics of intensity rating. That is, when subjects rate the odor intensity, they are likely to be attentive to the intensity of odor stimulation. Thus, relatively little attention is paid to the background sound, which may reduce the impact of background sound on odor intensity. By contrast, when subjects rate

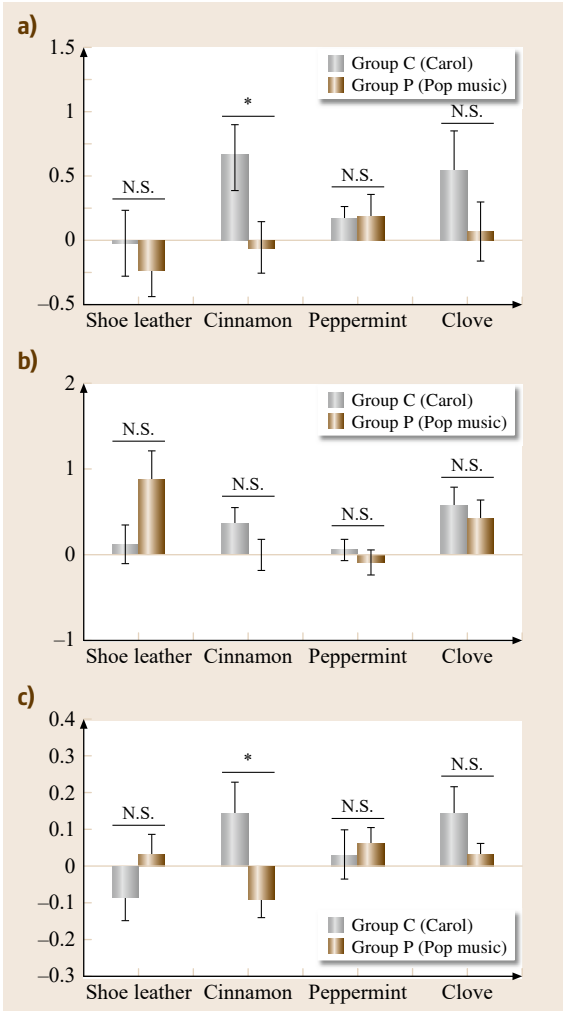

Fig. 47.4a–c Comparisons between Groups C and P in mean ratings for (a) sound-induced odor pleasantness, (b) sound-induced odor familiarity, and (c) sound-induced odor identification (after [47.133])

odor pleasantness, they tend to judge their hedonic impression for the odor stimulation in a *holistic* manner with consideration of the background sound given during the test, which may result in augmenting the impact of background sound [47.133].

47.5.2 Cultural Background

As addressed in the odor-color interaction [47.74], there is additional evidence that culture affects the cross-modal interaction between olfactory and auditory cues. *Seo* et al. [47.133] asked German and North American (the US) subjects to rate the degree of congruency between background sounds and orthonasal odors. Both German and American subjects judged that cinnamon and clove odors are matched well with the Christmas carols because foods and drinks contained cinnamon and clove are very common during the Christmas season in both countries. However, there was a cultural difference for the peppermint odor. Peppermint odor was rated congruent with the Christmas carol by American subjects, but not by German subjects. During the Christmas season, a candy cane flavored with peppermint is very popular in the United States. Especially, December 26th is called *National Candy Cane Day*. Likewise, culture plays an important role in formulating a culture-specific cross-modal interaction. Based on their experience and associative learning, people are likely to show specific cross-modal expectation, determining whether the pair of bimodal cues is matched or not. If people judge a pair of bimodal cues as congruent, the effect of congruent sound on olfactory perception would be obtained.

47.5.3 Background Sound

People are exposed to everyday odors in the presence of various background sounds. Earlier research has highlighted the effect of background sound on taste perception [47.136–138] or texture perception [47.139], but little is known about its influences on odor perception.

It has been reported that background sound interfered with the ability to discriminate odors compared with a silent condition [47.140]. Furthermore, the effect of background noise on an odor discrimination task was dependent on the type of background noise. For example, subjects showed more deleterious performance on the odor discrimination task in the presence of verbal noise (audio book) than nonverbal noise. It is known that an odor discrimination task is highly dependent on cognitive function [47.141]. Since verbal noise relative to nonverbal noise needs more cognitive load, selective attention to the background noise might reduce, to a higher degree, the ability to discriminate odors [47.140].

Olfactory sensitivity tasks are less dependent on cognitive function [47.141]. Thus, the question arises whether background noise would not interfere with the olfactory sensitivity. The answer appears to be *yes* or *no*. *Seo* et al. [47.142] asked subjects to conduct odor sensitivity tasks in the absence and the presence of the same types of background noise (i. e., verbal and nonverbal noise) which was used in the aforementioned research [47.140]. Overall, odor sensitivity was not different across the three background noise conditions: verbal noise, nonverbal noise and silence. However, with regard to odor sensitivity, the effect of verbal background noise was observed in a different manner between extrovert and introvert groups. For example, verbal noise, in comparison to silence, improved or impaired subjects' odor sensitivity in the extrovert or introvert group, respectively. These results suggest that the type of background noise and the degree of extraversion should be considered when investigating the effect of background sound on odor perception.

47.6 Influences of Trigeminal Cues on Olfactory Perception

People are exposed to odors/flavors with trigeminal stimulation, which is characterized as irritation, tickling, or burning, when they consume spicy foods or carbonated beverages. The cross-modal integration between olfactory and trigeminal cues has been reported with a focus on 1) congruency between bimodal cues and 2) odor localization elicited by trigeminal sensation.

47.6.1 Congruency Between Bimodal Cues

Little is known about whether certain combinations of olfactory and trigeminal cues can be judged as congruent. Recently, *Bensafi* and his colleagues [47.143] provided empirical evidence that a trigeminal sensation can be matched with a specific odor. For example, subjects judged orange odor as congruent with intranasal carbon dioxide (CO_2) stimulation, whereas they judged rose odor as incongruent with carbon dioxide stimulation. This result seems to be due to their exposure to the mixture of orange odor and carbon dioxide in soda drinks [47.143]. By contrast, the incongruent mixture of rose odor and carbon dioxide is not common in everyday life.

A congruent mixture of olfactory and trigeminal cues (orange odor and CO_2 stimulation) can be perceived as more pleasant than an incongruent mixture (rose odor and CO_2 stimulation) [47.143]. Further, neuroimaging (fMRI) revealed that the congruency-enhanced pleasantness was associated with increased neural activities in the hippocampus and anterior cingulate gyrus [47.143].

47.6.2 Trigeminal Cue-Induced Odor Localization

It is generally understood that humans have difficulties in purely lateralizing olfactory stimuli when they are presented to the right or left nostril ([47.144–147]; but [47.148]). However, it is known that trigeminal stimulants such as carbon dioxide can be lateralized with a high degree of accuracy [47.145, 147]. Notably, high concentrations of most known odorants evoke, in addition to olfactory sensation, trigeminally mediated sensation [47.149, 150]. *Doty* et al. [47.149] reported that subjects who lost olfactory, but not trigeminal, nerve function were unable to detect only 2 odorants (i. e., vanillin and decanoic acid) among 47 odorants. In that the human nasal mucosa contains not only olfactory receptors, but also cells that respond to mechanical, thermal, or nociceptive stimulation [47.147, 151, 152], odorants eliciting also trigeminal sensations can be localized [47.144–147, 153]. *Kleemann* et al. [47.147] demonstrated that subjects were not able to localize odorants exciting only the olfactory system, independent of their concentration, but they were able to localize the odorants activating both olfactory and trigeminal systems when the odorants were presented to either the right or left nostril.

47.6.3 Trigeminal Cue-Induced Odor Perception

The cross-modal integration between olfactory and trigeminal stimulations has been observed at several sites [47.154]: for example, at central nervous sites such as mediodorsal nucleus of the thalamus where olfactory and trigeminal afferents information converges [47.155, 156]; at the olfactory bulb [47.157]; at the olfactory epithelium [47.158]; and indirectly through nasal trigeminal reflexes [47.159].

It has been reported that trigeminal stimulation (pungency or irritation) suppresses orthonasal odor intensity [47.160–163] when the two stimulations were simultaneously presented. *Cain* and *Murphy* [47.160] also examined whether sequential presentation of trigeminal stimulus (CO_2) before odor stimulus (amyl butyrate) can alter the pattern of olfactory suppression. Trigeminal stimulation reduced perceived intensity of the subsequently presented odor, but its impact was less pronounced compared to the influence of simultaneous presentation. By contrast, *Jacquot* et al. [47.158] found that former trigeminal activation resulted in an increase in olfactory sensitivity. These inconsistent results reflect that both time and intensity of olfactory and trigeminal stimulations modulate the cross-modal integration between the two bimodal cues [47.156].

The effect of trigeminal cues on odor perception has also been investigated in food matrices. Trigeminal stimulation, such as carbonation, irritation and spiciness, appears to reduce perceived intensities of aroma and/or flavor [47.164, 165]. Conversely, it was also reported that trigeminal sensation enhanced aroma or flavor intensity [47.166–168]. *Saint-Eve* et al. [47.167] found that the presence of carbonation in beverages enhanced perceived intensity of retronasal odors compared to noncarbonated beverages. Furthermore, no significant effect of trigeminal stimulation on retronasal odor intensity has been observed in food matrices [47.165, 169, 170].

Likewise, for interpreting the effect of trigeminal stimulation on odor perception, key factors should be considered: for example, stimulus type, stimulus concentration, stimulation duration, inter-stimulus interval and environmental temperature [47.156, 165].

47.7 Influences of Tactile Cues on Olfactory Perception

While eating and drinking, tactile stimulations dynamically interact with olfactory cues (particularly retronasal odors). Nevertheless, little attention has been paid to the effect of congruency between olfactory and tactile cues in olfactory perception. Especially, the two tactile cues, *viscosity* and *hardness* (or *firmness*), have been used to investigate the cross-modal integration between olfactory and tactile cues.

47.7.1 Congruency Between Bimodal Cues

Only a few studies have reported the influence of congruency between olfactory and tactile cues on olfactory perception. It was found that certain aromas and texture can be matched [47.171]. For example, *Harthoorn* et al. [47.171] asked subjects to rate the degree of congruency between nonaromatized creamy custard

and each of seven aromas (vanilla, lemon, strawberry, chocolate, buttery, rubbery and lavender). The creamy custard showed high congruency scores with vanilla and buttery aromas, but it produced low congruency with rubbery, chocolate and lavender aromas. Overall, in the presence of creamy custard, congruent odors (vanilla and buttery) were preferred, whereas incongruent odors (rubbery and chocolate) were not appreciated. That is, this study demonstrated that a texture cue can enhance pleasantness of its congruent odors.

47.7.2 Effects of Viscosity and Hardness

It is generally observed that increasing viscosity results in a decrease in perceived intensity of retronasal odor (flavor) in various liquid matrices: for example, juice [47.172], coffee [47.172] and thickened flavor solutions [47.173–175].

In semi-solid and solid matrices, many studies have emphasized the effect of hardness (or firmness) on flavor intensity. Overall, earlier research has shown that perceived flavor intensity decreases with increasing hardness of semi-solid and solid matrices such as gels [47.24, 176–180], custard [47.24], milk [47.181] and candy [47.182].

The modulatory effects of viscosity and hardness on the perceived odor intensity can be explained by 1) physicochemical interaction of volatiles in the sample matrix and 2) tactile, cue-induced selective attention. Firstly, physicochemical properties, such as air/sample partition, diffusion coefficients and chemical bindings of the samples may influence odor intensity [47.182, 183]. For varying viscosity or hardness of the samples, structuring agents such as hydrocolloids and gelatine were used in previous studies. These structuring agents interact with volatiles of the sample, resulting in variations in the amount and the rate of odor release [47.176, 180, 182–184]. In this way, as a certain sample is composed of higher concentration of structuring agents (gelatine), the structuring agents of the sample may entrap more volatiles in the matrix, thereby reducing perceived odor intensity. However, this idea seems insufficient to explain relationships between viscosity/hardness and odor intensity because 1) structuring agents do not bind all kinds of volatiles [47.178, 182] and 2) concentration of the structuring agents was not linearly related to the perceived intensity of odor, as well as to the amount of odor release [47.178, 182]. When the amount of the odors released in the in vivo condition was compared among the samples with varying hardness/viscosity, the amount of odor/flavor release from the samples was not different, while perceived intensity of the odor/flavor was decreased [47.185, 186]. Another plausible explanation for the modulatory effect of viscosity/hardness on the perceived odor intensity is the tactile cue-induced selective attention. Oral behaviors (force, frequency and duration of biting, chewing and swallowing) vary depending on the first perception of sample texture in the mouth [47.182, 186, 187]. For example, a hard cracker needs greater biting force and longer chewing duration than a soft cracker, which may lead consumers to pay attention to the tactile cue more than aroma/flavor cue. As previously mentioned, consumers' olfactory performance may decrease (decreases in odor intensity and discrimination) when they are highly attentive to other sensory cues or tasks [47.99, 109, 142, 188]. In this way, it can be thought that certain tactile cues may be more distinctive (e.g., very hard or very viscous), which may induce more attention to the tactile cue, thereby reducing perceived odor/flavor intensity in the hard or viscous sample [47.183, 187].

47.8 Conclusion

In this chapter, the cross-modal integration between olfactory and other sensory cues was reviewed. In that odors are frequently experienced with accompanying other sensory cues such as visual, gustatory, auditory, trigeminal and/or tactile stimulation, olfactory perception should not only be understood as the result from a single sensory input, but also within the framework of multisensory interaction.

This chapter shows that *congruency* between bimodal cues plays a major role in modulating the cross-modal integration of odorous cues with visual, gustatory, auditory, trigeminal, or tactile cues. Furthermore, the modulatory effect of congruency on olfactory perception is affected by many factors including odor delivery route (orthonasal and retronasal pathways), selective attention to specific sensory cue, experience (associative learning), cultural background, type of a given task (analytical versus synthetic perception) and characteristics (hedonic tone and intensity) of a given stimulus.

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Part F Human B

Part F Human Body Odor, Chemo-Communication and Behavioral Implications

Humans predominantly live in environments and with habits that minimize exposure to their own physiological smell. The human habit of applying measures for body hygiene, as well as scenting themselves, their clothes and their homes, can be traced back to early civilization. On the other hand, science provides increasing evidence that human beings not only each possess a unique olfactory signature, making it easy for relatives to (subconsciously) assign them to their company, but that many influencing factors can modify these smells. Amongst others, dietary habits, hormonal variations or diseases may change the smell signature of an individual. Such changes might be recognized by others and initiate specific responses, even if these recognition and response patterns follow unconscious mechanisms. Accordingly, humans not only communicate via spoken words, body language or other acoustic or visual signs (e.g., gestures), but also via chemostimuli. Close relationships in social human behavior, such as in partnerships or parent–infant relationships, are influenced by olfactory cues, which strengthen or even weaken the social bonds between individuals. The underlying biochemical and physiological processes in body odor generation and smell evolution throughout the human lifespan are complex, as are the influencing factors, and are either endogenous or exogenous to the human body. Thus, it is important to keep in mind that the major part of our organism represents a complex microbial assembly, and the outer surface of our body is also far from sterile but is rather inhabited by myriad microorganisms. Depending on the species composition of this flora and their respective numbers, these microorganisms may cleave precursors of smell substances in characteristic ways, further adding another dimension to our individual smell signature. Changes in this microcosm, e.g., due to infections, can then be quickly recognized by others, which leads to changes in their behavioral responses, and potentially leads to social distancing and exclusion. In view of these considerations, future research on diagnostics and medico-physiological prediction will surely be of great benefit if both the influencing factors as well as the changes in individual body odor signatures are better understood, and adequate tools are developed or adapted to monitor these changes instrumentally.

48. Analysis and Chemistry of Human Odors

Christian Starkenmann

The analysis of volatile organic compounds (VOCs) is now accessible to almost any laboratory using gas chromatography coupled to mass spectrometry (GC-MS). With mass spectrum libraries now being well populated with thousands of molecules, structure elucidation is no longer a hurdle in most cases. Two-dimensional GC-MS and high-resolution MS systems facilitate the interpretation and accuracy of VOCs analysis (Chap. 17). Sample preparation techniques have also significantly improved. VOCs extraction can be performed using polymer absorbents, but the drawback is that the equilibrium of VOCs between the matrix and the polymer may reflect an analytical profile that does not correspond to reality. This chapter focuses on the common VOCs profiles in body odors, human urine odors and fecal odors. Analytical strategies are discussed. These VOCs are classified by chemical functionalities and their importance to the smell is discussed in detail.

48.1 Human Sweat

The human body is covered by eccrine and sebaceous glands having an important role for thermoregulation, skin protection, and other beneficial functions. Their distribution is not the same on the whole body; this will influence the quality of the skin microbiota. The skin bacteria will transform the lipids from sebaceous glands or amino acids from eccrine glands in short-chain carboxylic acids. Ammonia and lactic acid are also emitted by all body parts [48.1]. Personal habits, hygiene, disease, and living conditions can alter the overall body odor [48.2]. From the gland distribution, their densities on the human body and the understanding of different body part microflora, we can postulate that an odor mapping of different body parts is possible [48.3]. But the typical human sweat odor that we can smell in exercise room or in public transportation is only produced by apocrine glands and bacteria.

The apocrine glands are present massively in underarm regions but we can also find them in ears wax, eyes, nipple, and in pubis area (Fig. 48.1). These glands become active only at puberty; they excrete a complex juice containing some compounds which are unique to humans. The human smell is unique, except in those people who have a recessive ABCC11 gene variant, in which case the apocrine glands are inactive (Chap. 49). Under stress, the apocrine glands are stimulated which generate stronger underarm malodors.

48.1.1 Analysis of Human Axillary Odors

Since 1953, it has been known that fresh, sterile axillary sweat is odorless and that bacteria transform water-soluble chemical precursors to smelly volatile organic compounds (VOCs) [48.4]. Very soon after this discovery, in 1963, a description of the use of antibacterial substances to prevent the formation of malodor was published [48.5].

Sample preparation is critical for unbiased analysis. In early studies, sweat was collected by using a grooved rubber brush that was mechanically rotated in the axillary vault. Apparently, this procedure was *moderately discomforting* [48.5]. The smell was described as putrid and was due to microbial degradation of the stratum corneum from the eroded skin; therefore, it can be concluded that this procedure did not lead to the recovery of a representative human axillary smell. Furthermore, most studies on human body odors were performed on worn T-shirts or cotton-wool pads worn in the armpit region for 5–7 days and extracted with an organic solvent [48.6, 7] (Fig. 48.2a), allowing plenty of time for the microflora to perform an abundance of transformations and for chemical reactions to occur. The solvent extraction of these T-shirts generated background contamination, originating from fabrics mainly, as well as a loss of trace sulfur compounds by irreversible trapping on fabrics. This was an important limitation. To further avoid using solvent extractions, an individual sat or lie down in a glass box and the headspace was collected as it passed through the cabin in a gas stream [48.8] (Fig. 48.2b). From these studies, over 300–400 individual chemicals were detected, among which 135 were identified, but it could not be demonstrated that any of these were relevant for axillary odors [48.9]. Other devices for in-vivo underarm VOCs analysis by confining the underarm emanation with a polyethylene form are described without giving clear answers of which VOCs are responsible for the underarm malodor [48.8] (Fig. 48.2c). A stir bar sorptive extraction in connection with thermal desorption gas chromatograph-mass spectrometry (GC-MS) was also used to analyze the body fluids of 197 adults. A total of 373 compounds were selected for individual discrimination, such as methyl-*N*-methylanthranilate, α-ionone and other compounds [48.10]; however, these were not representative of the authentic human axillary smell but of an overall human emanation. In other words a subject who smokes or who comes back from a French fries place will smell differently from a subject just having a shower and wearing new clean cloths. Trained dogs may choose between these molecules to build an olfactive image used to track a subject.

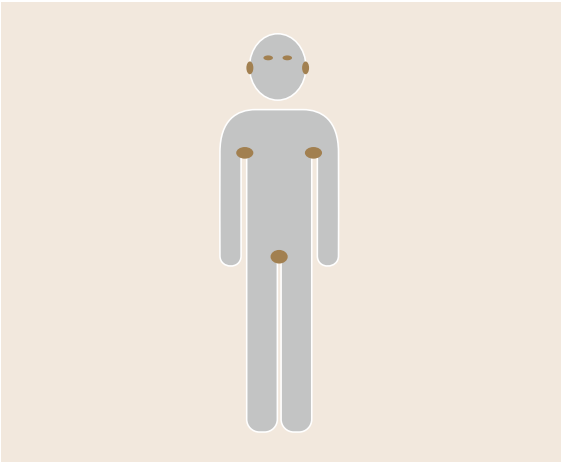

Fig. 48.1 Distribution of apocrine glands *in brown* on the whole human body (after [48.1])

The controversy about which are the most relevant compounds that cause axillary odors was mainly due to the sampling methodology [48.11, 12]. Now the role of skin microbiota in the production of body odors is better understood, mainly due to a founded understanding of the biochemistry of nonvolatile precursors, which will be explained in the following [48.13, 14].

48.1.2 Carboxylic Acids

The structure of (*E*)-3-methyl-2-hexenoic acid **1** was elucidated for the first time in the sweat of patients with schizophrenia; by 1969, researchers reported a concentration of 0.1 μg/ml of this acid in axillary sweat [48.15]. The occurrence of (*Z*)-3-methyl-2-hexenoic acid **2**, which represents 10% of the (*E*)-**1** isomer, and a possible difference in the *E*/*Z* ratio between men and women was discussed [48.16]. (*R*/*S*)-3-Hydroxy-3-methyl-hexanoic acid **3** was mentioned for the first time in 1975 as anticonvulsant [48.17], but the clear analytical occurrence of **3** in fermented human axillary sweat was reported in 2003 [48.18].

The understanding of organic acids excreted in axillary sweat occurred in two phases. The first phase involved the collection of axillary sweat on T-shirts or cotton pads sewn onto T-shirts (Fig. 48.2a), followed by solvent extractions. The second phase was based on the understanding of the biochemical pathways.

During the first phase, the major acid **1** was discovered as well as the straight chain aliphatic acids **4**–**7** and the branched acids in C2 **8**–**12**, C3 **13**, and C4 positions **14**–**18**. The occurrence of 7-octenoic acid **18** and other terminally unsaturated homologs **19** [48.19] were also observed. In this first phase, the background noise was important due to the contaminants extracted from

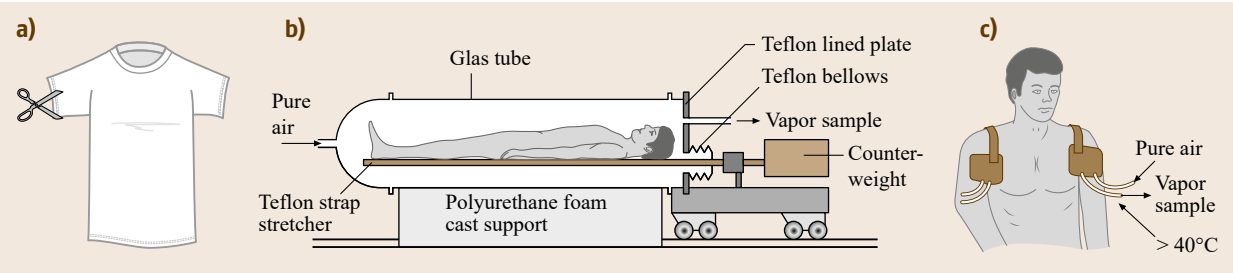

Fig. 48.2 **(a)** Many underarm malodor samplings were performed on T-shirts by solvent extraction of the underarm regions; **(b)** and **(c)** are two examples of headspace sampling devices described by *Dravnieks* et al. [48.8], reprinted with permission

the T-shirts. An example is given in Fig. 48.3, where we can see the background noise in the blank and the differences of excreted acids by individuals. Even if this study was performed only on four subjects some very interesting conclusions were made. The subjects A and D demonstrated a consistent acid profile. The four subjects presented differences in relative acid concentrations, for example the subject A produced about 30% of **1** and his T-shirts were described having the most human sweat smell and the T-shirt of the subject Joe was the less typical, it smelled more like dirty, rancid, therefore the acid profile influences the body odor.

The second phase focused on understanding the biological pathways in the formation of the acids. The sterile sweat does not smell and it is constituted only of nonvolatile water-soluble compounds. The analysis of sterile sweat led to the discovery of N^{α}-3-hydroxy-3-methylhexanoyl-L-glutamine and N^{α}-3-methyl-2-hexenoyl-L-glutamine [48.18]. The odorless sterile sweat is transformed by bacterial enzymes into odorous volatile compounds. From *Corynebacterium striatum* Ax20, present in the axilla region, it was possible to characterize a metal-dependent dipeptidase, N^{α}-acylglutamine aminoacylase. This enzyme cleaves the amide bond between the acid moiety and the glutamine. The structural gene of this enzyme was cloned for heterologous expression of a functional enzyme in *Escherichia coli* [48.18, 20]. Thanks to the availability of the enzymes, it was possible to incubate the sterile sweat and to discover a range of new hydroxy acids and unsaturated acids like **16**, **22–32** (Fig. 48.4) [48.21]. The relative abundance of acids confirmed that in fermented sweat, the major acid is **3** [48.21]. The acids **16**, **22–32**, **36–38** were present in much lower concentration compared to **3**, signifying that their contribution to the overall smell is minor. The ratio of **20** and **21** in sterile sweat, collected from 25 women and 24 men over a period of 3 years, was 10 : 1 [48.22, 23].

When odorous sweat was analyzed without knowing the precursor biochemistry, compound **3** was not

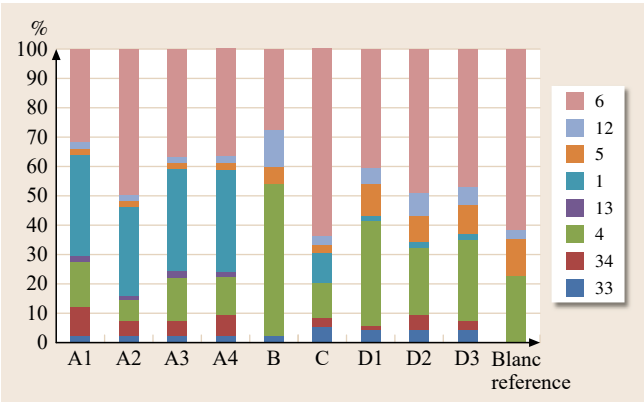

Fig. 48.3 Acids extracted from T-shirts. Cotton T-shirts, previously washed with unperfumed detergent and extracted with ethanol and chloroform were worn by volunteers in a sports club during 2 h exercise. The underarm T-shirt areas were cut. After acid base extraction, methylation with diazomethane, and clean-up via a Likens–Nickerson extraction, the GC-FID peak area of the acids were normalized to 100

discovered. Possible explanations could be a bad elution pattern of **3** on GC [48.24], or compound **3** is not stable on the GC injector port. It can be dehydrated or undergo a retro aldol reaction, leading to the formation of 2-pentanone and acetic acid. This was not verified, as synthetic **3** is stable in the injector port up to 280 °C. Another explanation could come from the bacteria. In vitro incubation of a mixture of synthetic **20** and **21** with *C. striatum* DSM 20668 produced only a mixture of **1** and **2**, whereas **20** remained in solution. Compounds **20** and **21** have different kinetics: **3** is better degraded by microorganisms further down and is more polar compared with **1** and **2**. In addition, its analytical recovery can be predicted to be less favorable; overall, this may explain why **3** was not discovered until 2003 by *Natsch* et al. [48.18].

Chiral-GC analyses revealed **3** to be a 72 : 28 mixture of the (*S*)/(*R*)-isomers. A difference in smell was described between *R*-**3** and *S*-**3** enantiomers, with *S*-**3**

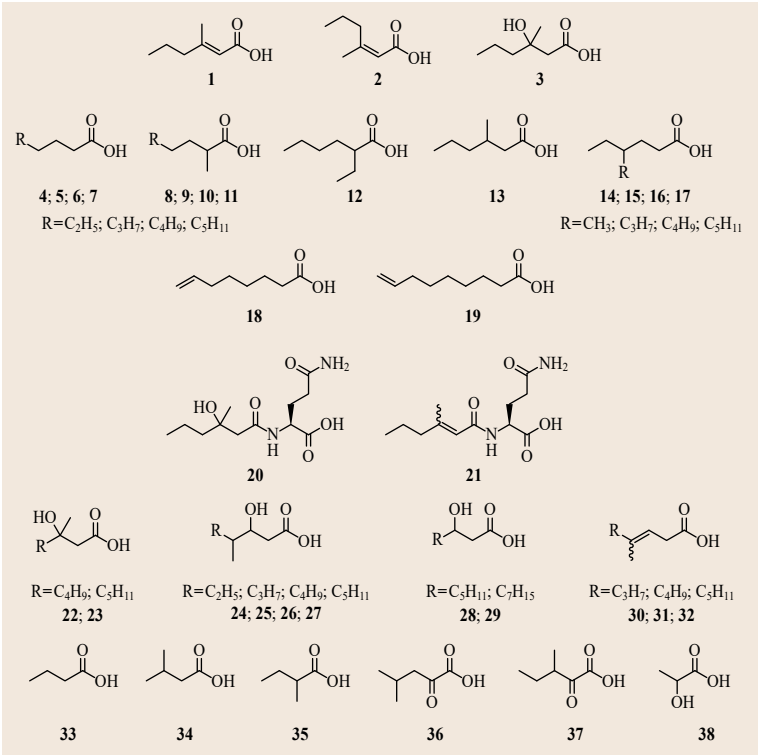

Fig. 48.4 Carboxylic acids detected in human axillary sweat

being described as more spicy (Table 48.1). The spicy odor descriptor was associated with cumin (*Cuminum cyminum*) seed odor or white *Piper nigrum*, even though these spices do not contain these exact same compounds, and the *R*-**3** enantiomer was described as weak animalic.

48.1.3 Sulfur Compounds

All of the investigators in the field of axillary malodors, in the year 2000, suspected that sulfur compounds also had an important role in axillary malodors. Unfortunately, these compounds are not easy to isolate for two reasons: they occur in very low concentration and free thiols stick to surfaces such as cotton T-shirts or metals. For the acids, one grain of table sugar would be enough to make an Olympic swimming pool smelling. For the sulfur compound, this grain of sugar would be enough to make 100 Olympic swimming pool smelling.

The discovery of thiol odorants in sweat occurred also in two phases in our laboratory. A perfumer noticed that clary sage (*Salvia sclarea* L.) (Fig. 48.5a) and *Ruta chalepensis* L. (Fig. 48.5b) in full blossom elicit a peculiar repulsive olfactory signal, somewhat reminiscent of human axillary perspiration [48.26, 27]. The sulfur compounds of the plant, refined in a 15 g fraction, were obtained from the distillation of 300 kg of clary sage. The sulfur compounds were concentrated by affinity chromatography. Then the chemical structures of most potent sulfur compounds were elucidated by gas chromatography coupled to mass spectrometry and an olfaction port (GC-MS-O). Both flower extracts contained many sulfur compounds, such as 3-sulfanyl hexan-1-ol **39** and 1-methoxyhexane-3-thiol **40**. Chiral analysis demonstrated that *S*-**40** has sulfury, alliaceous notes and evoked the smell of human axillary perspiration. *R*-**40** smelled sulfury, herbaceous and onion-like, but the principal sulfur compound detected by -olfactometry (GC-MS-O) in sweat extracts did not correspond to any of the sulfur compounds discovered previously in *R. chalepensis* L. or *S. sclarea* L. (Fig. 48.5) [48.26, 27].

For this reason, we decided to collect enough human sterile sweat for incubation. An exercise room equipped with steady bicycles and a sauna was installed in the basement of our research center. Volunteers were asked to exercise during at least 45 min plus 15 min sauna. The underarm area was scrubbed with a plastic cup, filtered on sterile filter and frozen. An average male gave 12 ml per session and females 2 ml per session (Fig. 48.6). The sweat pool used for the discovery of the sulfur compounds and other analyses represented 191 h of male efforts and 113 h of female efforts to

Table 48.1 Odor thresholds of sweat odorant compounds (after [48.23, 25–27])

Organic carboxylic acid	Abbreviation	Odor detection threshold (ng/l air)
3-Methyl butanoic acid	**34**	0.2
(*E*/*Z*)-3-Methyl-2-hexenoic acid	**1** and **2**	0.1
(*E*)-3-Methyl-2-hexenoic acid	**1**	0.7
(*Z*)-3-Methyl-2-hexenoic acid	**2**	0.7
(*R*/*S*)-3-Hydroxy-3-methyl hexanoic acid	**3**	0.2
(*S*)-3-Hydroxy-3-methyl hexanoic acid	*S*-**3**	0.08
(*R*)-3-Hydroxy-3-methyl hexanoic acid	*R*-**3**	0.2
(*R*/*S*)-4-Ethyl-octanoic acid	(*R*/*S*)-**16**	0.1
(*S*)-1-Methoxyhexane-3-thiol	*S*-**40**	4×10^{-5}
(*R*)-1-Methoxyhexane-3-thiol	*R*-**40**	1.09×10^{-3}
(*R*/*S*)-1-Methoxyhexane-3-thiol	(*R*/*S*)-**40**	3.6×10^{-4}
(*R*/*S*)-3-Methyl-3-sulfanyl hexan-1-ol	(*R*/*S*)-**41**	2×10^{-3}

give, respectively, about 2.5 l of male sweat and 0.2 l of female sweat. Additionally, we cultivated underarm bacteria and then incubated each colony with the sterile sweat. We first discovered that the more intense sulfury odor came from sweat incubated with one specific bacterial strain of the cultivated underarm bacteria, namely *Staphylococcus haemolyticus*. Then the incubation was repeated on 300 ml of sterile male sweat and finally after purifications and concentrations, the structure of 3-methyl-3-sulfanyl hexan-1-ol **41** (transpirol) was elucidated. This compound was discovered simultaneously by three groups [48.28–30]. In the same fraction, the compound **39** and the odorant 2-methyl-3-sulfanyl pentan-1-ol **42** (Fig. 48.7), the key odorants of fresh onion [48.31] were detected.

As it became evident that the identified substances are extremely odor potent but still low in concentration, the odor-detection thresholds of these volatile sulfur compounds were measured in air by olfactometry [48.25, 32, 33]. Thereby, the threshold of *S*-**40** at 0.04×10^{-3} ng/l air is 25 times lower than that of *R*-**39** (1.09×10^{-3} ng/l air). The racemic thiol (*R*/*S*)-**40** has an odor threshold of 0.36×10^{-3} ng/l air [48.23, 25]. The odor detection threshold of racemic transpirol **41** was measured at 2×10^{-6} µg/l air. Also, women seemed to be slightly more sensitive compared with men, as

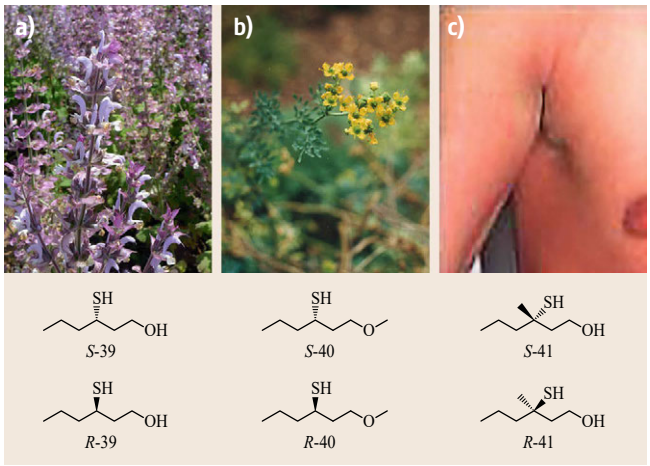

Fig. 48.5a–c From right to left, **(a)** *Salvia sclarea* L in Provence (France) (Picture Mrs V. Gervason). **(b)** Picture of *Ruta chalapensis* L. took in Chinque Terre (Italy) and **(c)** Human underarm. Chemical structures of compounds **39** and **40** discovered in these flowers, and in human **39**, **41**, and **42** were discovered

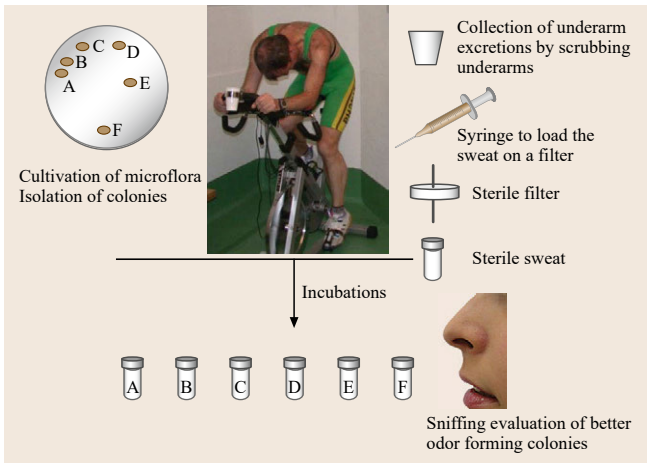

Fig. 48.6 Analytical approach to understand the biochemistry of underarm malodor formation. The microflora was analyzed. The odorless sterile sweat was incubated with isolated underarm bacteria colonies and the most efficient odor forming bacteria were detected by sniffing the fermented sweat (after [48.22])

shown by the values measured for 15 male subjects and 15 female subjects, but this needs further investigations to be confirmed (Table 48.1) [48.23].

All living organisms have biological systems to protect them against aggressive chemical substances. A tripeptide, constituted of the γ-glutamic, cysteine, and glycine amino acids, named glutathione **43** reacts with free radicals or any xenobiotics to prevent them from damaging the cell functions. The glutathione is an important scavenger for the living cells. From an un-

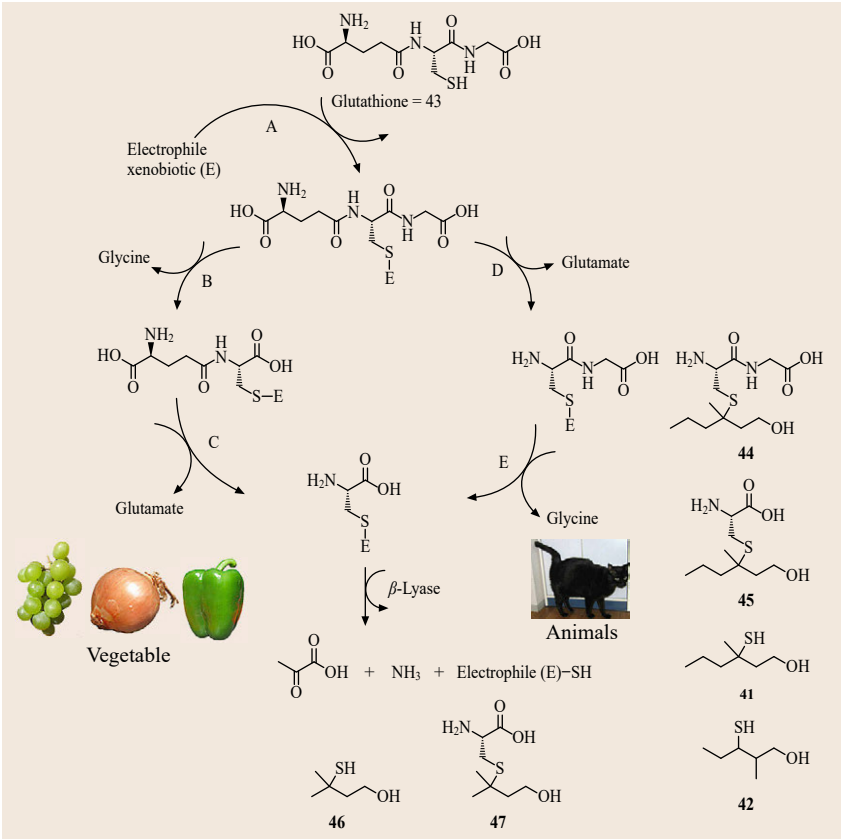

Fig. 48.7 Glutathione detoxification pathway. The glutathione free thiol is nucleophilic. Its main role is trapping the free radicals. In the case of human body odors and in plants-like *Alliaceous* sp. the soft nucleophilicity of the sulfur atome reacts with α,β-unsaturated carbonyls. Then enzymatic reactions can cleave the peptidic bond to release the glutamine or to release the glycine. The final step is the excretion of the cysteine-*S*-conjugate. In *Alliaceous* sp. most of the cysteine conjugates are stored under sulfoxide and cleaved when plant cells are disrupted. In human urine they are excreted under mercapturate (*N*-acetyl-cysteine-*S*-conjugate), which are good biomarkers of exposure to pollutants

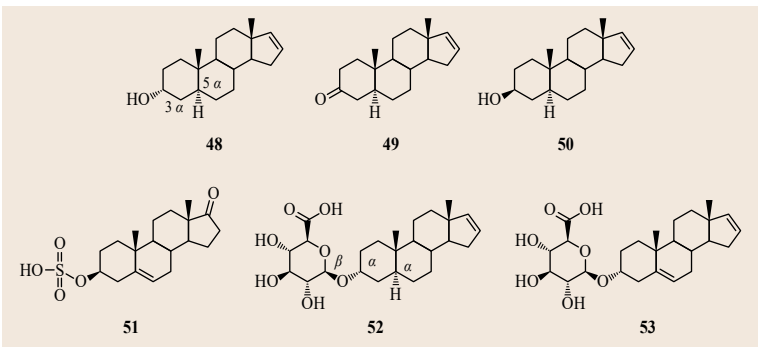

Fig. 48.8 Major odorous steroids and their respective precursors found in axillary sweat (after [48.34])

derstanding of glutathione **43** detoxification pathways (Fig. 48.7) [48.35, 36], the corresponding cysteine-*S*-conjugate **45** was the obvious candidate as being the precursor of sulfur compounds. The sterile sweat was then fractionated and a small portion of the fraction (only 1/10) was sacrificed for incubation with *S. haemolyticus* in order to identify by smelling which fraction was the odorless precursor. This fraction was fractionated further until it was possible to detect relevant masses by LC-MS-MS. This approach led to the discovery of 1-[(2-hydroxyethyl)-1-methylbutyl]-(L)-cysteinylglycine (cys-gly-*S*-transpirol) **44** [48.22]. The biochemistry of **41** in humans is close to 3-methyl-3-sulfanyl-butan-1-ol **46** found in domestic cats (*Felis domesticus*) and in bobcats (*Lynx rufus*) [48.37, 38]. The precursor is a cysteine-*S*-conjugate (2-methyl-3-hydroxy-butyl-*S*-cysteine) **47**.

48.1.4 Steroids

In 1944, Prelog and Ruzicka isolated 5α-androst-16-en-3α-ol (α-androstenol) **48** from 181 kg of hog

testes [48.39]. It was described as having a musk-like odor. When the alcohol was oxidized to 5α-androst-16-en-3-one (androstenone) **49**, the odor was described as being stronger and like *vessels which had been used for storing urine for prolonged periods* [48.40]. The perception of androstenone **49** can be offensive, pleasant, or odorless, depending on the individual and possibly hormonal state [48.41–43]. Compared with α-androstenol **48**, β-androstenol **50** is noticeably weaker in odor [48.44]. Androstenone **49** is a pig pheromone that was also found in the human armpit [48.45, 46]. The apocrine glands become active at puberty, and so these odorous steroids were intensively studied because of their possible role in human communication [48.7, 47–49]. α-Androstenol **48**, but not androstenone **49**, was detected in human male axillary sweat [48.6]. α-Androstenol was previously detected in human urine [48.50]. Because androstenone is a boar sex pheromone, an abundant literature focused on the importance of androstenone in axillary sweat, but to our knowledge, no solid documented work proved its occurrence in sterile sweat. However, *Bird* and *Gower* demonstrated the occurrence of androstenone in one subject [48.46], and *Claus* and *Alsing* confirmed the occurrence of androstenone after 24 h of collection on pads placed in the axillary region, via radioimmunoassay [48.7]. They estimated the production as being 14 ng/h per armpit. During the numerous incubations and analysis of sterile sweat in our laboratory, it became clear that androstenone **49** is not present in sterile sweat and that it seems to be the result of the oxidation of α/β-androstenol **48**, **50**. Here, it is especially interesting to note that some of our subjects had very efficient microflora to cause this oxidation while others did barely transform the substance [48.34].

The apocrine glands excrete water-soluble derivatives of odorants in sweat that are themselves odorless. For example, most of the steroids are excreted as sulfate derivatives such as 3β-hydroxyl androst-5-en-17-one (DHEA) sulfate **51** found in the range of 2–90 µg/ml in axillary sweat [48.34]. The precursor of α-androstenol is α-androstenol-β-glucuronide **52**, as in human urine [48.50], and in sterile sweat we measured α-androstenol-β-glucuronide **52** at 0.2 µg/ml for men and 0.08 µg/ml for women [48.34, 50]. If 5α-androst-5,16-dien-3α-ol β-glucuronide **53** is present in sterile sweat, the concentration is below 0.02 µg/ml (< 20 ppb) (Fig. 48.8).

When *Gilbert* and *Wysoski* [48.51] issued a questionnaire called *The Smell Survey* (1987), they obtained 26 200 replies and found that androstenone posed a greater identification and detection problem compared with other odors and that 70% of women could smell it, whereas only 63% men could [48.51]. Based on this observation, the genetic basis of variation in odor perception between individuals at the human receptor OR7D4 was studied, along with associated interindividual genetic variations; the main finding of this comparison was that genotypic variation in OR7D4 correlates with the perception of α-androstenone [48.52, 53].

In conclusion, apocrine glands start functioning when humans become adults. They excrete acids **1**–**3** that have a structure that is unique to humans. Thiol **41** is also unique to humans. The biochemistry associated with **46** in cats is similar and the unusual amino acid **47** was considered as a semiochemical in cats, but no proof of its action as a pheromone has yet been demonstrated [48.53]. Androstenone **49**, a pig pheromone, is also excreted by humans, primarily as α-androstenol **48** under the β-glucuronide conjugate **52**, and is then oxidized in androstenone **49**. This excretion starts at puberty. Together, these facts suggest that compounds **1**–**3**, **41** unique to human and **48**, **49** are odorant signaling compounds of humans.

48.2 Volatile Organic Compounds of Human Urine

The smell of urine is recognized by everyone, especially when the urine is not very fresh any more. However, the smell of fresh urine is mainly due to highly volatile compounds such as methyl mercaptan and amino compounds that result from the degradation of urea. More substantive molecules are later formed to produce the smell of stale urine. In relation to this process, we now better understand the role of microorganisms and chemical reactions that bring about these odors and their associated aversive responses in humans. The smell of felids urine was reported to be caused mainly by sulfur compounds **46** [48.54], which is not the case for human.

48.2.1 Analysis of Human Urine Odors

Everyone has at some time experienced the typical odor of stale urine downtown, in remote places or around train stations. In contrast to this, fresh urine has little odor. Moreover, dietary influences can modify the smell of urine; the most obvious case being the typical sulfury odor of urine after someone has eaten asparagus [48.55–57]. The modulation of urine odor by diet has been documented [48.58–60]. Most of the studies on the specific VOC composition of urine have been carried out in relation to urinary tract infections [48.61] and on changes in urine odor related to a specific

disease [48.62–66]. Other studies on the volatile and odorous fraction of human urine and their conjugates were performed to obtain insights into common excretion processes [48.63, 67].

However, also the common nuisances associated with urinary smell attract researcher's interest, not only because of their scientific craziness but also with the real goal of improving the quality of our everyday life. To understand the smell of urine in a latrine, to develop tools against such nuisances, investigators need to design an aging protocol from healthy subjects. However, few options are possible: collecting odors on site, boiling urine to mimic-aged urine during water evaporation in an open-air environment, or aging urine with microorganisms.

Fresh urine has a faint odor described as ammonia, floral, and sweet, but even if variations are noticeable, the overall odor descriptor is still *urine*. In this context, it is interesting to note that the term *urine* as descriptor for smell is more often used by perfumers than by flavorists, and not necessarily in a negatively associated context. When looking at the individual constituents of urinary smell, the ammonia-like odor quality is due to the presence of trimethyl amine [48.65, 66], which has a lower odor threshold than ammonia itself which is also related to pH effects: a water solution of NH_4OH at pH 7–8 is odorless, but at the same pH, a solution of Et_3N at 1 mg/l has an odor.

A boiled urine organic extract smells like urine but has a more syrupy, medicinal, and cooked-food type of odor, resulting from Maillard-type degradation products (Chap. 5) such as pyrazines, sugar degradation products such as maltol (3-hydroxy-2-methyl-4(4*H*)-pyranone), furaneol (4-hydroxy-2,5-dimethyl-3(2*H*)-furanone), and methyl nussol (2-hydroxy-3,4-dimethyl-2-cyclopenten-1-one). The occurrence of odorant molecules thermally generated was observed by GC-MS-O (Chap. 17) [48.67].

In comparison to that, a fermented urine organic extract smells like authentic stale urine, is acrid, and is more representative of stale urine than boiled urine, an odor that is noticeable when urinals are dirty. To obtain this sample, we left sterile fresh urine in the open air for 5 days during the summer. The urine was spontaneously contaminated by bacteria. From this aged urine, 73 bacteria colony isolates were incubated separately with sterile urine. Only 13 colonies produced a repulsive strong urine odor, from which five odor-generating bacteria were identified: *Morganella morganii*, *Escherichia fergusonii*, *Enterococcus faecalis*, *Citrobacter koseri*, and *Streptococcus agalactiae*. When sterile urine was incubated with *M. morganii*, the pH rose to 9 and the fishy odor due to ammonia, trimethyl amine and dimethyl disulfide was repulsive [48.67].

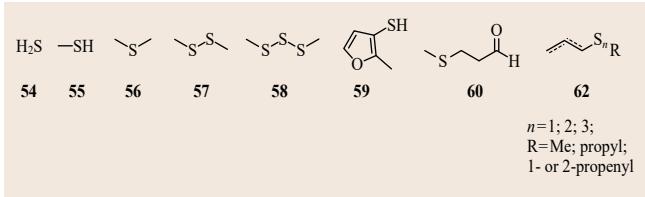

Fig. 48.9 Common sulfur compounds in urine and feces originating from food

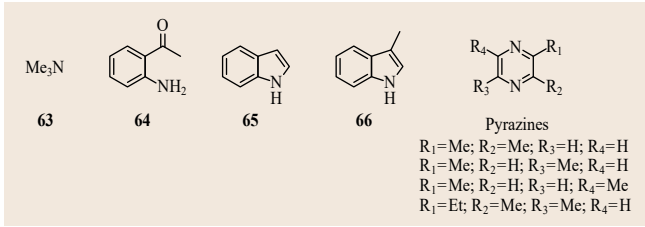

Fig. 48.10 Nitrogen-containing compounds in urine and feces

In the following, the respective compounds being pronounced in aged urine will be addressed in more detail.

48.2.2 Carboxylic Acids

The carboxylic acids present in fresh and aged urine do not contribute to its smell [48.68–71]. When we used gas chromatography-olfactometry (GC-O), an odor in the background reminiscent of sweat triggered our curiosity, and after a specific acid–base extraction and analysis, we confirmed the occurrence of traces of **3**. The acid **16**, main odorant compound of the goat, was always well detected by GC-O and it may contribute to the odor descriptor *animal*.

48.2.3 Sulfur Compounds

Hydrogen sulfide **54** does not seem to be reported in urine, but methyl mercaptan **55** is a key odorant. Dimethyl sulfide **56**, dimethyl disulfide **57**, smelling like garlic and rubber and dimethyl trisulfide **58**, smelling more sewage-like, are the other important contributors to urinary smell. 2-Methyl-3-sulfanyl furane **59**, a strong meaty odor, coming mainly from flavored food, was detected in all GC-O analyses of urine extracts; 3-(methylthio)-propanal (methional) **60**, the Strecker aldehyde of methionine, which smells of boiled potatoes, was detected in fermented urine by all panelists during GC-O evaluation [48.67] and is a potent odorant in urine.

When a urine donor eats garlic or onion, 1-propenyl, 2-propenyl mercaptan, or the corresponding polysulfides **62** are detected in the analysis and

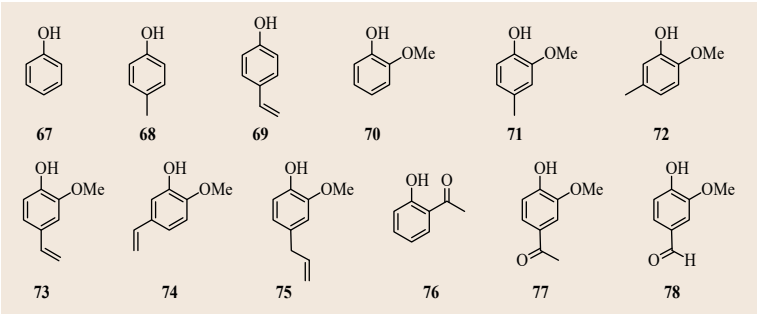

Fig. 48.11 Phenolic compounds in urine, feces, and in pit latrine sludge

can influence the onion-like, sulfury smell of urine (Fig. 48.9).

48.2.4 Nitrogen-Containing Compounds

Amino compounds are affected by a low pH. An ammonia solution at pH < 9 is odorless as its concentration in the headspace (air above the solution) is very low because it is present in protonated form. Trimethyl amine **63** is also affected by the pH but to less extent, because it is more hydrophobic due to the carbons attached to the nitrogen. Trimethyl amine is a very important odor contributor to urine smell and its odor threshold is thousand time lower compared to ammonia, but its odor is more fishy. 2-Aminoacetophenone **64** has a floral tonality, a smell-like Concorde grape, that was clearly detected by GC-O, having a nasal impact factor (NIF) above 50% [48.67]. For a representative odor of fresh, aged, or boiled urine, indole **65** is an important contributor. Indole has a pleasant odor and a good jasmine perfume cannot be made without using indole. But indole in the presence of other compounds can be associated to a malodor. The skatole **66** present in urine [48.60, 64] is too fecal, has a mothball-like odor, and its concentration is too low to be a typical impact odorant for urine. Boiled urine contains many pyrazine derivatives, originating from the condensation of ammonia from dicarbonyl compounds that stem from sugar degradation products [48.60]. The occurrence of pyrazine itself in urine has rarely been reported (Fig. 48.10) [48.70–74].

48.2.5 Phenols

In fresh urine, phenol derivatives are present, and their concentration and impact on the smell of urine even increases over time because of the action of enzymes originating from microorganisms. The core substance phenol **67** itself has a medicinal or ink-like smell [48.60, 62, 72]. The most important contributor to the smell of urine, however, is *para*-cresol **68**, which smells horse-stable-like and fecal. 4-Vinyl-phenol **69** was also identified in fresh urine and its odor is reminiscent of urine, leather. Apart from that, the concentration of the smoky-smelling guaiacol **70** increased when fresh urine was incubated with the bacteria *Citrobacter koseri* [48.67]. Also, the methyl guaiacols **71**,**72** smell smoky, with the clove-like, smoky tonality, and contribute together with the spicy smell of 4- and 5-vinyl guaiacol **73**, **74** or 4-allyl-2-methoxyphenol (eugenol) **75** to the smell of urine [48.61, 67]. 2-Acetylphenol **76** was detected in fresh and boiled urine (NIF = 75%), and its smell was described as old urine, representing the same odor impression as elicited by vanilloketone **77**. These compounds are impressive in terms of overwhelming odor impression when they elute from the column in GC-O analysis (NIF > 75%), but in water at 1% and pH 7, their odor impact are weak. Apart from that, vanillin **78** also contributes to the sweet smell of urine in some cases (Fig. 48.11).

48.2.6 Miscellaneous Odorant Compounds

The number of volatile organic odorant compounds in urine is higher compared to volatile compounds in fecal material. Diacetyl **79** contributes to the buttery smell of urine [48.61, 69, 71]. 2-Phenylethanol **80** is also an important contributor to honey and to the floral smell of urine; it is formed after aging with microorganisms [48.67]. Other compounds that have an impact on urine odor are also common in food, including 2*H*-chromen-2-one (coumarin) **81**, 3-hydroxy-4,5-dimethyl-2(5*H*)furanone (sotolone) **82**, and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (furaneol) **83** [48.61, 69, 71].

Monoterpenes present in urine commonly do not contribute to its smell, nor do ketones and many other compounds that often occur in urine and are linked to food, such as (+-)-3-methyl-6-(prop-1-en-2-yl)cyclohex-2-enone isopiperitenone **84** and menthol **85** (Fig. 48.12).

α-Androstenol **48** is excreted in the conjugated form as α-androstenol-β-glucuronide [48.50] and is responsible for stale urine odor after cleavage [48.44,

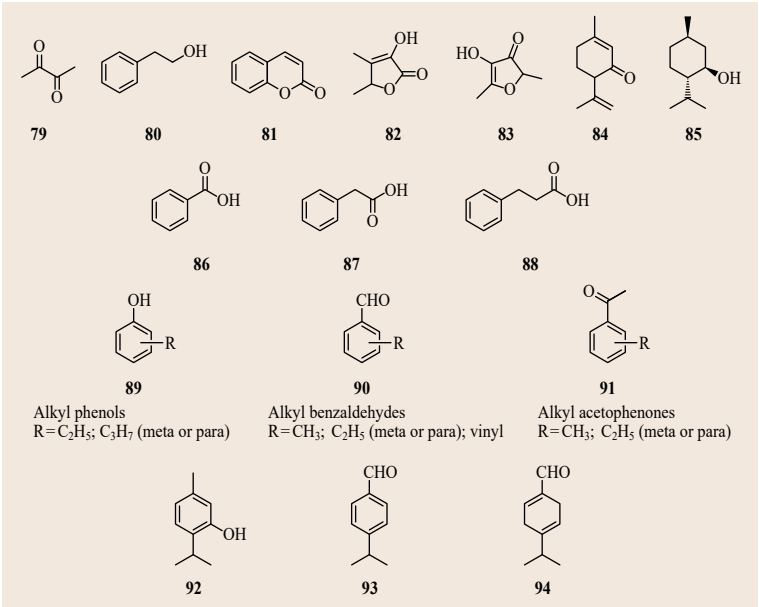

Fig. 48.12 Miscellaneous structures of compounds found in urine or in fecal material

71]. It is then oxidized to androstenone, described as having a urine smell [48.72]. *Streptococcus agalactiae* contains an efficient glucuronidase to liberate α-androstenol **48**, as well as menthol [48.65]. On the other hand, α-androstenol is not liberated during boiling of urine, as apparently the glucuronide conjugate is too stable under heat treatment [48.67].

In conclusion, the smell of urine is influenced by microflora and in the early stage of urine degradation, the pH increases and the smell of ammonia dominates, mainly due to the presence of trimethyl amine. After a few hours, the pH slowly returns to neutral and then more substantive molecules like *para*-cresol, indole, androstenol, and its conversion product androstenone, change the fresh urine smell into stale urine. Overall, the smell of urine is a more complex system compared with axillary body odors, or fecal odors. This is predominantly due to the fact that urinary excretion of odorants and volatiles is strongly linked to primary elimination pathways of exogenic and endogenic compounds from the food, and the smell of feces originates from further microbial biodegradation of fibers, sugars, and proteins, basic food constituents, as will be discussed in the following.

48.3 Volatile Organic Compounds in Human Feces and Pit Latrines

Understanding the smell of feces is not a new topic: the archives show that by the 19th century, scientists already had a good idea of which compounds are important to the smell of feces [48.78]. In medieval times, fecal smells were obviously a commonly tolerated and integral part of everyday life, for example, in the early cities with excretions having been simply deposed of in the streets. Nowadays, the smell of feces, as well as the analysis of the respective odor compounds currently gains momentum as modern humans increasingly less tolerate such human emissions. The fecal smell is rated as repulsive, but with regard to its molecular composition it is relatively simple, especially when being compared with that of urine. The importance of single constituents, such as acids, sulfur compounds, and nitrogen-containing compounds such as indole, skatole, and phenolic compounds are discussed in the following.

48.3.1 Analysis of Fecal Odors

The analyses of volatile compounds in human feces were mainly done to understand the relation between the gut microbiota and the food intake. Few methods were developed to understand the composition of malodorous compounds in human feces and the main motivations were not obvious to understand. The most pertinent analyses were performed on the headspace analysis using a tenax trap. In one case, the odor was sucked during the stool expulsion using a pump in

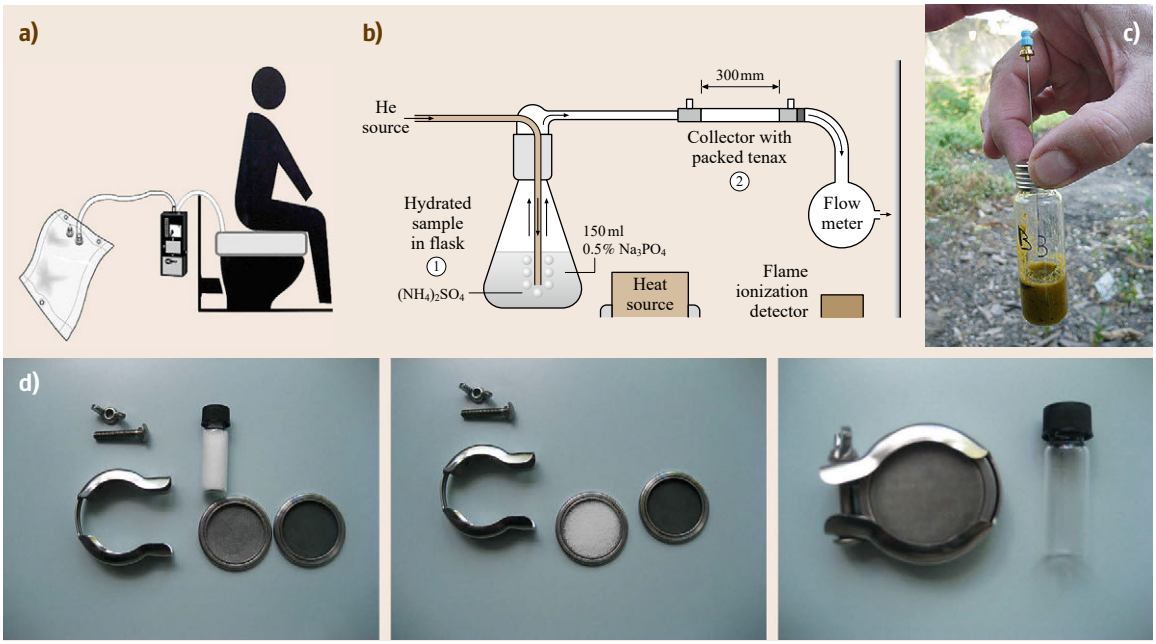

Fig. 48.13a–d Analytical devices used for sampling the smell of fecal malodor. **(a)** To analyze immediate odor, adapted from [48.75]. **(b)** Example of dynamic headspace sampling (after [48.76]). **(c)** Example of SPME pit latrine sludge sampling [48.77]. **(d)** Device used to analyze pit latrine odors on site overnight [48.77] ((**c**) and (**d**) reprinted with permission from [48.77]. Copyright (2013) American Chemical Society)

a sampling bag and then the gas phase from the bag was pushed onto a tenax trap [48.75] (Fig. 48.13a). More commonly, the stool was placed in an Erlenmeyer flask and a gentle air flux was used to push the headspace in a tenax trap [48.76] (Fig. 48.13b). Analyzing a single stool by direct extraction is challenging because after a few days, even with centrifugation, it is difficult to eliminate and discard the fine residual suspension. Acidification of the suspension, in the presence of an organic solvent, can form a stable gel which poses another obstacle to the extraction of volatiles from such samples. Headspace analysis is a good alternative, but method validation must be carefully designed [48.79–85]. The fecal sludge from African pit latrine was analyzed by SPME using internal standards (Fig. 48.13c) [48.77]. The limitation was the stability of some compounds on the fiber. The dynamic headspace using tenax cartridges is a good alternative to SPME, but due to the breakthrough volume, we must choose if to trap highly volatile compounds or to trap heavier compounds. This means that pumping pit latrine headspace during a short period of time, about 1 h, methyl mercaptan is well detected but indole and scatole are not detected. The benefit of using static headspace is that we can leave the device on site overnight and it can be hidden easily in the ventilation port of the pit latrine. During our African pit latrine analysis, we used an organic polymer (Porapak Q), which was conditioned in our lab and dispatched in many small glass flasks. On site the polymer was loaded on a filter commonly used for filtration in organic chemistry. The disc was exposed to the headspace and then the polymer was loaded back in the flask to be shipped back in ice-cooled pack to the lab (Fig. 48.13d) [48.77]. The solvent extraction of the polymer allowed to obtain an extract which can be smelled and injected multiples times, but highly volatile or unstable compounds like methyl mercaptan **55** are lost.

48.3.2 Carboxylic Acids

Short-chain fatty acids are major products of microbial oligosaccharide breakdown in the large intestine, a pathway that leads not only to the formation of products that supply the intestinal cells with energy but also goes along with the generation of smelly compounds. The extent of formation of individual acids in relation to the metabolism of these oligosaccharides or arabinoxylans from maize, rice, and wheat has been investigated in vitro and in vivo [48.86, 87]. Thereby, acetic acid, propionic acid, and butyric acid **33** are the most abundant acids. Isotope labeling studies using [$U^{13}C6$] glucose were used to show that $^{13}C_2$ and

$^{13}C_4$ acids are the major labeled butyrate species produced from glucose fermentation via [^{13}C2] acetate-acetyl coenzyme A as the intermediate [48.88, 89].

Specifically, butyric acid is an important contributor to fecal odors. The odor threshold of butyric acid in air is $< 0.1\,\mu g/l$, but in water or sludge, pH influences its partitioning in air. Overall, the impact of all organic acids becomes less important at neutral pH. Furthermore, the composition or the ratio between acid concentrations also plays a role in the resulting odor profile. For example, stool with a ratio of **33** and **34** of 2 : 1 will be significantly different from that with a ratio of 10 : 1. Other acids contribute to fecal smell, for example, benzoic acid **86**, phenylacetic acid **87** (odor threshold $1\,\mu g/l$), and 3-phenylpropionic acid **88**. It is also known that people with diarrhea produce stools that consist mainly of acids, which explains the vomit odor. Over time, stool smells less and less of rancid cheese and vomit; this can be explained by the faster bacterial metabolism of the acids compared with the slower bacterial catabolism of phenolic compounds [48.77].

48.3.3 Sulfur Compounds

H_2S **54** and methyl mercaptan **55** are primarily responsible for the odors associated with bad breath, feces, flatulence, and sewage. Dimethyl sulfide **56**, dimethyl disulfide **57** and dimethyl trisulfide **58** are the second most important contributors to an eggy, sewage, cabbage smell [48.86]. The sulfur atom originates from proteins and the consumption of vegetables, such as onion or garlic. The consumption of albumin as present in eggs increases the formation of methyl mercaptan [48.87]. Fermentation in anaerobic conditions and nutritional substrates that originate from waste both favor the formation of sulfur compounds, such as **54** and **55**, to produce typical sewage, egg-like odors [48.86, 88–92].

48.3.4 Nitrogen-Containing Compounds

In 1878, a Swiss doctor from Bern hospital, *Brieger* [48.78], distilled 50 kg of feces from healthy men. He chose only those patients who were in hospital after an accident and did not suffer from any other diseases. In the course of these investigations, he discovered skatole **66** and described its contribution to the odor of feces. He also mentioned the occurrence of butyric acid **33**, branched C-5 acids, phenol **67**, and indole **65**. As in the case of urine, the analysis of fecal odor was at this time primarily conducted in the medical sciences as a diagnostic tool for the detection of specific diseases.

The first GC-MS-O analysis on human feces was performed in 1984 [48.93]. About 25 g of fecal material was placed in an Erlenmeyer flask suspended in an Na_3PO_4 buffer and $(NH_4)_2SO_4$ and then stored for 24 h before dynamic headspace sampling was performed and the odorants trapped in a Tenax collector trap (Fig. 48.13b) [48.76, 93]. The Tenax absorbent was then thermally desorbed on GC-flame ionization detection (FID) coupled to an olfaction port or to a GC-MS. The pH was not displayed, but the solution must have had a pH > 8. These conditions favored the analysis of nitrogen compounds, which are not strongly protonated at this pH. Indole **65** and skatole **66** were well detected and were described as being mothball and napthalene-like, but not considered as being important to the foul odor of feces. The fishy odor, characteristic of volatile amines, was also not detected, and the author concluded that trimethylamine was not important for fecal odors [48.93]. These observations are still valid today and have been confirmed by recent investigations on this topic.

48.3.5 Phenols

The major phenolic odorants in fecal material are para-cresol and also to some extent meta-cresol. Phenol is also always detected, but its bandage smell is not a typical attribute for fecal smell. Ethyl and propyl phenols in meta and para positions **89** (Fig. 48.12) are always detected by GC-O and twist the fecal smell into one that is more barnyard like and outdoor associated. Although guaiacol **70** can be present, it was not detected in a single stool and can be ruled out as a contributor to human fecal odor (Fig. 48.12) [48.77].

In conclusion, the stool odor is mainly due to short chain fatty acids, *p*-cresol, sulfur compounds, indole, and skatole. The odor profile is simpler compared to urine because these compounds result from proteins and fibers and not from detoxifications pathways, with soluble molecules of odorant molecules excreted in urine. Only in extreme situations when the subject eats heavily flavored food, terpenes can be detected in fecal material.

48.3.6 Miscellaneous Odorant Compounds

Benzaldehydes and substituted benzaldehydes with methyl, ethyl, or vinyl residues **90** and acetophenone derivatives **91** may contribute to the fecal smell to a lesser extent and were frequently detected by GC-O. Thymol **92** was smelled and identified in stools from India, as well as 4-isopropylbenzaldehyde (cuminaldehyde) **93**, 1,4-*para*-menthadien-7-al (phellandrenal) **94**, and vinylguaiacol **73**. They were more abundant than

para-cresol **68**, and the smell was spicy and rancid, atypical of fecal material. This observation reflects a rich spicy food that affects the smell of Indian stools (Fig. 48.12) [48.83].

48.4 Conclusions

The human axillary sweat has a unique odor due to the occurrence of compounds **1**, **2**, **3**, **41**, not found yet in any other natural species. This is the signature of human adult smell. The urine smell is mainly due to trimethyl amine, ammonia, dimethyl disulfide, indole, and skatole. But in urine, many other organic compounds originating from food are excreted in soluble form like glucuronide or sulfate form. The α-androstenol **48** is an important contributor to the stale urine odor as well as its oxidation product **49**; **48** is also found in underarm regions and it is excreted by apocrine glands, when they become active at the puberty. The fecal material smell is mainly due to the acids **33**, **34**, **35**, phenol **67**, para cresol **68**, plus indole **65** and skatole **66**. The type of the food intake plays an important role in fecal smell. Eating garlic, onion, or cabbage will produces a lot of powerful volatile sulfur compounds.

The microflora is very important to generate malodor, cleaving the chemical bond between the soluble part of the molecule to the hydrophobic part, for example, the glucuronide of α-androstenol **48**, the glutamine conjugate of the acids **1**–**3** or cysteine-glycine conjugate of the sulfur compounds like **41**. The beauty of nature is the capacity of modulating the generation of odors; if the apocrine glands are very active to excrete the α-androstenol glucuronide **52**, the microflora must include bacteria producing the right β-glucuronidase enzyme to release the odorant molecule. Understanding this chemistry is very important to develop strategies to prevent the malodor formation without disturbing the microflora. What was said for the gut microflora, as *Zimmer* reported [48.94]:

> *For a century, doctors have waged war against bacteria, using antibiotics as their weapons. But that relationship is changing as scientists become more familiar with the 100 trillion microbes that call us home – collectively known as the microbiome,*

is also valid for the skin microflora.

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49. Biochemistry and Genetics of Human Axilla Odor

Andreas Natsch

Human axilla odors are only formed once skin secretions come into contact with the skin microflora. Axilla odors are thus a product of an intricate interplay between skin bacteria and axillary gland secretions. The bacterial populations in the axilla are dominated by *Staphylococci* and *Corynebacteria*. The magnitude of odor formation is associated with the population density of *Corynebacteria* on the skin of human panelists.

In recent years, amino-acid conjugates were identified as the key secreted odorant precursors: Different odorant acids are secreted as glutamine conjugates, whereas sulfur volatiles arise from the bacterial degradation of conjugates related to glutathione. Specific enzymes were identified in *Corynebacteria* cleaving these amino-acid conjugates and thus releasing the odors.

Based on this molecular understanding, questions on the evolutionary significance of these odors could be asked. The pattern of secreted odor precursors appears to be stable within an individual and it is genetically determined as was shown in a twin study. Based on behavioral studies, an association between axilla odors and the human leukocyte antigen (HLA) genotype had been proposed, but the chemical nature of potential HLA-associated body odors remained enigmatic. A family study on siblings with identical HLA-genes could not identify a link between genetically inherited patterns of glutamine conjugates and HLA-types.

Axilla odors are largely absent in a significant fraction of the human population in the Far East. This has been associated with a single nucleotide polymorphism (SNP) in the *ABCC11* gene. Indeed, human subjects lacking a functional ABCC11 allele are not able to secrete the amino-acid conjugates of the key odorants, which confirm the relevance of these biochemical mechanisms of odor formation identified in the last decade.

49.1 Importance of Bacteria in Odor Formation

The human axilla is covered by a particularly dense array of secretory glands. These mainly belong to two types: eccrine glands secrete an aqueous solution, containing salts, and water soluble components such as lactic acid and amino acids. Apocrine channels associated with the hair follicles secrete a more hydrophobic mixture containing fats, steroids, and proteins belonging to the lipocalin family. Lipocalins are small proteins binding a variety of hydrophobic ligands. They include the major urinary proteins (MUP) in mice, which

bind odorants in mouse urine, as well as odor binding proteins (OBP) in humans and other vertebrates, which can bind to a variety of lipids and odorants. The major lipocalin in human sweat is apolipoprotein D. In addition to the two major types of glands mentioned above, apoeccrine glands were discussed as a third type with a high abundance in the axillary region [49.1]. The secretions provided by all these glands serve as ideal growth medium for a number of bacterial species – combined with the partial occlusion provided by the anatomy of the axilla and the dense coverage by hair (which delay water evaporation and thus help to provide a moist environment), the axilla is an almost perfect habitat for bacterial colonization. It was therefore early recognized that the density of the bacterial flora is particularly high within the axillary region [49.2] and that this bacterial habitat is strongly differing from the dry surface of most other skin sites. This was recently summarized in the statement [49.3]:

> *The moist underarms lie a short distance from smooth dry forearms, but these two niches are likely as ecologically dissimilar as rainforests are to deserts.*

In parallel to the observation of a high bacterial population density in the axilla, it was shown that there is a direct association between bacteria and body odor formation. Fresh apocrine secretions were found to be odorless, but they developed the typical pungent axilla odor if contacted with skin bacteria [49.4]. This initial study sets the scene to search for the mechanisms of body odor formation, which could only be understood by focusing on both the skin microflora and the axilla secretions to finally understand the interplay between both contributors.

49.2 Specific Odor-Forming Bacterial Populations Colonizing the Axilla

In early studies, the bacterial population was investigated by culture-based methods. Human panellists were tested both for strength of odor formation and the presence and abundance of specific bacterial species. This approach led to the conclusion, that the two main genera in the axilla are *Staphylococci* and *Corynebacteria*, and that odor formation is particularly associated with the population density of lipophilic *Corynebacteria* [49.2, 5]. In these pioneering studies, it was not only shown that there is a correlation between density of *Corynebacteria* and odor formation, but the authors did also directly incubate bacteria with apocrine secretions and could show that only *Corynebacteria* were able to release the odors. This conclusion was later confirmed by a detailed correlation analysis between odor intensity and abundance of specific bacterial groups, and again the strongest association was observed between population density of *Corynebacteria* and odor formation [49.6]. More recent studies did not focus on odor formation, but generated a global inventory of bacterial species present at different skin sites. These studies applied more advanced culture-independent molecular methods and high throughput sequencing of the deoxyribonucleic acid (DNA) coding for 16s-RNA (ribonucleic acid). Among nine human panelists, the populations on seven were found to be dominated by *Staphylococci* and *Corynebacteria* by these culture-independent techniques whereas the remaining two carried a population dominated by β-proteobacteria [49.3]. *Propionibacteria* were also found to be present in significant densities in some panelists. A similar result was found in a study on 16s-RNA sequences of pooled samples from five panelists [49.7]. A study on three panelists also indicated a population dominated by *Staphylococci*, with *Corynebacteria*, and *Propionibacteria* as the next most abundant genera [49.8]. Similarly, in a further recent study *Staphylococci*, *Corynebacteria* and *Propionibacteria* were found as forming the typical axilla flora, in this case additionally a high number of sequences clustering with *Streptococci* was found [49.9]. Overall these recent studies using the latest high throughput sequencing technologies confirmed the taxonomic view reached with culture based methods 30 years earlier and added some more details on the interindividual bacterial diversity. However, they have not added further information on the importance of different bacterial populations for odor formation as they only provided an inventory of sequences and putative species but asked no functional questions: Therefore, the *Corynebacteria* remain the prime suspect.

49.3 Specific Odorant Precursors Secreted in the Human Axilla

Given the knowledge on the bacterial populations and their importance in odor formation, for a long time the key missing link was what kind of constituents in the gland secretions are transformed by bacterial ac-

tion into the odorant principles. Significant progress was made in this area in the last decade by the use of liquid chromatography-mass spectrometry (LC-MS) technologies – but next to this analytical tool, the studies successfully isolating odor-precursors had to combine the chemical analysis with targeted degradation (by hydrolysis or bacteriolysis) of the precursors, followed by sensorial analysis.

49.3.1 Precursors for Acids

Two key, structurally closely related, odorant acids were described as typical body odorants in acidic fractions of sweat: 3-methyl-2-hexenoic acid (3M2H) [49.10] and 3-hydroxy-3-methyl-hexanoic acid (HMHA) [49.11]. The quest for a potential precursor for 3M2H started early. In a pioneering study, it was shown that this compound is bound to a water-soluble precursor and can be liberated therefrom both by hydrolysis catalyzed by NaOH or by bacteriolysis using corynebacterial isolates [49.12]. Due to the high protein content in apocrine secretions, it was proposed that 3M2H is likely to be bound to proteins, either by a covalent bond or by noncovalent associations. Following these early studies, evidence for the latter hypothesis was presented showing that there appears to be a noncovalent linkage between apolipoprotein D and 3M2H [49.13]. However, no hypothesis could be presented, how *Corynebacteria* then would dissociate these noncovalent associations to release the odorants. A covalent linkage to the terminal glutamine of apolipoprotein D was more recently proposed [49.14], but the relevance of this observation was questioned, since with this 1 : 1 association between the small acid and the large protein, an extremely high level of apolipoprotein D would be needed to explain the high observed amounts of odorant acids which can be released from axilla samples [49.15].

Using size exclusion chromatography in combination with LC-MS, hydrolysis of sweat fractions and sensorial analysis it was later shown that axilla secretions contain large amounts of glutamine conjugates, in which the acids 3M2H and HMHA are covalently attached to the N_α-atom of glutamine (Fig. 49.1) [49.11]. This observation was confirmed by an independent laboratory quantifying the HMHA-Gln conjugate in a longitudinal study for 3 years on 49 volunteers [49.16]. Male axilla sweat samples were shown to contain on average 794 µg of the HMHA-Gln conjugate, whereas female samples contained 365 µg. Based on all available evidence, these glutamine conjugates appear to be the key precursors for odorant acids, and an alternative proposal for odorant acid formation was recently abandoned [49.15, 17].

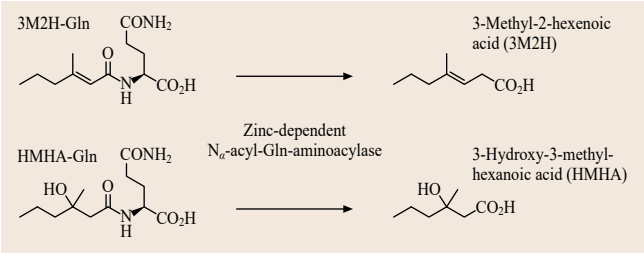

Fig. 49.1 The structure of the precursors for the two key odorant acids and the enzymatic activity in *Corynebacterium Ax20* performing the precursor cleavage

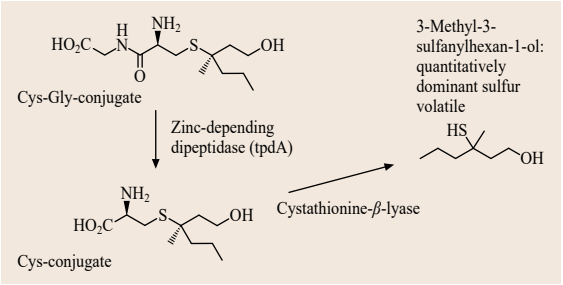

Fig. 49.2 The structure of the key precursor for odorant sulfanylalkanols and the two enzymes isolated from *Corynebacterium Ax20* involved in odorant release

49.3.2 Precursors for Sulfanylalkanols

Next to the acids, sulfanylalkanols were identified as key contributors to body odors by three laboratories in the same year [49.18–20], see for a detailed review in this volume (Chap. 48). Originally we proposed that the sulfanylalkanols are secreted as cysteine conjugates based on the finding that (i) *Corynebacteria* isolated from the axilla can release the odorants from such cysteine conjugates, and that (ii) a cystathionine-β-lyase from a strain of *Corynebacterium* can release the odorants from both the synthetic cysteine conjugates and from authentic sweat. However, it was later shown that the quantitatively dominating precursor is a cysteine-glycine conjugate (Fig. 49.2) [49.21]. This finding was confirmed by our laboratory [49.22] and later in population studies [49.16, 23]. A minor level of the originally proposed cysteine conjugate was then also analytically detected [49.22], although it is not clear whether this compound is directly secreted by the glands or whether it is an intermediate formed on the skin by the bacterial peptidase activity.

49.3.3 Precursors for Steroids

The odorant steroids androstenol and androstenone had been identified as the first human axilla odor-

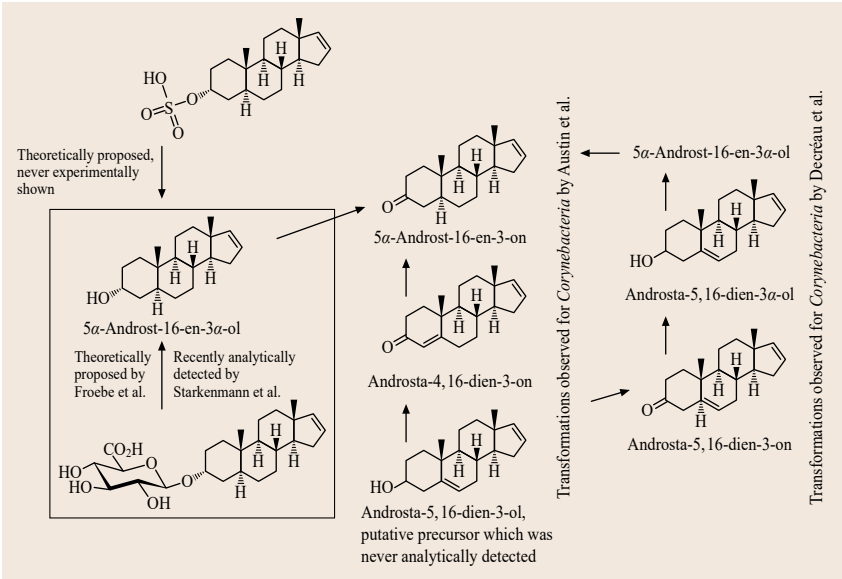

Fig. 49.3 The structure of the proposed precursors for odorant steroids and the associated biotransformation reactions

ants [49.24, 25], following their description as key odorants and pheromones in the pig. However, the analytically detectable levels are extremely low in human sweat and it was recently proposed that their contribution to axilla odor is much less than was originally believed [49.15]. At the same time, there is much less analytical information on secreted precursors for these steroids.

Based on analogy with the soluble, transported forms of other steroids in the human body, it was proposed that androstenol could be secreted as sulfate or glucuronide conjugate [49.26], although such precursors were not analytically detected in sweat when this hypothesis was first presented. Indeed, cleavage of some synthetic steroid sulfates by isolates of *Corynebacteria* was later shown [49.27], but the sulfate conjugates could never be detected in sweat. Only very recently it could be shown, that sweat contains the glucuronide conjugate of androstenol (Chap. 48).

An alternative hypothesis proposed the formation of the odorant steroids from other nonodorant steroids (which are not conjugated to a carrier molecule) [49.28, 29]. Evidence for a number of steroid oxidations and reductions by axilla bacteria was indeed presented [49.29, 30] – but these studies did not detect analytically the potential odorless precursor androsta-5,16-dien-3-ol in the axilla secretions, which then would be transformed to the odorant principles by the proposed mechanisms. These alternative pathways are summarized in Fig. 49.3. They appear currently rather unlikely.

49.4 Malodor Releasing Enzymes in Axilla Bacteria and Corresponding Genes

Although the importance of bacteria and the contribution of specific taxonomic groups to odour formation had long been known, the quest to identify odor-releasing enzymes could only start once the odor-precursors were analytically identified and synthetic samples of these compounds could be prepared. Using such synthetic references, we could identify particular bacterial strains with the ability to cleave the conjugates. A highly odor forming strain, *Corynebacterium* sp. Ax20 [49.11] was selected as model organism. Based on recent sequence analysis of the DNA coding for 16s ribosomal RNA, it is most closely related to *Corynebacterium glaucum* [49.15].

49.4.1 Acid Releasing Enzyme

The first focus was to explain the release of the most dominant odorants, the carboxylic acids. Using classical biochemical tools, a single enzyme from Ax20 involved in cleavage of the glutamine conjugates was purified to homogeneity and then used for amino acid sequence analysis. The corresponding gene could be cloned and expressed in *Escherichia coli* [49.11]. This recombinant enzyme, biochemically speaking it is an N_{α}-acyl-glutamine aminoacylase, did cleave both the synthetic precursors and it released the acids from axilla secretions [49.31], thus verifying its role in odor re-

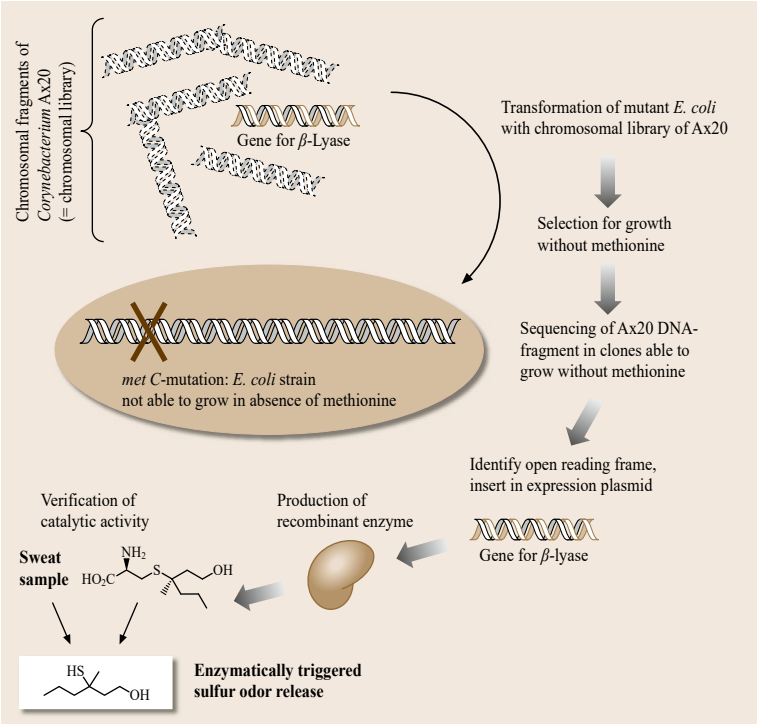

Fig. 49.4 The experimental approach to isolate the β-lyase gene from Ax20 and verification of its involvement in release of odorant sulfanylalkanols from cysteine conjugates

lease (Fig. 49.1). This enzyme was also coined AMRE (axillary malodor releasing enzyme) [49.32]. It has a very high specificity for the glutamine residue in the substrate, not accepting other amino acid residues. Thus it has a high specificity to the glutamine conjugates secreted in the axilla. At the same time, it has a high tolerance to different acyl-side chains. As discussed in the following, there are indeed many different acyl-glutamine conjugates present in the axilla, but no conjugates with other amino acids were found. Thus, the substrate specificity of this enzyme (high specificity for Gln, but not acyl part of the substrate) is perfectly adapted to the available substrates, and presents a fascinating example of coevolution of the skin bacteria with the human host. A direct evolutionary benefit for the bacteria would then be the utilization of the released glutamine as a nutrient source.

49.4.2 Enzymes Releasing Sulfanylalkanols

Based on the assumption that sulfanylalkanols might be linked to cysteine in the secreted form, cysteine conjugates were first synthesized. They are indeed cleaved by the same bacterial isolate Ax20. Thioethers of cysteine are often cleaved by cystathionine-β-lyases, which are encoded in many bacteria by the *metC* gene. The corresponding enzyme could therefore be cloned from Ax20 by a genetic approach instead of the tedious classical biochemical approach of enzyme purification: Chromosomal fragments of Ax20 complementing *E. coli* *metC* mutants were isolated. These fragments contained a common open reading frame, which was expressed in *E. coli* and the recombinant protein was purified. This experimental approach is schematically depicted in Fig. 49.4.

This enzyme was then shown to cleave both synthetic cysteine conjugates and it was able to release sulfanylalkanols from sweat (Fig. 49.4) [49.18]. A phylogenetically related cystathionine-β-lyase, which is also able to release the sulfanylalkanols from cysteine conjugates, was later cloned from *Corynebacterium jeikeium* K411 [49.15], showing that this enzymatic activity is present in several species of *Corynebacteria*. In the latter bacterial isolate, a detailed analysis of the regulatory mechanisms was also made, and a repressor protein was cloned, supressing the expression of the β-lyase gene in the presence of high methionine concentrations [49.33]. This indicates that the primary biological function of this enzyme is still in the biosynthesis of methionine (rather than in the cleavage of cysteine conjugates, which is a secondary function), and that its production is regulated by the bacterial need for methionine. On the other hand, detailed analysis of *Staphylococcus hemolyticus* led to the cloning of a *metC* gene, again coding for a cystathionine-β-lyase. However, this enzyme had an extremely low

activity on the cysteine- and on the Cys-Gly conjugates and it did not release odors from native sweat. It was therefore concluded that this enzyme is not the key enzyme for odor release in this bacterial strain or that the concerted action of several enzymes is needed [49.34].

Upon the report that the key precursor for sulfanylalkanols is as a Cys-Gly conjugate rather than a simple cysteine conjugate, the question arose whether this new compound could be cleaved by the same β-lyase from Ax20, but this was not the case. Indeed, complete enzyme extracts of Ax20 were able to release the odor from the Cys-Gly conjugate, but this ability was lost as soon as the extracts were fractionated, pointing to the involvement of at least two enzymes. Indeed, if all individual fractions of the bacterial lysate were complemented with the recombinant β-lyase, only then a fraction was identified which was able to release the odor from the Cys-Gly conjugate. This indicated that a second enzyme is needed to first release the Gly-residue. This conclusion proved correct and led to the isolation, by classical purification and activity assays, of a novel dipeptidase named *tpdA* which cleaved the glycine from the Cys-Gly conjugate. This enzyme then generates the substrate for the previously identified β-lyases and the sequential action of both enzymes is needed for odor release [49.22]. These findings are summarized in Fig. 49.2.

49.4.3 Formation of Steroids

As regarding the formation and transformation of odorant steroids, so far no enzyme was isolated and characterized in axilla bacteria, which would catalyze the hypothetical routes summarized in Fig. 49.3. Thus, to advance this field it would now be necessary to study the glucuronidase activity in axilla bacteria to verify the cleavage of the recently identified odor precursor. Currently, however, there are no reports in the literature on this highly interesting topic.

49.5 Fingerprints of Released Odors in Human Individuals

There are three key, quantitatively most dominant, odor precursors identified so far, as summarized in Figs. 49.1 and 49.2. These could be identified in all human individuals in the longitudinal study cited earlier [49.16]. However – human individuals have different odors, and only three chemicals cannot account for this interindividual variability. With the availability of the recombinant enzymes, it became possible to look in more detail into the volatiles released from native sweat, and it could be shown that a broad variety of different acids can be released by the same N_α-acyl-glutamine aminoacylase [49.31]. The variability of the pattern of these different acids was then studied in more detail. One key question to ask was whether the odors are inherited and stable in an individual, thus giving each person his/her specific odorprint. This could best be addressed with a twin study. Pairs of genetically identical twins contributed sweat samples, which were then treated with the aminoacylase and the released odor pattern was analyzed with a comprehensive 2-D (two-dimensional) gas chromatographic method. With this approach, an influence of the micro-

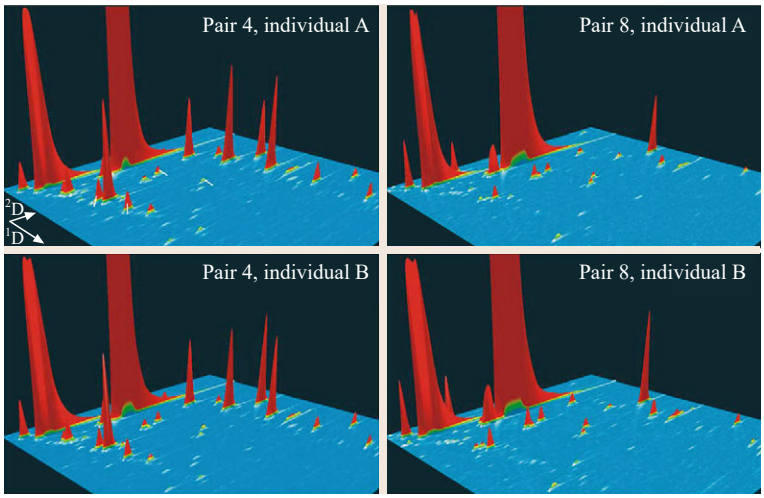

Fig. 49.5 The pattern of carboxylic acids in two pairs of monozygotic twins. The acids were released from sweat samples by the bacterial N_α-acyl-glutamine aminoacylase and analyzed in the form of their methyl esters by comprehensive two-dimensional GC (after [49.35])

bial population could be excluded, as the fingerprints generated from the released pattern of odor precursors were actually evaluated. Indeed it could be shown that monozygotic twins had highly similar patterns of acid precursors, and that these patterns were stable within individuals between different days and between the samples from the left and the right axilla, but there was a high variability between nonrelated individuals (Fig. 49.5). No standardization of the diet was needed in order to observe these stable odorprints, indicating that diet has a minor impact on these typical axilla odors. This study showed for the first time genetically inherited, individual-specific odor patterns in human beings at the analytical level [49.35], although inherited odors in twins were shown before in sensorial studies [49.36]. It is known that apocrine glands, and hence body odor, only form with puberty [49.1], but currently there is no information whether the body odor then changes with age or hormonal changes such as the menopause.

49.6 Effect of the Human Leukocyte Antigen (HLA) on Precursor Release?

In mice, urine odors appear to be influenced by genes in the major histocompatibility locus (MHC). Urine samples from mice with different MHC-genotypes can be discriminated by trained mice [49.37]. This seems to have important implications for mate selection, parent-progeny recognition [49.38] and inbreeding avoidance [49.39]. Later, evidence for human leukocyte antigen (HLA) associated body odors were also reported in humans [49.40] (HLA is the human pendant of the MHC). However, a more recent study did not replicate the same findings [49.41]. Nevertheless, we wondered whether the genetically inherited patterns of odorant acid precursors identified in twins could be associated with an individual's HLA-genotype. Therefore, families with four siblings of identical sex were recruited and tested for both HLA-genotype and pattern of released acids in the sweat. Siblings have a 25% chance of exhibiting the same HLA-genotype, and thus in these larger families many pairs with identical HLA-genotype could be identified, which, given the high diversity in the human HLA region, is rare in the general population. Again, individuals had similar patterns of released acids if sampled on different days, thus verifying the stable patterns of acid precursors. However, whether two siblings shared none, one or two HLA-haplotypes had no significant effect on the similarity in their odor patterns (Fig. 49.6). Thus we could not show a clear effect of the HLA-genotype on patterns of odorant acid secretion [49.42]. On the other hand, these acids were the most likely known candidates for such an HLA-dependent effect for the following reasons:

1. In mice, the HLA-dependent signal could be isolated from urine in the acidic organic fraction [49.43].
2. This fraction contained no MHC-specific acids, but an MHC-dependent pattern of common acids.
3. The acids are the only human odorants previously shown to have a both a high diversity and an individual specific, genetically inherited pattern. Given the negative results in this study, the open question remains, whether other, genetically and HLA-determined odorants can be isolated from human body secretions, or whether potential HLA-associated body odors in humans remain a myth as their chemical nature cannot be resolved by current analytical means.

In a very recent report, a new potential HLA-dependent odorant principle in human body odor was proposed. Based on the earlier findings in mice that synthetic peptides with specific motifs binding to the MHC receptor can trigger a response in mouse olfactory neurons [49.44], it was hypothesized that humans may also perceive nonapeptides, which are known as HLA-ligands. Such synthetic peptides were thus tested both in a behavioral study and in a neuroimaging study. Statistically, significantly different results were obtained

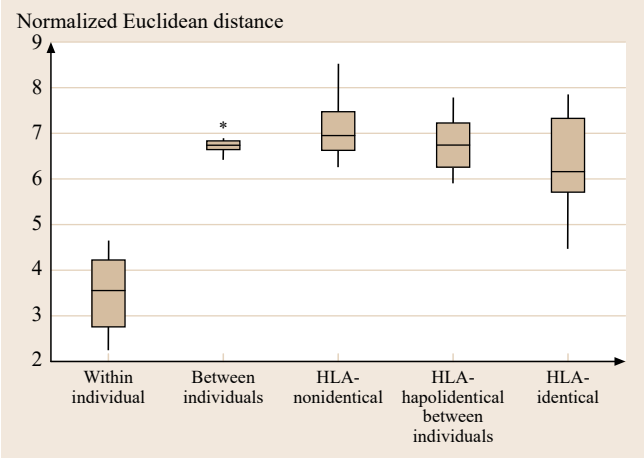

Fig. 49.6 Similarity between odorprints obtained from pairs of unrelated individuals or siblings sharing one or two HLA-haplotypes (after [49.42])

in both approaches depending on the receiver's HLA-genotype, which led to the conclusion that such HLA-binding peptides indeed are the HLA-dependent body odorants in human body secretions [49.45]. However, the study left many questions unanswered, as neither there was analytical verification of the HLA-dependent presence of such peptides in sweat nor evidence for conscious detection by human panellists – all parameters firmly established for the human body odorants described earlier. Finally, the question how nonvolatile nonapeptides may reach the human olfactory epithelium remained unanswered for the time being [49.46].

49.7 Ethnic Effects on Odor Formation and the ABCC11 Polymorphism

There is little analytical evidence whether body odors are affected by ethnic origin. However, there is one exception. A significant fraction of the population in the Far East is known to produce dry and white earwax, as opposed to the yellow and wet earwax dominant in the remaining global population. Individuals with the *dry and white* phenotype were reported not to have the typical axilla odors [49.47, 48]. This phenotype could be linked to a single nucleotide polymorphism (SNP) in the gene coding for the efflux pump protein ABCC11 [49.49]. We thus wondered whether this SNP does equally affect secretion of odor precursors, as it does affect the formation of the yellow earwax phenotype. Panelists with ethnic origin in the Far East were thus typed for their ABCC11 genotype and their axilla secretions were sampled and analyzed with LC-MS for the content of the odor precursors [49.23]. A 100% association between a functional allele of the *ABCC11* gene and the presence of the odorant precursors depicted in Figs. 49.1 and 49.2 could be shown, suggesting that this efflux protein is directly involved in secretion of these precursors in the apocrine glands. Moreover, this strong association between an odorant phenotype and the ability to secrete these odorant precursors gives a very clear indication that the findings from the biochemical and sensorial analysis reviewed earlier are relevant, as the odorant genotype and phenotype are closely linked to the presence of these precursors. In addition, also the amount of a number of steroids is affected by the ABCC11 phenotype. However, these steroids were detected with specific antibodies, and the analytical evidence for the exact structures of these analytes is therefore less strong as compared to results obtained for the odor precursors by mass spectrometry.

It may also be worth to look at the evolutionary context of this SNP. Based on detailed analysis, it was concluded that it is present as a specific extended haplotype in all individuals [49.49], that is, there are other, completely linked SNP's in close proximity of this mutation. This indicates that (i) the mutation is relatively young in human evolution, as no crossing over took place between these linked SNPs and (ii) this mutation occurred only once and all carriers have inherited this same allele from this single mutation event. Given the fact that the allele frequency reached close to 100% in several human populations, one has to speculate that there was a strong positive selection pressure for the dominant recessive, ABCC11-negative phenotype (Fig. 49.7). Whether there was a positive, odor-dependent selection for this nonodorant phenotype in partner choice, or whether the ABCC11-negative phenotype contributed another, positively selected advantage is a challenging question.

49.8 Outlook: Toward More Specific Deodorants

The detailed biochemical studies do have practical implications, as it is now possible to develop specific chemicals targeting any of the three key malodor releasing enzymes described in this chapter. Whereas classical underarm products attempt to reduce secretion of sweat (antiperspirant mode of action) or to reduce the growth of the bacterial flora on the skin (deodorant mode of action), it has now become possible to try a more specific intervention by targeting the enzymes. One class of such molecules was developed in our laboratory – based on the finding that the N_α-acyl-glutamine aminoacylase has a high specificity for Gln, but a high tolerance for the acyl-part of the substrate, we designed conjugates, which upon bacterial action release a fragrant molecule instead of a malodorant molecule. Such alternative substrates were tested in in vitro studies, and shown to reduce malodor release in bacterial incubations by competitive inhibition of the enzyme [49.32]. One substrate was then further taken into a series of clinical tests in deodorant formulations not containing classical deodorant principles. Interestingly, this substrate did reduce malodor formation in vivo, and this effect was most

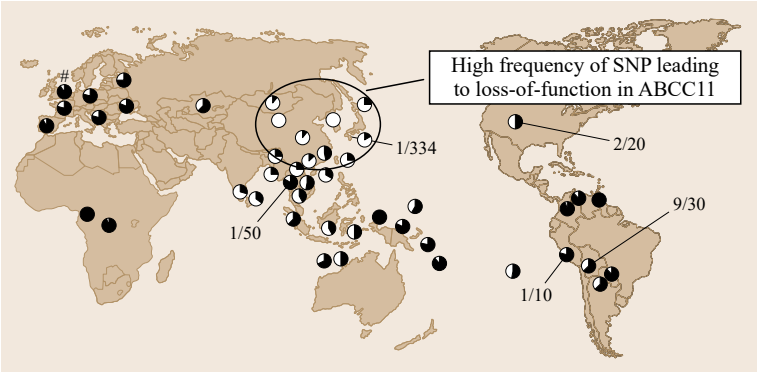

Fig. 49.7 Global frequency of the 538G ⇒ A allele in the *ABCC11* gene in the human population. Gray indicates frequency of the mutated allele. Individuals carrying this loss-of-function mutation on both chromosomes have completely lost the ability to secrete the odor precursors listed in Figs. 49.1 and 49.2, whereas these compounds are detectable in all individuals carrying one or two of the functional G-allele (after [49.49])

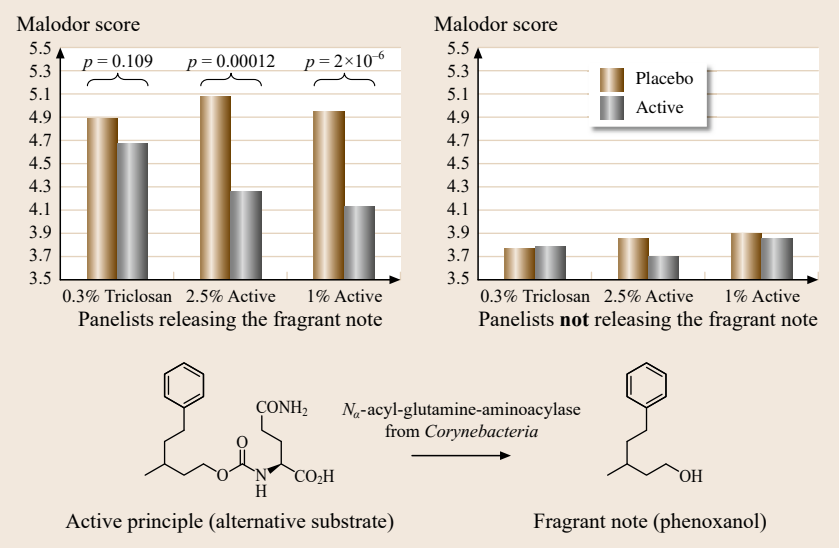

Fig. 49.8 In vivo test of an alternative substrate targeting the N_α-acyl-glutamine aminoacylase (after [49.50])

prominent on individuals with high malodor scores – notably these were also the panellists having high N_α-acyl-glutamine aminoacylase activity as determined by a noticeable release of the fragrant principle from the alternative substrate in vivo [49.50] (Fig. 49.8). Shown in the figure are malodor scores 8 h after application of deodorants containing either the classical principle Triclosan or the new active shown below. Panelist results were separated for those on whom the fragrant note could be perceived by the assessors and those who did not release the fragrant note. The odor level was low in the *negative panelists* putatively not carrying a bacterial population with high aminoacylase activity (right panel). At the same time no further malodor reduction could be observed in these panelists, but a clear effect was noted on individuals with high odor and carrying a bacterial flora able to cleave the substrate (left panel). Next to the ABCC11 study described earlier, this observation further substantiates the relevance of the biochemical routes to odor discovered before: Strength of odor is clearly linked to the presence of bacteria able to cleave Gln conjugates. Thus, both the basic genetic study and the applied deodorant research yielded further in vivo proof of the biochemical work performed earlier.

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50. Individual Variation in Body Odor

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Humans produce numerous volatile compounds from different areas of the body, either as a direct result of metabolic processes or indirectly via metabolism of resident microflora. Body odors vary between individuals, partly due to genetic differences, but odors of the same individual also vary across time due to environmental influences. We discuss how at least part of the genetic influence appears to be related to certain personality characteristics and to sexual orientation. We then review the current state of the art in terms of intraindividual variation, including effects of intrinsic factors, such as hormonal influences on body odor and environmental factors, namely effects of diet and certain diseases. Some of these changes can be perceived by other individuals and might therefore provide social cues of current motivational, nutritional, and health status. Finally, we discuss how specific odor profiles associated with certain infectious diseases and metabolic disorders can be used as a cheap and efficient medical screening tool.

In common with other animals, humans constantly produce a cloud of volatile chemicals which can potentially be perceived by others. The majority of these compounds are direct by-products of body metabolism or products of the metabolism of either commensal or pathogenic microflora. Human body odors are emitted from various areas of the body notably from the mouth, the anogenital region, the scalp, and the axillae. In healthy adults, axillary odor appears to be the most distinctive, due to a relatively high concentration of both eccrine and apocrine glands in this area. Interestingly, most compounds in fresh apocrine sweat are odorless and these are converted to odoriferous molecules by the action of the residential bacterial microflora (Chap. 48). Body odor appears to be individually specific and relatively stable [50.1], perhaps due to genetic influences. This is supported by three lines of evidence:

1. Body odor of monozygotic twins show high resemblance [50.2].
2. Unacquainted individuals can match relatives (e.g., offspring and parents) based solely on body odor [50.3].
3. People show odor preferences associated with the genes of the major histocompatibility complex [50.4].

Further, people can identify others based on their body odor (for details of this kind of evidence, see Chap. 51). Apart from genetic influences, there are also numerous intrinsic and extrinsic factors shaping individual variation in human body odor. Here, we first review two factors contributing to interindividual odor variation, namely personality factors and sexual orientation. We then turn our attention to intrinsic factors of intraindividual variation in body odor, namely hormonal influence (emotion-related fluctuations in body odor are reviewed elsewhere; see Chap. 49), and to environmental factors, such as effects of diet and disease.

50.1 Personality

People tend to spontaneously attribute a range of psychological characteristics to others based simply on their appearance or on *thin slices* of their behavior. At least in some characteristics, such attributions are to some extent accurate; that is, they correlate with the target's personality profile. These attributions have been described as having a *kernel of truth* [50.5, 6]. Although less well-known, body odor could also contribute to such attributions based on first impression, as some personality traits are correlated with social perception of body odor. For example, women in the fertile phase of their menstrual cycle find the axillary odor of relatively dominant men more attractive [50.7], and in a series of studies it was recently shown that strangers can accurately attribute levels of neuroticism and dominance in others based solely on their axillary odor with women showing more accurate judgments compared to men [50.8, 9]. Furthermore, prepubertal children can accurately judge neuroticism [50.10]. The precise mechanism responsible for the association between personality traits and axillary odor quality is not well understood. In the case of dominance, both traits may be underpinned by levels of testosterone. The picture might be more complex in the case of neuroticism, but a potential indication lies in the observation that some emotional states (anxiety) have impact on odor quality and in turn affect other people exposed to such odors [50.11, 12]; as neurotic individuals tend to be more frequently distressed, this might also affect their body odor.

50.2 Sexual Orientation

The effects of one's sexual orientation extend beyond the sex of preferred romantic partners. There is robust evidence that it also influences various psychological (e.g., verbal fluency [50.13]) and morphological characteristics (e.g., second to fourth digit ratio, which is considered a marker of prenatal exposure to testosterone [50.14]), perhaps due to shared biological machinery, such as prenatal exposure to the level of androgens [50.15]. Several studies have consequently tested whether sexual orientation also has an impact on the quality of body odor, although results of these studies are somewhat inconsistent. In one study, odor samples taken from both heterosexual and homosexual men and women were judged for pleasantness by groups of heterosexual and homosexual men and women. There was a complex pattern of significant between group differences, although one relatively consistent pattern emerged: All groups except homosexual men showed lower preference for the odor of homosexual men [50.16]. In contrast, another study, which tested only the preferences of heterosexual women, reported that they found the odor of homosexual men more, not less, appealing than those of heterosexual men [50.17]. We therefore await further research before being able to draw sharp conclusions on this fascinating topic. Furthermore, the underlying mechanism linking sexual orientation and the quality of body odor is currently unknown.

50.3 Hormonal Influences

The endocrine system controls a very wide range of physiological processes and contributes to the motivation systems. Hormonal action can thus also influence body odor quality, either as a by-product of hormonal metabolism or by metabolism of the affected tissue. Furthermore, hormonal action might also target the apocrine glands in order to directly communicate motivational state to other individuals. The main focus of research on endocrine influences on body odor has been on steroid hormones.

In women, for example, there is relatively robust evidence showing that attractiveness of axillary body odor rated by men varies across the menstrual cycle, peaking in the follicular phase when the probability of conception is highest [50.18, 19]. No such changes are observed in women using hormonal contraception, suggesting that this effect is steroid hormone dependent [50.20], presumably as a result of changing amounts or ratios of estrogen and progesterone. One early study also found significantly higher pleasantness of vaginal odor in the follicular phase of the cycle [50.21]. Although the magnitude of these cyclic changes is substantially lower than the differences in odor attractiveness among individual women [50.22], they are nonetheless perceivable and might play a role in coordinating sexual activity. In line with this idea,

men exposed to women's axillary odors collected during the fertile phase of the cycle experience elevated levels of testosterone [50.23, 24] although another study was not able to replicate this effect [50.25]. Similar increases in testosterone and cortisol are invoked by vulvar odor collected in the women's fertile phase [50.26]. In a series of follow-up studies, it was found that exposure to fertile phase axillary odors specifically activates mating-related concepts in men (e.g., generating more sexually tinged words), increases their judgments of women's sexual arousal, and leads to more risky decisions (assessed by a computerized blackjack card game) [50.23]. Furthermore, women seem to be similarly reactive to fertility-related odors as they showed increased testosterone levels after exposure, although this is presumably a consequence of intrasexual competition rather than attraction [50.27].

One might also expect changes in body odor related to pregnancy, based on the specific hormonal profiles which occur during this time. This includes elevated levels of human chorionic gonadotropin during the first trimester and continuously rising levels of progesterone and estrogens during the course of pregnancy. In one study, several specific compounds were detected in axillary and areolar samples taken from pregnant women. Some of these were also found in lactating women after delivery, but not in a control group of nonpregnant women. Two of the identified chemicals, 1-dodecanol and oxybis octane, showed systematic fluctuations during the pregnancy [50.28]. Furthermore, changes in breath volatiles of pregnant women have been found using an electronic nose, although no specific compounds related to pregnancy were identified [50.29]. These analytical results are also supported by subjective ratings, such that men rate axillary odor of women in the second trimester as most pleasant [50.30]. Finally, several studies on attractiveness of human body odor to mosquitoes showed higher bite rates in pregnant women [50.31–33]. Interestingly, the attractiveness of body odors to mosquitoes appears to be affected by levels of short-chain fatty acids, and this might explain higher bite rates observed in pregnant women [50.34].

In contrast, investigations into potential links between the quality of body odor and levels of other hormones present more inconsistent results. In one study, it was found that attractiveness of axillary body odor is positively associated with cortisol levels but not with testosterone [50.35]. Another study, based on a larger sample of both odor donors and raters, showed that males whose odor samples were judged as attractive show higher levels of testosterone but not cortisol [50.36]. Finally, one more study found a negative association with cortisol levels [50.37]. Thus, clearly it is currently difficult to draw any robust conclusions on relationships between these steroid hormones and odor, and further investigations of potentially modulating factors responsible for these inconsistent findings are required.

50.4 Diet

Some authors consider diet as the most salient environmental factor shaping our body odor as humans consume a high variety of aromatic foods [50.38]. Several volatile compounds may subsequently emanate in breath odor. Further, some components of the diet might produce volatile compounds only after being metabolized by the digestive system. As volatile molecules are relatively small, they can pass through the epithelium and be distributed across the body via the blood stream. In this way, they can consequently affect axillary odor or odor of urine and feces. The studies on effect of diet are summarized in Table 50.1.

Evidence from animal studies indicates that diet might be a potent modulator of body odor and that in some species, females can use odor cues to assess the quality of potential mates by the quantity and quality of ingested food. *Pierce* and *Ferkin* [50.49] investigated the effect of food deprivation on odor of female meadow voles (*Microtus pennsylvanicus*). It was shown that the odor of starving animals was less attractive compared to individuals fed *ad libitum*. This effect disappeared 48 h after re-feeding. The crucial factor for nutrition appears to be not only the availability of food, but also its quality, such as the amount of dietary protein. It was found that both male and female meadow voles preferred the odor of opposite-sex individuals on a high-protein diet, and spent the least time investigating the odor of individuals on a low protein diet [50.50]. Similarly, attractiveness of urine odor was positively linked to high quality food in guinea pigs (*Cavia porcellus*) [50.51]. In an analogous manner, red-backed salamander (*Plethodon cinereus*) females assess territory quality by examination of male fecal pellets and prefer pellets from individuals fed on high-quality food [50.52]. Other social interactions might be affected by diet as well. For instance, in spiny mouse (*Acomys cahirinus*) pups, preferences are formed early in life, and they subsequently prefer the odor of females fed on the same diet as their mothers [50.53].

The effect of diet on human body odor was first demonstrated in twin studies. Humans were able to discriminate the hand odors of monozygotic twins on

Table 50.1 Summary of studies on effect of diet on human bodily odors

Authors	Food	Odor source	Odor quality/hedonicity	Volatile compound(s)
Fialová et al. [50.39]	Garlic	Axilla	↑ Attractiveness, pleasantness, ↓ intensity	
Hauser et al. [50.40]	*Amba* (mango, saffron, curry)	Skin, amniotic fluid	Foul, curry	
Hauser et al. [50.40]	*Khilba* (fenugreek)	Skin	Fenugreek	
Hauser et al. [50.40]	*Shug* (cumin, garlic, salt, oil, pepper)	Skin	Cumin	
Havlíček and *Lenochova* [50.41]	Red meat	Axilla	↑ Attractiveness, pleasantness, ↓ intensity	
Korman et al. [50.42]	*Hilbe* (fenugreek)	Skin, urine	Maple syrup	3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolone)
Lefèvre et al. [50.43]	Beer	Skin	↑ Attractiveness to malarial mosquitoes *(Anopheles gambiae)*	
Pelchat et al. [50.44]	Asparagus	Urine	Sulfurous, cooked cabbage	Methanethiol, carbon disulfide, dimethyl disulfide, dimethyl sulfide, dimethyl sulfone, dimethyl trisulfide, S-methyl-2-propenthioate
Suarez et al. [50.45]	Pinto beans, lactulose	Flatus	Rotten eggs, decomposing vegetables, sweet	Hydrogen sulfide, methanethiol, dimethyl sulfide, hydrogen sulfide
Suarez et al. [50.46]	Garlic	Breath	Garlic	Hydrogen sulfide, methanethiol, allyl mercaptan, allyl methyl sulfide, allyl methyl disulfide, allyl disulfide
Tamaki et al. [50.47]	Garlic	Breath	Garlic	Methanethiol, dimethyl sulfide, allylthiol, allyl methyl sulfide, dimethyl disulfide, methyl propyl sulfide, diallyl disulfide, 3-(allylthio) propionic acid
Yalcin et al. [50.48]	Fenugreek	Skin, urine	Maple syrup	3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolone)

a different diet, but their performance was not higher than chance when assessing odor of twins on the same diet [50.54]. This task appears to be too difficult even for trained dogs. They successfully discriminated between the odors of both dizygotic and monozygotic twins on different diets, but not the odors of monozygotic twins on the same diet [50.55].

Perhaps predictably, the main source of bodily odors that is affected by diet is breath odor. Breath malodor could have a profound impact on everyday social interactions [50.56] as numerous volatiles emanate from consumed food due to mastication and digestive processes in both the oral cavity and the stomach. Nevertheless, only some parts of the diet produce specific odor profiles. Garlic odor would be a representative example. It has been demonstrated that the typical garlic odor in breath is more intense after ingestion of raw garlic compared to cooked garlic [50.47]. The characteristic odor consists of distinctive sulfur-containing compounds (allicin, mono-, di- and trisulfides, ajoene, and vinyldithiines). Moreover, this odor lasts for several hours even despite oral hygiene, especially due to the unique derivation of allyl methyl sulfide from the gut. Thus, *garlic breath* initially originates from the mouth and subsequently from the gut [50.46].

Another source of bodily odors originates from digestive processes. Action of bacteria on endogenous sources produces gases within the digestive system [50.57]. These eventually emerge as flatus that consists of both nonodorous compounds, such as oxygen,

Fig. 50.1 An odor sample rating session (courtesy of J. Fialová)

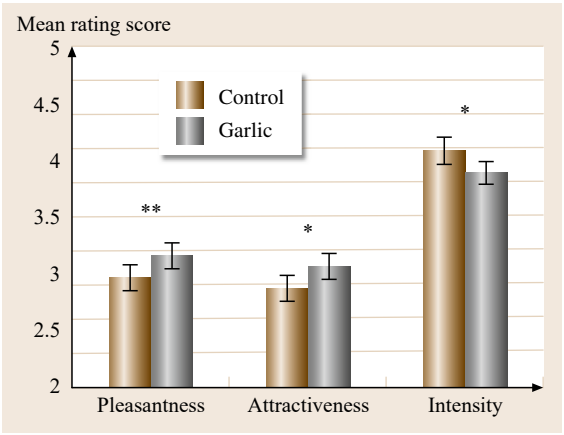

Fig. 50.2 Mean ratings (± SE) of 16 pairs of axillary odors on pleasantness, attractiveness, and intensity in the experimental (*garlic*) condition (*gray bar*) and control (*non-garlic*) condition (*brown bars*) by 40 women. Ratings were on 7-point scale (e.g., 1 – very unpleasant and 7 – very pleasant). *Asterisks* indicate level of significance in paired *t*-tests. $^{*}p < 0.05$ level; $^{**}p < 0.01$ level (after *Fialová* et al. [50.39])

nitrogen, carbon dioxide, hydrogen and methane, and odorous ones containing sulfur, the production of which could be affected by dietary habits [50.45]. Higher levels of sulfur occur in some breads, dried fruits, brassicas and soy flour. A study where flatulence was increased in participants due to consumption of pinto beans and lactulose found that flatus malodor correlates with the concentration of hydrogen sulfide (reminiscent of rotten eggs) and methanethiol (decomposing vegetables) [50.58]. Similarly, urine of people who have recently eaten asparagus has an unusual sulfurous odor similar to cooked cabbage [50.44].

Several case studies show that the mother's diet might also affect the body odor of the newborn baby. For instance, in one case, a newborn baby had body odor and urine that smelled of maple syrup. The baby was therefore suspected of having maple syrup syndrome, but subsequent laboratory tests did not confirm this diagnosis. It was subsequently discovered that, prior to delivery, the mother ate fenugreek-spiced food which was responsible for this distinctive odor [50.42, 48]. The *maple syrup* odor that appears after fenugreek consumption was recently analyzed and several compounds which could be responsible for the distinctive odor were found in human sweat [50.59]. The same odor may also be detected exuding from the mother's skin and may be transmitted to the infant via the mother's breast milk [50.42]. In other cases, the mother consumed *shug* (a dish containing cumin, garlic, salt, oil, and pepper) and her baby consequently smelled of cumin. In a similar way, a newborn baby and its amniotic fluid was found to be yellowish, with an odor reminiscent of curry, after the mother ate *amba*, which consists of mango, saffron, and curry [50.40].

The evidence on the effects of the diet on axillary odor is comparatively limited. One study [50.41] investigated whether consumption of red meat affects human body odor, because people from some predominantly vegetarian cultures say that people who eat meat smell bad because of it. The results of the study showed that the axillary odor of individuals on a nonmeat diet was perceived as more attractive, more pleasant, and less intense than the odor of the same individuals on a diet containing meat (at least one meat dish daily for 2 weeks). These results might appear counterintuitive, as meat consumption is thought to play a significant role in human evolution, and because they might be at odds with studies on effects of high protein diets in rodents (see above). The explanation may be that the amounts of meat consumed in contemporary populations, and in Havlicek and Lenochova's experiment, may be higher than would normally be experienced in traditional or ancestral societies. In this way, body odor changes after consumption of relatively large quantities of meat could in fact resemble a metabolic disorder [50.41]. Another surprising finding resulted from a series of studies that examined the effects of garlic consumption on axillary odor. Samples of body odor from the same individuals were obtained in both an experimental

(high garlic consumption) and control condition. Axillary odor of the participants after ingesting garlic was perceived as more attractive, more pleasant, and less intense (Fig. 50.1). In contrast to the effects of garlic on breath odor, the positive influence on axillary odor might be explained by longer term health benefits of garlic consumption, including antioxidant action and antibacterial activity [50.39] (Fig. 50.2).

Interestingly, dietary effects might also affect attractiveness of human body odor to blood sucking insects. *Lefèvre* et al. [50.43] found that beer consumption increases human odor attractiveness to malarial mosquitoes (*Anopheles gambiae*). Exposure to the body odor of participants who consumed beer caused an increase in mosquito activation (take-off and up-wind flight) and orientation (flying toward volunteers' odors).

50.5 Diseases and Disorders

The profile of volatile compounds found in human body odor can be affected by health and disease. This was recognized by ancient medical authorities, such as Hippocrates, Galen, and Ibn Sina, who advocated the use of olfaction in medical diagnostics. Recent technological advances and availability of highly sensitive techniques like gas chromatography-mass spectrometry (GC-MS) makes volatile compounds an increasingly significant part of early disease diagnostics. Generally, such changes in body odor might be either a result of altered metabolism and/or more direct effects of infectious agents. For this reason, metabolic disorders and infectious diseases are reviewed separately in the following paragraphs, where we present some representative examples of the effects of disease on body odor (Table 50.2).

50.5.1 Metabolic Disorders

The main cause of metabolic disorders is deficiency in enzymes or transport systems. Such deficiencies frequently lead to the accumulation of specific metabolites and in some disorders, to its further conversion to other compounds. If these are volatile, the metabolite or its products may lead to a characteristic odor profile in affected individuals. These metabolic disorders are often a consequence of simple Mendelian inheritance.

Isovaleric Acidemia

The disorder is caused by a deficiency of the isovaleryl-CoA dehydrogenase, which is involved in leucine metabolism. Due to the disorder, isovaleric acid accumulates in the tissues and leads to serious ketoacidosis which may subsequently result in coma [50.83]. Patients with isovaleric acidemia produce high levels of isovaleric acid in body fluids and urine, which is characterized by the distinctive odor of sweaty feet [50.64].

Maple Syrup Urine Disease

This is an autosomal recessive inherited disorder caused by deficiency in the enzyme 2-oxo acids dehydrogenase complex, which results in the accumulation of branched-chain amino acids, such as leucine in tissues and body fluids [50.84]. If not recognized early after birth and treated by a branched-chain amino-acid-free diet, the disorder can result in mental retardation. Body odor and urine odor of affected individuals smell relatively pleasant, resembling maple syrup. The compound responsible for the odor appears to be sotolone (3-hydroxy-4,5-dimethyl-2(5H)-furanone) [50.63].

Phenylketonuria

This disorder is caused by a recessive mutation in a gene coding for phenylalanine hydroxylase. The enzyme is expressed in liver tissue where it converts the amino acid phenylalanine into tyrosine. Due to the phenylalanine hydroxylase deficiency, the phenylalanine is converted to phenylpyruvic acid and phenylacetate which are excreted in sweat and urine. The phenylacetate gives affected individuals a musty odor, resembling sweaty lockers [50.61].

Trimethylaminuria

The disorder is characterized by a deficiency of the flavin containing monooxygenase 3 which converts trimethylamine to trimethylamine *N*-oxide. Trimethylamine is produced by gut bacteria from choline rich food, such as eggs or legumes. In unaffected individuals, most of the odorous trimethylamine is converted in hepatic tissue to odorless trimethylamine *N*-oxide. However, in people suffering from trimethylaminuria, trimethylamine emanates from their breath, sweat, and urine, with an odor which resembles that of decaying fish [50.60].

Diabetes

An example of a metabolic disorder with an etiology involving multigenetic as well as environmental factors (e.g., dietary habits) is diabetes. Type I diabetes is characterized by insufficient secretion of insulin, and the lack of insulin leads to an increase in the level of ketones including acetone in the blood. As a consequence, people suffering from diabetes with elevated

Table 50.2 Summary of studies on disease-related body odors

Authors	Disease/Disorder (Pathogenic agent)	Pathology/Symptoms	Odor source	Odor quality	Volatile compound(s)
Metabolic disorders					
Chalmers et al. [50.60]	Trimethylaminuria	↓ Flavin monooxygenase 3	Breath, sweat, urine	Decaying fish	Trimethylamine
Cone [50.61]	Phenylketonuria	↓ Phenylalanine hydroxylase	Sweat, urine	Musty, wolf-like, barny, sweaty locker-room towels	Phenylpyruvic acid
Laffel [50.62]	Diabetes	↓ Insulin secretion	Breath	Sweet	Ketones (acetone)
Podebrad et al. [50.63]	Maple syrup urine disease	↓ 2-oxo acids dehydrogenase	Skin, urine	Relatively pleasant, maple syrup like	3-Hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone)
Tanaka et al. [50.64]	Isovaleric acidemia	↓ Isovaleryl-CoA dehydrogenase	Urine	Sweaty feet	↑ Isovaleric acid and its derivatives
Infectious diseases					
Anderson et al. [50.65] *Landers* et al. [50.66] *Wolrath* et al. [50.67]	Bacterial vaginosis (Gram-negative bacteria *Gardnerella vaginalis, Mycoplasma)*	Abnormal vaginal discharge (color, consistency, amount), itching, burning, dysuria	Vagina	Cheesy, fishy, foul	Trimethylamine
Finlay et al. [50.68]	Skin ulcers *(Bacteroides, Propionibacterium)*	Cutaneous lesions	Skin	Offensive, foul	
Garner et al. [50.69]	Cholera *(Vibrio cholera)*	Watery diarrhoea, vomiting, dehydration	Feces	Sweetish	Dimethyl disulfide, *p*-menth-1-en-8-ol
Honig et al. [50.70]	Scarlet fever *(Streptococcus pyogenes)*	Red-colored rash on the body, sore throat and fever	Skin, breath	Foul	
Liddell [50.71]	Typhoid fever *(Salmonella typhi)*	High fever, drenching sweat, gastroenteritis	Skin	Baked brown bread	

Table 50.2 (continued)

Authors	Disease/Disorder (Pathogenic agent)	Pathology/Symptoms	Odor source	Odor quality	Volatile compound(s)
Phillips et al. [50.72], *Syhre* and *Chambers* [50.73] *Syhre* et al. [50.74]	Tuberculosis *(Mycobacterium tuberculosis)*	Cough, chess pain, weight loss, fever, night sweats	Breath	Foul	Methyl nicotinate, methyl phenylacetate, methyl *p*-anisate, *o*-phenylanisole, cyclohexane, benzene derivatives, decane, heptane
Shirasu and *Touhara* [50.75]	Diphtheria *(Corynebacterium diphtheriae)*	Sore throat, fever, difficulty breathing	Breath	Sweetish, putrid	
Tumors					
Jobu et al. [50.76]	Bladder		Urine		Ethylbenzene, nonanoyl chloride, dodecanal, (*Z*)-2-nonenal, 5-dimethyl-3(2*H*)-isoxazolone
Phillips et al. [50.77]	Lung		Breath		Alkanes, alkane and benzene derivatives, isoprene, benzene
Phillips et al. [50.78]	Breast		Breath		2-propanol, 2,3-dihydro-1-phenyl-4(1*H*)-quinazolinone, 1-phenyl-ethanone, heptanal, isopropyl myristate
Psychiatric disorders					
DiNatale et al. [50.79] *Phillips* et al. [50.80] *Phillips* et al. [50.81], *Smith* et al. [50.82]	Schizophrenia	Hallucinations, delusions, cognitive deficit	Breath	Peculiar, unpleasant	*Trans*-3-methyl-2-hexenoic acid, carbon disulfide, pentane

ketones produce acetone from their breath, which gives off a characteristic sweet smell [50.62].

50.5.2 Infectious Diseases

The pathogenic activity of many infectious agents also produces various volatile compounds which are emitted from skin, breath, sweat, vaginal fluid, urine, and feces. In contrast to odor-producing metabolic disorders, which are frequently characterized by specific volatile molecules with distinctive odor as we have just described, effects on odor of patients with infectious diseases are more complex and therefore more challenging to characterise. This can be attributed to three main reasons:

1. Bacteria of one strain/species may metabolize different substrates producing a complex mixture of volatiles.
2. Different bacteria overlap in the specific volatiles they produce.
3. Some diseases are frequently characterized by multiple infections which may lead to less characteristic odor.

Several infections of the digestive system are characterized by distinctive fecal odor. This involves infection by *Vibrio cholerae* which causes acute watery diarrhoea with a distinctive sweetish odor. The volatile compounds responsible for the odor were identified as p-meth-1-en-8-ol and dimethyl disulfide [50.69].

Infections of the respiratory system frequently affect breath odor. For instance, people suffering from lung tuberculosis, caused by infection with *Mycobacterium tuberculosis*, are reported to have foul breath odor. A specific mixture of volatile compounds was reported from the breath of infected patients, with a similar volatile profile found in in vitro cultures [50.73]. The biomarkers of tuberculosis infection were proposed to be nicotinic acid, cyclohexane and some benzene derivatives [50.72, 74]. Similarly, individuals infected with *Corynebacterium diphtheriae* are characterized by sweetish and putrid breath odor, resulting from effects of the diphtheria-causing bacteria on the upper respiratory system, generating other symptoms including sore throat and swollen tonsils [50.75].

The vagina is a major source of body odor in adult women. It is rich in residential microflora which play a part in odor production. Changes in vaginal odor might reflect infection by pathological agents and it is frequently used by gynecologists in differential diagnostics [50.65]. For instance, bacterial vaginosis is frequently accompanied by a cheesy or fishy odor which is caused by the production of highly odorous trimethylamine [50.67]. Women diagnosed for an infection by the protist *Trichomonas vaginalis* also frequently complain about malodor [50.66].

Perhaps most common are changes in skin odor caused by infections. These may derive from infection in other parts of the body, such as infection of the intestinal tract by *Salmonella typhi*, the agent of typhoid fever. People suffering from typhoid fever are said to smell like baked bread [50.71]. Infections directly affecting the skin include scarlet fever caused by *Streptococcus pyogenes*. The disease manifests in the form of a rash, strawberry-colored tongue, and fever, but patients also emit a distinctive foul odor from their skin and breath [50.70]. An offensive smell is also associated with anaerobic infections (e.g., by *Bacteroides*, *Propionibacterium*) which cause skin ulcers. Patients often complain about the strong smell which can be significantly reduced by cutaneous application of metranidazol [50.68].

50.5.3 Tumors

Oncological disorders are characterized by abnormal cell growth, mostly caused by mutations in genes (or epigenetic factors) controlling for cellular growth and division. However, neoplasia might be caused by various genes and further development depends on affected tissue. Nevertheless, affected cells might show specific metabolic changes, partly attributable to oxidative stress, and production of distinctive patterns of volatile molecules. Recently, there has been increased interest in the analysis of various substances in patients with different carcinomas. Air exhaled by individuals with lung cancer form a specific pattern of volatile molecules including alkanes, alkane derivatives, and benzene derivatives [50.77]. Similarly, people diagnosed with breast cancer emanate a specific profile of volatiles in their breath. Five biomarkers for breast cancer have been detected including 2-propanol, heptanal, and isopropyl myristate [50.78]. In addition, the urine of people suffering from bladder cancer and prostate cancer has been analyzed. Volatile metabolites reported to be related to bladder cancer include dodecanal, 2-nonenal, and ethylbenzene [50.76]. The specific odor profile associated with several carcinomas has been confirmed by studies using dogs as cancer detectors. Dogs can be trained to differentiate between breath or urine odor samples taken from people suffering from lung, bladder, and prostate cancer [50.85].

50.5.4 Psychiatric Disorders

For a long time, it has been noted by psychiatric hospital personnel that certain psychiatric conditions can be as-

sociated with a peculiar odor. Schizophrenia, in particular, has attracted most attention. Early studies claimed to identify *trans*-3-methyl-2-hexenoic acid as a reliable marker of schizophrenia-associated odor [50.82]. These results were subsequently questioned [50.86], but a further study found that schizophrenic patients may indeed show elevated levels of this compound [50.79]. More recently, analysis of breath volatiles indicates that compounds like carbon disulfide, pentane, and several other volatiles might be associated with schizophrenia [50.80, 81]. Interestingly, other patients treated with neuroleptics did not share the same pattern of volatiles. This suggests that compounds associated with schizophrenia are not a by-product of the medical treatment, although further studies are needed.

There is accumulating evidence showing that some affective states, such as anxiety, influence axillary body odor (for review see [50.11]). One may therefore speculate whether some affective disorders (e.g., major depression) are also associated with changes in the body odor. To our knowledge, there has not yet been a systematic investigation on this subject.

50.6 Conclusion

Seen from various perspectives, human body odor is a highly complex biological system. First, it consists of several sources, such as the axillae, skin, mouth, feet, anogenital region, and the scalp. Each of these sources is characterized by sets of dozens or even hundreds of different volatile compounds. Second, most of the volatile compounds are not directly produced by the human body, but mainly result either from residential or pathogenic bacterial metabolic activity. Third, each human is characterized by an individual odor profile, which is partly due to the genetic influences. This profile is relatively stable across the life span and contributes to individual olfactory identity and may affect social interactions. On the other hand, individual body odor can also be altered by various intrinsic and extrinsic factors.

The main aim of this chapter was to review selected factors contributing to the inter- and intraindividual variation in body odor. We first focused on differences in body odor associated with between-individual differences in personality and sexual orientation (other sources of variation include factors, such as genotype at the major histocompatibility complex (Chap. 49). We then described within-individual changes in body odor due to hormonal influences, in which odor seems to be intimately associated with hormonal fluctuations, although it must be said that most research has focused on steroid hormones, such as estrogens or testosterone. It is noteworthy that other humans can perceive hormone-related changes in body odor and that odor might therefore provide important social cues, perhaps especially those relevant to reproduction, such as actual or potential fertility. Nevertheless, most of the chemicals responsible for hormone-related effects are currently not identified and await further investigation.

One of the major influences on body odor quality is considered to be diet, which contains numerous aromatic chemicals of mainly plant origin. As expected, various volatiles consumed in the diet affect breath and fecal odor. However, some compounds might also be emitted from the skin surface, or can influence body odor indirectly via several possible mechanisms, including oxidative metabolism, nutritional status, and antibacterial action. The effect of diet might also show an idiosyncratic pattern as a result of the interaction between digested food and individual genetic make-up. Unfortunately, these interactions are currently poorly understood.

Finally, various disorders and diseases are characterized by specific odors which are often used in clinical diagnostics or may be at least increasingly utilized in the future as a diagnostic mean. This is pronounced in the case of inherited metabolic disorders which often results in the production of unusual volatiles or their metabolites. Several carcinomas, such as lung or bladder cancer, are also known to be associated with changes in produced volatiles, and these can be used in early screening. More complex odor profiles are associated with some infectious diseases. The potential for using these changes in screening looks likely to increase in the near future.

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51. Processing of Human Body Odors

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Human chemosensory signals are able to transmit a wide range of social information to conspecifics. Resulting from the interaction of several genetic and physiological processes (e.g., metabolic, immune, nervous), each individual produces a unique odor signature. The central processing of such chemosignals by conspecifics modifies physiological, behavioral, and psychological responses. To illuminate the importance of this mode of communication, we describe how humans produce, decode, and respond to warning chemosignals. Behavioral evidence highlighting the cognitive and emotional consequences of body odor communication will be discussed. Special attention will be devoted to the current understanding of human body odor neural processing. After an overview on the topic, we discuss the role that social chemosignals may have in our everyday life in health and disease.

Social communication is a central aspect in the life of animals and human beings alike. Despite the considerable research conducted on the topic of how social information is communicated among humans [51.1], the multisensory character of such communication has often been misrepresented. In humans, most of the literature is characterized by evidence originating from the visual and auditory modalities, uncovering how facial expressions or body postures as well as linguistic features (e.g., prosody) affect social communication [51.2]. Although olfaction is widely used across species for social communication [51.3], little attention has been given to chemosensory communication in humans. This is even more surprising in light of the intrinsic and unique advantages of this form of communication. For instance, chemosignals can easily escape

the restrictions imposed by physical and time barriers where chemical molecules are able to freely disperse in air or water, and thus can be transported over long distances and remain a signal for several days. According to the physical features of the molecules, in particular volatility, chemical communication can outlast the presence of the sender (low volatile molecules) or quickly dissolve and facilitate the transmission of messages in a rapid time scale (highly volatile molecules) [51.4]. Among the far senses, olfaction remains functional when vision and audition are unavailable (e.g., in the dark, in noisy environments) and even when multiple chemosensory stimulations are simultaneously present. As an example, despite the efforts of western cultures to mask body odors with fragrances [51.5], chemosensory communication is still possible [51.6].

Aside from this ability of chemosignals to travel in space and time, and to persist within sensory overloaded environments, chemosensory communication is rather effortless for senders, as suggested by the low amount of energy required to produce and release a chemosignal [51.3]. The energy investment necessary during decoding is also limited, as suggested by the fact that receivers often elaborate the message outside of their conscious awareness [51.7–9].

This series of advantages is further strengthened by the specificity of the communication. Chemosignals transmit detailed social information related to both stable and transient states. Chemosignal communication successfully conveys information regarding personal identity [51.10–14], kins [51.15–17], partners [51.11, 12, 18], relatives [51.15, 17, 19, 20] and friends [51.15]. Furthermore, age [51.21], gender [51.21, 22], and personal predispositions [51.23] can be gleaned via human chemosignals. In addition, humans transmit information of transient states such as health status [51.24], sexual availability [51.25] and emotions [51.26–39] through chemosignals. This cumulative evidence suggests that the olfactory modality is a reliable medium through which social communication can occur among humans.

In the following sections, we will first briefly present the rationale for the terminology that we will be using throughout the chapter. Second, we will discuss the often mentioned, though questionable, concept of humans as microsmatic individuals, trying to review the basis for this belief, and the consequences for research and interpretation of data. Third, we will review how human chemosignals are produced and which are the experimental methods used during collection. Fourth, we will discuss the effects of chemosignal communication in recipients with special attention to central neural processing. Because such neural underpinnings are still incomplete and/or unaccounted for, we will advance speculative, yet fact-based, arguments in the hope of stimulating future discussion and research. Fifth, among all types of olfactory-mediated information, we will focus on the transmission of chemosignals promoting harm avoidance, for the critical survival benefits they serve. Please note that when using the word *signal* throughout the text we do not differentiate whether the signal is beneficial to the sender or not. Finally, challenges in the field as well as outstanding questions that warrant further investigations will be emphasized.

51.1 The Microsmatic Fallacy

Over the years, among both laymen and scientists, the concept of humans being microsmatic animals has wrongfully taken ground (Chap. 32). The term *microsmatic*, used in reference to primates and then extended to humans, traces back to *Turner* [51.40] who described animal species with differential olfactory skills ranging from acute in macrosmatic animals, such as dogs, to those without an olfactory system, i. e., anosmatic animals such as dolphins. Many authors have then characterized primates and humans as microsmatic in light of the concomitant increased emphasis on their use of vision [51.41–43] and based on morphological aspects. Often highlighted is the fact that the relative size of the olfactory epithelium and the olfactory bulb is reduced in primates as compared to other species [51.44, 45]. A direct comparison with vision has been gathered by the investigation of olfactory skills in nocturnal primates, who – unable to rely on visual cues – show a greater portion of peripheral and central structures dedicated to the main olfactory system as compared to diurnal primates [51.46]. Such morphological approach, coupled with direct comparisons between vision and olfaction, has rendered the erroneous conclusion that animals primarily relying on vision exhibit a poor sense of smell. This notion has been extended to humans by Pierre Paul Broca, best known for his discovery of the speech processing area subsequently named after him. On the basis of the available measures of the relative sizes of the olfactory system and estimates of the centrality of olfaction in daily life, *Broca* and *Pozzi* [51.47] suggested that humans (as other mammals) were microsmatic. Data collected using more accurate techniques in the subsequent years deemed this classification system rather obsolete. *Keverne* [51.48–50] challenged the

concept that microsmia can be explained on the basis of a reduced number of olfactory receptors alone. Each receptor is part of a combinatorial code that can respond to different odors and even a limited number of receptors can therefore form many distinct patterns [51.51]. With respect to genetics, the biggest part of the human genome is dedicated to olfaction, with its approximately 400 olfactory receptors [51.52]. Besides functional genes, modern techniques have revealed that the number of pseudo-olfactory genes is higher in humans as compared to other species, macrosmatic included [51.53–55]. This notion, often used as an argument for human microsmia, does not, however, take into account the new discoveries that these *non-coding* regions have an essential role in gene regulation and ribonucleic acid (RNA) transcription [51.56]. The jury is still out on whether having more pseudogenes is advantageous or not.

Additionally, direct correlations between olfactory structures' morphology (e.g., anatomical size), the percentage of expressed olfactory genes, and olfactory performance cannot be found in either humans or other species investigated [51.57–61]. Direct behavioral testing of the human sense of smell also repeatedly contradicts the microsmia concept where humans have been demonstrated to possess a superbly keen sense of smell [51.62, 63], as confirmed by studies assessing detection thresholds and discrimination performance [51.58–60]. For example, ethyl mercaptan (or ethanethiol), an additive used to make odorless gases (such as propane) perceivable, can be detected by untrained humans at concentrations less than 1 ppb (part per billion) and perhaps as low as 0.2 ppb [51.64]. This ability was directly translated to a real-life example by *Yeshurun* and *Sobel* [51.65]: Humans can discriminate between two olympic-size swimming pools, of which only one contains three drops of ethyl mercaptan. Provided that the chemosensory message is of sufficient relevance, it appears that humans are able to detect very subtle olfactory cues [51.57].

A final testament towards the notion that odors indeed are important to us is the fact that a large portion of western countries' commercial interests revolves around the cosmetic, fragrance, and food industries. Capitalizing on the emotional advantages of odors to evoke memories and emotional transfer (Chap. 39), the fragrance industry has in 2015 a projected annual global sales revenue of approximately 29 billions US dollars [51.66], almost equivalent to the gross domestic product (GDP) of Paraguay in 2014 at USD market prices.

Altogether, these facts indicate that humans have a sense of smell that cannot be considered functionally microsmatic. Indeed, *Zelano* and *Sobel* [51.63] have even suggested that humans constitute an ideal model to study the olfactory system, providing access to introspective information that would be otherwise inaccessible in animals.

In the next sections we will provide evidence that human behavior – and in particular social behavior – cannot be fully understood without having a good knowledge of olfactory functioning.

51.2 Human Chemosignals

Chemosignals in social communication often comprise complex molecular mixtures, some of which are volatile and produce an odor [51.3]. These chemicals are interpreted as a signal actively conveying information from a sender and potentially influencing the behavior of receivers, rather than a cue, a passive biological trait that provides an observer with information [51.67]. In light of the still unresolved debate on whether human chemosignals transmitting social information should enter the domain of pheromones [51.3, 68], we will use the terms chemosensory signals, social chemosignals or body odors interchangeably in the present chapter (Chap. 52). Such signals are produced by the human body, making an individual the sender of a message. They are made of chemicals, part of which are characterized by odorous substances and contain socially relevant information. These chemical messages can be transmitted to a human receiver, who decodes the message and uses that information to adjust his/her responses in the environment.

51.3 How do Human Senders Produce Chemosignals?

Several systems across the human body can produce volatile odorous chemosignals with communicative potential. Here, we briefly present known chemical pathways that have been suggested to be involved in communicating social information in humans. For a more detailed overview of the chemical composition and production of human body odors, please see Chap. 49.

51.3.1 The Axillary Glandular System

The majority of studies on human body odors to date have been conducted using axillary secretions with a few noticeable exceptions [51.69–75]. Besides the increase in experimental feasibility, the biological reason for such over-representation is that the glandular system contained in the axillary area contributes consistent secretions.

Most of the social communication mentioned in the literature is thought to derive from the activity of apocrine glands, found in the ano-genital and underarm areas or its specialized variations, among which we can count mammary glands (producing milk), ciliary glands in the eyelids (Moll's gland, responsible for lacrimal secretions), and ceruminous glands (which produce ear wax) [51.76]. The number of apocrine glands changes in relationship with the specific area of the body in which they are retrieved. However, the number of glands seems to be unrelated to the ability to produce signals with socially relevant information. The areas of the cutaneous gland system, in which the number of apocrine glands are most prolific, are the axillae [51.76]. Besides apocrine glands, the axillary skin contains eccrine, apoeccrine, and sebaceous glands. In light of this diversity, the glandular system of the axillary region constitutes a specific habitat, differing from most other body parts, because it harbors hair follicles with sebaceous glands and a high density of sweat glands [51.77].

Apocrine glands, inactive before puberty, increase in size under hormonal influence and begin functioning [51.78]. Their secretions are characterized by milky odorless solutions, consisting of electrolytes, steroids, proteins, vitamins, and a variety of lipid compounds [51.71, 73–75]. The activity of apocrine sweat glands is pronounced during eustressing and distressing situations and reduced during emotionally neutral physical exercise [51.79].

Eccrine glands are located throughout the body, with only a few exceptions [51.76]. In the armpit areas, they coexist with apocrine glands. The clear secretion produced by eccrine glands is thinner as compared to that of apocrine glands, mainly consisting of water and electrolytes, derived from blood plasma, and sodium chloride [51.76]. The amount of sweat depends on the number of functional glands and the size of the surface opening. Neural and hormonal mechanisms are responsible for the total volume of secretions. When all of the eccrine sweat glands are working at maximum capacity, the rate of perspiration for a human being may exceed 3 l/h [51.80].

Apoeccrine glands, comprising up to 50% of all axillary glands, share features of both apocrine and eccrine glands, as the name suggests. They produce watery secretions, in higher quantity as compared to both apocrine and eccrine glands, thus playing a primary role in axillary sweating [51.81].

Sebaceous glands are responsible for the discharge of an oily, waxy substance called sebum, mainly involved in the waterproofing and lubrication of the human skin [51.82].

Altogether, these glands contribute with their activity to the production of water-based secretions, involved in thermoregulation processes and skin protection [51.77, 83]. Such secretions are initially near odorless and the characteristic sweat odor is the product of the incubation with the bacteria residing in the armpit area [51.84–88].

51.3.2 The Axillary Microbiome

The moist environment created by the glandular secretions in the axillary areas represents a moderately diverse ecological niche hosting specifically adapted organisms establishing a distinct microbial profile [51.89]. The presence of hair follicles and sebum creates a rather occluded environment in which nutrients are readily available at a temperature that facilitates bacterial colonization [51.85]. The dominant resident flora is composed of Corynebacterium, Staphylococcus, Streptococcus and b-Proteobacteria [51.85, 89–93]. Combined, this flora is responsible for the microbial biotransformation of the nutrients secreted in the human axilla [51.94, 95]. Specifically, the axillary Corynebacteria and cocci (e.g., *Staphylococcus epidermidis*) are largely responsible for the production of odorous substances, such as androgen steroids [51.96] and aliphatic acids (e.g., isovaleric acid [51.97]). Furthermore, they contribute to the conversion of weakly odorous steroids (androstadienone) into more intensely smelling androstenes with urineous and musky notes (androstenone and androstenol, respectively [51.98, 99]).

Even though it is well established that the microbial activity is responsible for body-odor formation, the active metabolic processes are still unknown [51.100]. Obtaining axillary meta-transcriptomics is necessary to identify how endogenous characteristics (e.g., sex, age, handedness, ethnicity, and individual host factors) in combination with exogenous features (use of cosmetics, detergents, etc.) contribute to the definition of individual chemosignals, opening up a better understanding of body odors in a personalized manner.

51.3.3 What is in a Chemosignal?

The combination of a personalized axillary microbiome [51.100] and both exogenous and endogenous

factors of the donor (or sender of the chemical message) interacts to form a unique production that constitutes the individuals body odor; an end product that lies behind the notion that each individual possess an *odor print* [51.22, 101] (Chap. 50). (The term *odor print* should not be confused with the newly coined term *olfactory fingerprint* [51.102]; the former relates to a biomarker for identification of an individual and the latter is related to the characterization of an individual's olfactory perception.)

The high chemical variability needed to allow such a large degree of individualization does not necessarily rely on different chemical compounds to form this signature. Rather, it seems like a greater dependence is put on a differential quantitative composition of similar compounds, thus creating a complex code consisting of both chemical components and magnitudes of each individual component present [51.103]. Four classes of chemical compounds have been consistently associated with the characteristic sweaty odor of axillary secretions [51.104]: unsaturated or hydroxylated branched fatty acids, thio-alcohols, short chain fatty acids, and volatile steroids. For a more specific overview of the chemical composition, please refer to Chap. 49.

Additional variability in the production of axillary body odor is associated with sexual dimorphism, which strictly interacts with genetic makeup, the endocrine system, the immune system, microflora peculiarities, and diet. Men and women show dynamic structural and functional differences at the level of the glandular system. As alluded to above, during puberty, apocrine glands grow and mature [51.105], eccrine glands' production increases [51.106–108] and the axillary microbiome augments and differentiates across genders [51.109–111]. This results in substantial differences in the olfactory profiles of axillary sweat in men and women, such as different concentrations of fatty acids (e.g., 3M2H), isomers (e.g., Z-isomer), thiol and acid precursors [51.86, 95], as well as androgen steroids in male odor samples as compared to women [51.99, 112]. Furthermore, differences in the genetic makeup alter the olfactory characteristics of the body odor. Here, we bring forth the examples of the impact of a gene (ABCC11) and of a gene cluster: The major histocompatibility complex (MHC), or in humans, the human leukocyte antigen (HLA) system. The ABCC11 gene, in its homozygous variant, is expressed by the apocrine glands and produces a very mild body odor [51.113] as an effect of a reduced production of odoriferous molecules [51.114]. MHC/HLA are the main determinants of immunological individuality and may contribute to the uniqueness of each *odor print* [51.115, 116]. For instance, as well demonstrated by transplant studies, different polymorphisms of the MHC/HLA between donor and receiver increase organ rejection rates. This rate is reduced when the similarity of the MHC/HLA is high, as among relatives [51.117].

Odor variability is also influenced by ecological factors, such as the health status [51.24, 118] and diet features [51.119]. Indirect evidence of a change in the composition of body odor is gathered from the analysis of odor preferences in the recipients. Activating the innate immune system in healthy individuals via injection of lipopolysaccharide results in more aversive body odors as compared to controls within a few hours [51.24]. Analogously, red meat consumption seems to have a negative impact on perceived body odor hedonicity [51.120].

All in all, a combination of endogenous as well as exogenous factors within the sender dynamically impacts the activity of the glandular system and of the axillary microbiome, determining unique and complex olfactory outcomes of the body odor production. Such olfactory signatures are complex messages containing information of social relevance, reflecting the activity of all the systems of the senders specifically involved in the production of the body odor. Signature odors contain information about particular individuals and, as a result of the processing of such information, they modulate the activity of different systems in the perceiver, prominently, his/her perception, physiological and neurophysiological state and behavior. Also, the outcomes promoted by olfactory signatures reflect the high variability that characterizes body odor production, creating a complex and dynamic multivariate pattern of changes in the recipient.

51.4 Human Axillary Chemosignals for Experimental Purposes

Recent data suggest that humans spontaneously sample chemical signals from their own body parts that have been in close proximity with an unknown individual using what could be considered an intuitive sampling method. Individuals sniff their hand more often in the period following a handshake with a conspecific relative to a similar timeframe lacking such contact [51.121]. However, in an experimental setup where temporal presentation is of importance, more controlled sampling methodologies, as those reviewed in the following paragraphs, are usually implemented. Unfortunately, even though different sampling methods

may have significant impact on the obtained results, the field still lacks a clear methodological systematization of the way odor stimuli are collected meaning that direct comparisons between individual studies are, at best, difficult.

We have characterized the methodological steps of sampling and preserving body odors by extending the framework proposed by *Lenochova* et al. [51.122]. Each of these steps can influence the quality of the donated chemosignals, and – by extension – the experimental measures of interest:

1. The restrictions placed on body odor donors
2. The medium of sampling
3. The type of sampling
4. The time of the day and length of sampling, to
5. Sample storage.

51.4.1 Restrictions on Body Odor Donors

Common lifestyle restrictions related to hygienic, dietary, and behavioral concerns are applied before and/or during body odor sampling. The stringency of such restrictions varies greatly across studies and research groups, but the rationale is always a reduction in the variability due to external factors of the body odor samples. Such external factors can be either removed (e.g., fragrance-containing body products, foods and drinks, etc.) or standardized (e.g., all donors are provided with the same odor-free products). As any contact of an exogenous material with the sampling area can be a source of contamination, it is nearly impossible to collect uncontaminated samples outside of the strictest laboratory settings. Therefore, standardization of inevitable contamination sources can be a valuable approach, provided that the experimental design is compatible (e.g., planned contrasts).

Hygienic restrictions combat the many fragranced products often applied to body odor sampling areas and to the materials that come into contact with those areas. Products (e.g., deodorants, antiperspirants, perfumes, etc.) usually applied to sampling areas, often the underarm or vicinities (e.g., trunk, face, hands) are often eliminated. Any materials that will contact sampling areas (e.g., clothes, bedding), are often selected with regard to minimizing contamination by exogenous chemicals. To address the variability of shower products, donors can be provided with a standard, odor-free body wash, shampoo [51.21], and – in some cases – deodorant. Hygiene restrictions are usually implemented before the sampling period starts and can last from a minimum of 1–2 days to as long as 10 days (especially in cases in which chemical analyses are performed [51.123]) presampling. Restriction involving armpit shaving has only rarely been taken into account. Experimental evidence suggests that, even though odor preference increases for unshaved relative to shaved armpits [51.124], such differences are minimal and, when present, transient [51.125].

Dietary restrictions, the most commonly implemented type of restriction, include the elimination of a collection of food items that, when metabolized, leave detectable traces in bodily fluids. A few examples are garlic, fruits, and asparagus [51.126–128]; however, many other reports on this topic are either anecdotal or subjectively ascertained by the experimenters, and there is a lack of controlled studies in this area. Alcohol consumption is commonly eliminated during presampling and sampling periods despite the fact that the effects of alcohol on the perception of body odor is still lacking. To date, one of the few controlled studies of how diet modulates skin secretions comes from a study investigating the olfactory traces of a diet rich in red meat [51.120].

Behavioral restrictions are a catch-all category of nonhygienic and nondietary regulations aimed at eliminating exogenous contamination. Sporadic or social smoking as well as second-hand smoking is often eliminated due to its strong contamination potential. Levels of physical exercise and exposure to highly emotional situations are often regulated, especially in studies examining the emotional chemosignals. Sexual intercourse and sharing a bed with another person and/or pet are also commonly prohibited during presampling and sampling periods of nighttime-collection studies. For female donors, hormone-based contraception and menstrual phase are commonly recorded – if not actively controlled for – to detect or avoid their effects on body odor perception [51.129, 130].

The most common means of ascertaining the usability of each sample is to have a small (e.g., two to three) panel of trained people determine the presence of uncontaminated body odor. While chemical analysis (e.g., gas chromatography-mass spectrometry) would provide a means of detecting and quantifying a priori-defined contaminants, obstacles of cost and practicality concerns often make this impossible.

51.4.2 Medium of Sampling

T-shirts alone, or cotton pads sewn into the armpit area of the T-shirt [51.131], are the most common means by which to collect body odor samples from the axillary areas. The T-shirts, with or without pads, are usually worn directly on the bare skin and are subsequently removed and subjected to olfactory ratings or additional analyses [51.13]. If the underarm area or cotton pad is not cut and separated from the rest of the T-

shirt, the ratings can be confounded by odors originating from extra-axillary areas, such as the torso [51.130]. The variability introduced by T-shirts can be counteracted by laundering each with the same odor-free detergent shortly before use and storing each in a similar manner until use. If the T-shirts are worn during the day without compensative strategies or are worn at any time with clothes laundered with a nonstandard or fragranced detergent, the odor variability of the sweat sample significantly increases. Prepared properly, T-shirts can serve as shields to external chemicals for cotton pads sewn to the T-shirt or anchored by other means to the underarm area. Cotton pads have also been used in isolation and fixed to the axillae with surgical tape [51.16, 130, 132]. This facilitates the adherence of the sampling medium to the collection area and maintains its position throughout the sampling period.

The large variability in sampling mediums and procedures has generated an equally wide array of criticisms. One common concern is with the use of tape as a means of securing pads to the underarm area, as the compounds found in the tape material and its adhesives pose a risk for contamination. Another concern is with the use of cotton as a sampling medium, which can trap the sulfurous compounds of axillary odors [51.133], removing the sulfurous compounds from the odor signature, when the collection is performed with cotton pads and/or T-shirts [51.95]. These concerns and others like it are important in that any sampling method that systematically alters body odor collection prevents studies from assessing the odor signature in its entirety. However, these concerns must also be balanced by feasibility considerations. In the case of cotton as a sampling medium, finding an alternative that simultaneously enables sample collection in situations mimicking day-to-day life and preserves the sample's sulfurous compounds has been very challenging. Furthermore, sulfurous compounds have been identified at very low concentrations; their detection required the collection of hundreds of milliliters of sweat in glass vials while participants were exercising in a sauna [51.95]. It seems therefore that although perceivable using a perceptual smell test – such as that performed by most behavioral and neuroimaging experiments – these molecules, by their own chemical nature, tend to disappear when extracted from a cotton medium and when chemically quantified in isolation. The chemical and physical considerations of the odorants, the materials and form of sampling media, as well as potential interactions are all questions ripe for systematic evaluations within future studies.

51.4.3 Type of Sampling

An additional factor that is seldom factored into experimental designs as a source of experimental variation is the direct comparison of different activities performed during collection. Recent research on the emotional effects of body odor communication raises this issue. Some experimenters used video clips with specific emotional content (e.g., horror clips for fear/anxiety, splatter video for disgust, cheerful videos for happiness [51.26–28]) to induce a vicarious experience of a transient emotional state. Other experimenters prefer more ecologically relevant conditions and collect emotional chemosignals of acute stress responses at an important examination [51.134], during a first-time skydiving event [51.33], or during a high-ropes course experience [51.30]. As a control condition for emotionally induced axillary secretions, groups have used sweat collected from participants at rest or during performance of aerobic or anaerobic physical exercise. The use of physical exercise might be experimentally problematic due to the increase in secretions originating from the eccrine glands (as opposed to the apocrine glands), which serve to cool the body during exercise. Published evidence from chemical analyses characterizing the differences between these control conditions is lacking [51.135].

51.4.4 Time of the Day and Length of Sampling

Various studies sample body odors at different times of the day – some daytime, others nighttime, and still others, both. Considering the differences in level of activity in many systems (skin microbiome, metabolic, immune, autonomic, and central nervous) during wakefulness and sleep [51.136], it is possible that such differences are reflected in the sampled body odors. However, no experimental evidence contrasting daytime and nighttime collection has been published. Furthermore, regardless of sampling time (of day), body odor samples can be affected by the length of the collection. Large disparities in collection duration have been reported across studies, ranging from 20 min [51.26] to 7 nights [51.11]. Considering sampling time and duration in conjunction, samples collected during the day for longer periods of time are likely to be more emotionally variable and more likely to include a greater variety of exogenous, contaminant compounds that nighttime samples do not share. Despite limitations in the control over activities performed by daytime donors, our own experience is that daytime-collected samples are more intense. Aside from the interference of night-

mares and other emotionally vivid dreams, nighttime-collected samples are considered less variable due to the decreased variability in participants' emotional and physical states and greater control over sampling conditions. To reach supra-threshold detection levels, multiple-night sampling periods or highly controlled daytime samplings are often used. One method of characterizing sample quantity is to weigh the pads used during collection and compare this weight to unused pads [51.26–28]. This method is particularly useful to determine the presence of chemosignals collection over short sampling periods. These differences in sampling time of day and length might lead not only to differences in the intensity of the chemosignals but also to differences in the co-varying odor pleasantness [51.132, 137, 138].

51.4.5 Sample Storage

Post-collection methodologies are another critical variable affecting the preservation of body odor samples. After sampling, bacteria transferred to the sampling medium will continue metabolizing the sample, thereby continually altering the chemical substrate over time. In an effort to limit this confound, some groups use freshly collected samples for each rating session [51.132, 139–141]. This approach prevents us from studying longitudinal effects of specific olfactory signatures. The most commonly used storage method is freezing [51.21, 142–144], though, like so many other aspects of sampling methodology, there is a great degree of variability; across studies and research groups, samples are frozen at variable temperatures in different types of containers for a variable amount of time and are thawed (and re-frozen for re-use) a variable number of times in different types of containers. A study conducted by *Lenochova* et al. suggests that freezing at −32 °C, the lowest temperature achievable with a regular freezer, for relatively long periods of time (up to four months was assessed) and repeated freeze-thaw cycles do not affect perceptual ratings of body odor samples [51.122]. However, the freezing temperature is relevant in maintaining the integrity of a chemosignal, as it is for other types of bodily secretions. Storing milk at temperatures around −20 °C resulted in degradation after a few months of storage, whereas conserving the samples at −80 °C did not change the chemical profile of the milk [51.145, 146]. Other parameters, such as whether the thawing is done within the storage container (e.g., a ziplock bag) or within a delivery container (e.g., a glass or plastic jar), are still untested. It is important that future studies consider the absorptiveness and permeability of the storage and delivery containers to limit both the loss of volatiles as well as the acquisition of exogenous contamination.

Finally, it is important to note that the aforementioned experiments assessed the effects of these freezing parameters only on the perceptual characteristics of the body odor stimuli, not on whether the chemical structure or composition, and therefore the chemosignals themselves, may have been altered. Moreover, it is not clear what the link is between perceptual ratings and chemosensory signal strength.

51.5 Central Processing of Human Chemosignals

Ever since the first olfactory neuroimaging study was published over 20 years ago [51.147], a plethora of studies have demonstrated the involvement of a range of brain areas in the processing of olfactory stimuli. Areas consistently reported as involved in processing of common odors are the piriform cortex, amygdala, entorhinal cortex, hippocampus, hypothalamus, thalamus, orbitofrontal cortex, and the insula [51.148]. For a detailed overview of the cerebral processing of olfactory stimuli, please see Chap. 38.

Stimuli with ecological relevance presented in other sensory modalities, such as vision [51.149–151] and audition [51.152], are processed via dedicated brain pathways outside of the main sensory system. The range of behavioral studies presented above supports the claim that human chemosignals are salient carriers of information about other individuals, along the same lines as visual and auditory stimuli. Empirical validation of this claim has been corroborated by a study by *Lundström* et al. [51.131]. To assess whether human body odors are subjected to non-olfactory processing recruiting a separate network from common odors, *Lundström* et al. [51.131] exposed recipients to different body odors as well as to a fake body odor, namely a mixture of common odorants (including cumin oil, anise oil, and indole) with perceptual characteristics similar to real social chemosignals. Direct comparisons of neural processing between the two perceptually similar stimuli, real and fake body odors, demonstrated that real body odors selectively activate a neural network located predominantly outside the main olfactory system and composed of the occipital cortex, the angular gyrus, and the anterior and posterior cingulate cortex (Fig. 51.1).

51.5.1 Occipital Cortex

The occipital gyrus is an area located within the primary visual cortex, responsible for the processing of visual information. Activations at the level of the occipital gyrus following exposure of body odors suggest that this area has a multimodal character. Indeed, rather than activating at the presentation of purely visual stimuli, it responds to the social relevance of the stimuli presented in different modalities [51.153]. It has previously been demonstrated that olfactory cues are able to activate visual processing areas in the absence of visual stimulation [51.154–158] and that emotional highly relevant stimuli induce increased activations in the primary visual cortex [51.159]. In the study by *Lundström* et al. [51.131] activations in primary visual areas are retrieved from participants who were not able to distinguish between real and fake body odors. This is in line with the fact that activations in the occipital cortex are commonly found in olfactory neuroimaging studies [51.145–149], and it suggests that the observed activity is not completely attributable to the social content of chemosignals. In other words, olfactory stimuli – independent of their social value – activate this visual area. Therefore, this finding has been interpreted as indicative of a preparedness mechanism [51.131]. An odor (as well as an image), whether ecologically relevant or not, indicates the presence of an object in the immediate vicinity and may trigger the visual system to be prepared for the entrance of a stimulus in the visual field, therefore requiring special attention.

51.5.2 Angular Gyrus

Located in the posterior part of the inferior parietal lobule, the angular gyrus activates in response to information related to the human body. Indeed, the functional role of the angular gyrus has been associated with a variety of tasks, including those involving social cognition [51.160] and multisensory integration if we also include the overlapping area of the intraparietal sulcus. Please refer to Fig. 51.1 [51.161]. Overall, recent meta-analytic evidence suggests that the angular gyrus is involved in the processing of concepts during tasks in which the simultaneous interface of perception/recognition and action is required [51.160]. Specifically, lesions at the level of this area alter (or fully impair) the ability to interpret the perception of one's and other individuals' bodies [51.162–165] and evidence indicates the angular gyrus serves as a cross-modal hub in which converging multisensory information is combined and integrated, including that relating to body

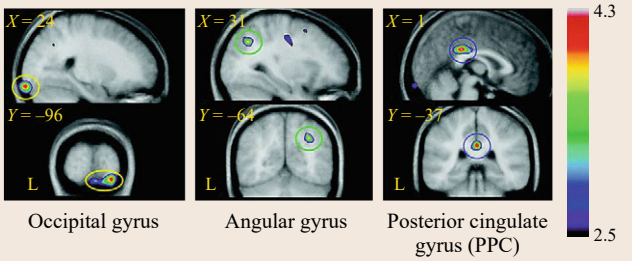

Fig. 51.1 Comparing the group-averaged regional cerebral blood flow (rCBF) responses to the processing of body odors versus fake body odors produced the depicted statistical parametric maps of *t* values thresholded at 2.5 superimposed on group-averaged anatomical magnetic resonance imaging (MRI). The *top panels* show a lateral view and the *lower panels* show a coronal view of the same areas. Coordinates refer to the center of activation and slice expressed according to the Montreal Neurological Institute (MNI) world coordinates system. From left to right, the *colored circles* mark an increased rCBF response in the right occipital cortex (*yellow*), in the left angular gyrus (*green*) and in the posterior cingulate cortex (*blue*) (after [51.131])

representations [51.165]. Given its multisensory nature, the role of the angular gyrus in the chemosensory context cannot be appreciated in isolation and requires the simultaneous account of the contribution of the connected regions.

51.5.3 Anterior and Posterior Cingulate Cortex

Smelling a body odor in contrast to common odors increases activity in both the anterior cingulate cortex (ACC) and the posterior cingulate cortex (PCC), which have been linked to the implementation of attentional, mnestic templates [51.166–168], emotion regulation [51.169, 170] and regulation of emotional actions [51.171, 172], respectively. Moreover, a recent meta-analysis identified ACC and PCC as critically involved in self-reflective processes [51.173]. PCC elaborates not only self-relevant information, but it is also included in the evaluation and decision-making process of whether a certain stimulus is applicable to the self, therefore in line with autobiographical memories [51.173]. In contrast, the ACC activity underpins the emotional aspects of the processing of self-relevant information [51.174] and it is involved in the comparison with other-reflective processes, to indicate self-specificity [51.173].

Although a definite mechanism of body odor communication has not been established, it may be that body odors, in contrast with common odors, receive

a preferential processing in virtue of their signal value [51.131]. Seen from an evolutionary perspective, signals carrying important information, such as those related to threats, might have been selected by evolutionary pressure to receive preferential processing (i. e., emotional and attentional prioritization).

51.6 Human Chemosignals of Harm Avoidance

Besides general differences in the neural processing of common and body odors, the body odor literature has focused on the characterization of the neural underpinnings of different messages with social relevance. These specific areas of social communication via chemosignals can be distinguished in two macro areas: chemosignals related to reproduction and chemosignals involved in harm avoidance. We will refer to the excellent review by *Lübke* and *Pause* [51.104] in reference to an analysis on the chemosignals of reproduction and we will here focus our attention on the chemosignals sustaining harm avoidance.

Harm avoidance is an umbrella term used to refer to all adjustments made in response to stressful events, involving demanding environments and/or dangerous individuals [51.90]. Stress is a response based on fight, flight, or tend-and-befriend behaviors, that occurs following the perturbation of an organism's homeostasis [51.175]. If stress requires severe adjustments over long periods of time, such as in the case of chronic stress, the organism faces collapse and eventually death [51.176]. Given the life-threatening power of stress, it is not surprising that mechanisms of harm avoidance have been favored during evolution and are presently used by the vast majority of animal species [51.3] as well as by plants [51.177]. Chemosensory signals have all the characteristics to act as efficient warning signals, as overviewed in the introduction of this chapter. Below, we will review chemosensory harm avoidance mechanisms, including strategies capitalizing on self-other recognition, discrimination among different types of *others* and transient harm-related signals.

51.6.1 Self-Other Recognition

The first step to identify what is safe and what is potentially harmful is the recognition of something different from oneself. Disposing of a system able to differentiate self from others might capitalize on the decoding of social information related to threat, including that coming from body odors. A self-reference mechanism, such as that used by rodents and referred to as the *armpit effect* [51.178], is supposed to be at the basis of the ability of people to identify their own body odor by sniffing [51.179]. However, *Pause* et al. [51.180] could not reveal a similar pattern of results when testing males and females with self and non-self odors. In the authors' words, the low accuracy in the identification performance might be a result of the low odor concentration. Alternatively, it might indicate that the ability to discriminate between subtle body odors does not necessarily involve conscious processing [51.131].

51.6.2 Different Types of Others: From Kin to Strangers

Recognizing whether a signal is similar or different to oneself is a binary decision. However, what is *other* can be categorized in many different ways. Other can be a kin, with whom the receiver shares genetic features; the individual can be a friend, with no genetic relationships with the donor of the chemosignal, yet familiar due to exposure to that individual; or the individual can be a stranger, with no genetic or learned connections and, given the lack of additional information besides the odor, considered potentially dangerous.

Recognition of kin via chemosignals is an important mechanism that is manifested very early in life. In virtue of the pre-post birth continuity of the chemosensory experience [51.69], body odors are supposedly the first signals used to prevent the occurrence of stress situations. In situations of high vulnerability, such as early in life when a newborn is totally dependent on maternal care to survive [51.181], olfactory cues from the mother and the newborn become the basis of parent–infant interactions, and they constitute a foundation of the development of the parent-child relationship and secure attachment [51.182, 183]. Besides the ability of newborns to recognize and prefer the odor of their mother's amniotic fluid and breast body odor, mothers – who have an active role in the protection of their offspring from stress and harm – are able to discriminate and prefer the odor of their own baby [51.139, 184, 185].

However, as mentioned above, experience has an impact on the processing of human social chemosignals. Evidence to this claim is suggested by *Lundström* et al. [51.131], who asked their participants to smell the odor of highly familiar individuals such as long-time friends. Interestingly, duration of friendship – which correlates with the neural activity in the right occipitotemporal cortex – did not increase the identification rate

or the confidence in the recognition of the kin's body odors, supporting the notion that social chemosignal communication is primarily mediated by nonconscious processes. This view of a specific reaction to familiar body odors and their relationship with the duration of friendship suggests that exposure to a specific body odor in the context of long-lasting positive experiences will specifically impact the social chemosignal communication. In the study the odors of some sisters were also included [51.131]. When compared to friends, no differences in the identification of body odors were found, suggesting a complex interplay between learned and genetic cues. This would be in line with evidence found in mice, for whom a common odor (e.g., peppermint odor) can produce the same social behavior as a kin's odor (i. e., maternal odor), when previously associated with care and protection [51.181].

How is chemosignal communication modulated by the origin of the body odor? Such highly relevant stimuli seem to embed a threatening message, as suggested by the perceptual ratings collected in the *Lundström* et al. study [51.131]. Body odors were rated as being more intense and less pleasant when rated by individuals to whom these donors were a stranger to than when the very same odors were presented as the body odors of kins, indicating that the relationship to the rater, and not the chemical composition of the odor, was responsible for the perceptual differences found across conditions.

51.6.3 Transient Harm-Related Chemosignals: Behavioral Evidence

Besides stable traits, more transient information related to harmful situations can be communicated via body odors. For instance, *Shirasu* and *Touhara* [51.118] reviewed a number of infectious diseases as well as metabolic disorders, toxins and poisons that had been associated with either a volatile organic compound and/or a more or less specific odor label. *Olsson* et al. [51.24] have recently demonstrated that healthy individuals use chemosensory cues to evaluate the health status of individuals in their surroundings. Indeed, the body odor of senders undergoing an innate immune response induced by a lipopolysaccharide injection is rated as more intense, unpleasant and unhealthy (in other words, aversive) than that of individuals exposed to a placebo. This finding, in line with the idea that perceived disgust promotes avoidance behavior [51.28], suggests that olfaction plays an important role in reducing the risk of contagion, thereby maintaining an individual in good health. This is in line with the idea that behavioral avoidance is a first-line defense against disease; which of course is more cost efficient than to involve the organism's immune system.

Other types of situational danger can also be communicated via chemosignals. When an individual faces the need to implement a fight or flight response, and therefore experiences a stress situation, it is of value for potential conspecifics in the surrounding to be warned that a critical event is occuring. A series of studies have recently explored the chemosensory communication of stress by using axillary sweat samples. Stress is a complex response characterized by an increase in physiological arousal associated with the experience of a variety of emotions, ranging from those related to eustress (e.g., excitement and surprise) to those coupled with distress (e.g., fear and disgust) [51.186]. The literature on chemosensory communication of stress includes chemosignals collected within a wide range of situations, potentially affecting the quantity and the quality of the axillary sweat samples donated. The less intense stress sampling conditions consist of a person watching movies with a predominant emotional character and vicariously experiencing the situation visualized, while the body odor sampling occurs. Although this method induces relatively weak stress responses, the advantage is in providing a rather precise characterization of the emotions experienced by the donor, and that can be mediated to recipients via chemosignals, of anxiety and disgust [51.27, 28, 37, 187] (for positive accounts see [51.26]). In an attempt to keep the emotional specificity of the body odor donation stable and to specifically focus on the collection of chemosignals in highly anxious situations – thus, increasing the intensity of the signal – *Pause* et al. [51.134, 188] collected the body odor from donors waiting for an examination needed to complete their academic degree. Other groups increased the intensity of the stress response at the expense of emotional specificity by collecting body odors in extreme conditions, such as a first-time parachute jump [51.31] and high-rope courses [51.30, 38]. These chemosignals were subsequently presented to receivers and the effects of the exposure were quantified. It was reliably demonstrated across studies that chemosensory sweat stimuli are difficult to detect perceptually [51.33, 134, 188] and as a result, participants have difficulties in reliably verbally reporting the emotions experienced by sweat donors during collection [51.31, 32, 37, 187]. However, there is mounting evidence suggesting that adults, and in particular women, are more accurate than chance in identifying the emotion of the donor during collection [51.37, 187], in visually evaluating facial expressions on the basis of the chemosensory prime [51.32], and in discriminating between emotional expressions especially when body odor samples from donors experiencing fear are the target [51.31]. Altogether these data suggest that the effects of chemosensory stress signals are largely

unknown to the perceiver and that perceptual acuity mechanisms specific for social information are at play. Indeed, detection of safety signals (e.g., positive emotions, [51.38, 134]) is reduced in favor of an increased prevalence of detecting threatening stimuli [51.31, 32]. This is in line with the idea that body odors originating from fearful individuals, in virtue of the highly relevant message they convey, receive prioritized processing [51.189, 190]. Indeed, anxiety chemosignals, but not emotionally neutral body odors, affect the acoustic startle reflex [51.36], an evoked preattentive reflex modulated by the affective and relevance value of the stimulus [51.191].

Further, the association between a neutral body odor and a threatening situation can facilitate the processing of body odors. *Alho* et al. [51.192] tested the idea that an offender, in parallel to eyewitness lineup identification tests, could be identified by their odor alone. Participants could do this fairly well. It was also demonstrated that when the encoding of a body odor is associated with the simultaneous presentation of threatening information (e.g., authentic and arousing videos of criminal activities), they were subsequently identified well above chance in body odors lineup tests and considerably better as compared to the body odors associated with neutral videos. In other words, a body odor of a threatening stranger was remembered significantly better than a nonthreatening stranger.

So far, the evidence suggests that the responses to threatening body odors are in line with the elicitation of the automatic, and rather nonconscious, responses to fearful stimuli [51.193]. In contrast with this view, *Chen* et al. [51.35] found that smelling threatening body odor stimuli increased the response time and response accuracy of participants in processing words with fearful content in a word association task. This finding, interpreted as the ability of body odors to modulate cognitive processes, could be seen as counterintuitive. In contrast to carefully deliberated, but not timely reactions, rapid and automatic reactions to threatening situations are thought to increase the chances of survival. In other words, the promotion of mechanisms facilitating fast detection of threat, possibly high in false positives, would maximize survival rates as compared to a precise and slow processing. Threatening stimuli commonly enhance speed (that is, lower reaction times) at the cost of accuracy, as previously demonstrated for fearful multimodal stimuli [51.189, 194–196]. Because few studies have investigated the trade-off between speed and accuracy in response to threatening body odors, it is too early to say whether the detection of these chemosignals is governed by other processing principles than visual signals of threat.

Besides the cognitive consequences of body odor communication, emotional information processing is also affected. Evidence suggests that threatening body odors are sufficient to induce – particularly in female receivers [51.31] – a reflection of the emotional state of the sender [51.28, 197]. This is particularly true for fear-related odors, which have been demonstrated to quickly establish (and maintain) synchrony between sender and receiver. Sweat samples, produced with the involvement of the sympathetic-adrenal medullary system contain a distinctive signature (not yet chemically specified), which triggers in receivers fearful facial expressions (i. e., co-contraction of corrugator supercilii and medial frontalis muscle) and vigilant behavior (faster reaction times when classifying facial expressions) [51.27].

Emotional synchronization also occurs in the presence of chemosignals of disgust [51.28]. Indeed, a body odor from a person experiencing disgust while watching a disgusting movie will induce in the recipient an experience of disgust, revealed via the analysis of sniff patterns and facial electromyogram [51.198]. As demonstrated by *de Groot* et al. [51.28], the chemosignal communication of different forms of harm is specific and therefore can be discriminated. On the one hand, fear chemosignals generate a fearful facial expression and promote sensory acquisition (increased sniff magnitude and eye scanning); on the other hand, disgust chemosignals evoke a disgusted facial expression and sensory rejection (decreased sniff magnitude, target-detection sensitivity, and eye scanning) [51.28].

One of the theories mainly used to explain the transmission effects from sender to receiver via chemosignals involves the idea of emotional contagion, a basic mechanism promoting coordinated thoughts and actions, mutual understanding, and interpersonal closeness [51.199, 200]. However, as demonstrated in other types of social interactions [51.201], the simple emulation of a social percept is not the only possible adaptive response. Indeed, complementary states can be triggered. Emotional complementarity occurs when one person's emotions evoke different (yet corresponding) emotions in others. A classic example is the fact that a person's distress triggers compassion in another [51.202]. In the domain of social chemosignals of harm avoidance, emotional complementarity can acquire different meanings. Let's imagine the example of an individual who donates his/her body odor while involving in an act of aggression, characterized by anger. Rather than the implementation of an emotional contagion mechanism, i. e., the social chemosignal triggers anger feelings in the recipient, it is also plausible that a fear reaction is experienced by the recipient. This

would be in line with the fact that angry expressions are perceived as threatening [51.203, 204] and are known to trigger adaptive actions in the observers [51.205]. Future research extending the limited knowledge of chemosignals of aggression, now mostly performed with indirect measures (e.g., competitiveness, dominance, [51.23, 206]) or limited to one odorous compound (e.g., androstenone) and not to a more complex odor signature [51.4], will shed further light on the issue.

51.7 Central Processing of Human Chemosignals Involved in Harm Avoidance

Behavioral and psychophysiological data suggest that chemosignals involved in harm avoidance can be preferentially processed. To further characterize this idea, the neural underpinnings of such body odor communication are explored below.

51.7.1 Self Body Odor Activates Areas in the Self–Other Recognition System

Lundström et al. [51.15] uncovered the neural mechanism of human self-recognition mediated by chemosignals, in line with the *armpit effect* [51.178]. They compared the body odor of oneself to the odor of a known friendly person and did not find any difference in cerebral activity. They suggested that humans, as had been suggested for rodents, use their own body odor as a template to assess the identity of another individual [51.178]. Further support to this interpretation seems to come from the neural underpinnings elicited by body odors when contrasted with common odors. As suggested, the PCC processes emotional stimuli via interaction with the ACC using *self* as reference frame [51.173]. Furthermore, the activity in the angular gyrus can be put in the context of self-referential space. Indeed, patients with an epileptic focus in the angular gyrus are known to report out-of-body experiences or perceptual distortions of other people's bodies [51.164, 165]. The self-reference matching mechanism is in line with results from studies using chemosensory event-related potentials, a temporally specific technique based on the averaged epochs of the electroencephalogram occurring in association with the presentation of chemosensory stimuli [51.180, 207]. These studies reveal that the human brain is able to discriminate between body odors coming from different sources, irrespective of conscious awareness of such differences. Specifically, the odor originating from oneself can be discriminated and processed faster as compared to the odor originating from someone else [51.180]. Further evidence reveals that more neural resources are required to process the body odors more similar to oneself (e.g., with similar HLA) [51.207], indicating a preferential neural processing for self-matching chemosignals. The lack of conscious awareness, present in both the behavioral and neural correlates of chemosignal self-other recognition [51.15, 18], might be explained by a unique anatomical feature of the olfactory system. Olfaction is the only sense characterized by the lack of a mandatory thalamic relay, meaning that the olfactory receptors project directly to the olfactory bulb and primary olfactory cortical areas [51.208]. Given that thalamic processing has been implied in conscious awareness [51.209], it seems likely that the contribution of this structure to the olfactory percept might make it more weakly associated with conscious processing. However, the thalamic involvement in conscious and unconscious olfactory perception has not yet been fully clarified [51.210].

51.7.2 The Neural Encoding of Familiarity in Someone Else's Body Odor

The first chance to use odors to recognize others comes as early as in utero [51.69]. The evidence on the behavioral preference of infants for their mother's odors and for parents of their baby's odor is also reflected at the neural level. Indeed, the odor of the mother and the child are both subjected to preferential processing, a strategy that in evolutionary terms facilitates the survival of the individual and of the species. Mothers exposed to the body odor of newborns, including their own infant, show selective activation of the thalamus [51.211] and of the orbitofrontal cortex [51.212]. Interestingly, the orbitofrontal cortex is activated in both mothers and nulliparous women when smelling body odors originating from men [51.212]. Moreover, as demonstrated by *Lundström* et al. [51.211], both mothers and nulliparous women respond with neuronal activity within the reward system to the exposure to unfamiliar baby odors. The authors interpret this finding in light of evolutionary theories suggesting that the general ability of infant chemosensory cues to prime affection in adult women serves the purpose to motivate them to care for the infant, i. e., an indication of attachment mechanisms at play [51.213]. Although untested, the fact that fathers are also able to identify

the odor of their infant's amniotic fluid and judge it as qualitatively similar to their infant's and mother's body odor [51.214], suggests that fathers too can present specific neural pathways elaborating body odors.

The individual learning history seems to modulate signal processing, especially in brain areas with social roles [51.215]. Smelling a friend [51.15] activates, among other areas, the occipital cortex including posterior parts of the retrosplenial cortex, previously involved in the processing of familiar faces and voices [51.216]. Furthermore, the duration of friendship – an indirect index of the familiarity of the body odor – significantly correlates with increased regional cerebral blood flow in the extrastriate body area, a region implicated in the multimodal processing of information related to the human body [51.217, 218]. In other words, the more familiar the smell of the body odor, the greater the neuronal involvement to process body-related information [51.15].

With respect to unknown individuals, *Lundström* et al. [51.15] demonstrated that smelling a stranger's body odor evokes cortical activations (i. e., inferior frontal gyrus and amygdala) similar to those described in response to viewing negative stimuli [51.219–221], including masked fearful faces [51.220]. This supports the idea that a body odor never encountered before is treated like any other highly relevant (and potentially dangerous) stimuli [51.222] and differently from perceptually comparable common odors.

In line with the idea that the strangers' body odors conveys a threat sociochemosignal is the activation of the insula, implicated in the processing of unpleasant chemosensory stimuli [51.223] as well as in the recognition of fearful and disgusting objects [51.150]. The simultaneous activation of amygdala and insula, two highly interconnected areas [51.224], suggests that the body odor of a stranger induces separate, yet related, activations of fear and disgust [51.222]. This reaction is not, however, specific to body odors, but is shared with all novel and suddenly presented stimuli [51.225].

In addition, the body odor of a stranger elicits activations at the level of the supplementary motor area and, right below threshold of significance, of the premotor area [51.15]. These structures are known to be part of a hierarchical executive network involved in the adaptation and optimization of motor behaviors [51.226]. The involvement of such a network suggests that the body odor of a stranger by communicating fear or disgust information prepares the recipient to react to the potential presence of an unknown individual in the vicinity. Such motor preparation involves fight or flight responses: basic reactions critically involved in the survival process of the individual.

51.7.3 Threatening Body Odors and Their Neural Correlates

In line with the idea of a preferential processing of chemosignals indicating threat or harm, smelling the body odor collected in anxious situations increased neuronal processing from medial frontal brain areas (P3 amplitude) [51.188]. This effect was more pronounced in women than in men, which is in accordance with the findings that females show a processing advantage for social emotional stimuli [51.227] and for perceptually weak threatening stimuli [51.228]. Preferential processing can also be inferred by the allocation of early attentional resources to threatening stimuli [51.229] and to ambiguous stimuli (e.g., neutral facial expressions [51.29]).

Studies conducted with body odor samples collected from individuals experiencing anxiety activate brain areas involved in the processing of social emotional stimuli (fusiform gyrus), and in the regulation of empathic feelings (insula, precuneus, cingulate cortex; [51.33]). This evidence is used in support of the idea that fear-related chemosignals produce emotional contagions [51.28], and increase the level of situational anxiety [51.30] in the receivers.

When the sweat presented in the scanner comes from individuals experiencing high levels of stress, it being eustress or distress, such as in the case of the parachute jump [51.31], activations were mainly demonstrated within the amygdala, a center encoding the relevance of a situation and stimulus, in a nonspecific fashion. This is indeed related to a negative affect compatible with fear. The amygdala has long been known to process negative emotional stimuli [51.150, 220, 221] including threat [51.230], although nonselectively [51.231]. In mice, the amygdala has been specifically identified as the main processing center of threat-related endogenous odors [51.232]. This might be taken as an indication of the fact that the amygdala should also be involved in the detection of threat-related olfactory stimuli in humans and not in the processing of fear itself [51.230]. As suggested by *Pause* et al. [51.188], amygdala activations are more pronounced in women than in men [51.233], indicating that stress-related chemosensory cues are more relevant to women.

However, not all evidence points towards a preferential processing of threatening human chemosignals. Indeed, the cognitive modulation induced by smelling threatening body odors suggests that smelling anxiety chemosignals makes the receiver more accurate and less fast in using that information [51.31, 32, 35], thus raising the issue of intermodal differences in the processing

of threatening stimuli. If, on the one hand, threatening visual and auditory stimuli are processed fast [51.195, 196], this is not in accordance with how the olfactory system generally works. Indeed, chemosensory stimuli take longer to be processed as compared to visual or auditory stimuli [51.234]. Furthermore, the estimated time difference between the onsets of the first perceptual and the first cognitive processing is approximately 200 ms in vision, and it is approximately doubled for olfactory stimuli [51.235, 236]. This evidence highlights a downside of olfaction as a warning signal: early detection of alarming olfactory stimuli might be delayed by the slower central processing that olfactory signals undergo. Nevertheless, the possibility of using such mechanisms in critical conditions (e.g., in the absence of visual and auditory functioning, at a distance), makes the olfactory warning system a good addition to the survival kit mechanisms, by allowing chemosensory stimuli to shape the slower and more deliberate processing rather than the initial and more rapid detection phase [51.35].

Interestingly, none of the published functional neuroimaging studies including threat-related body odors in their paradigms has reported activations at the level of the primary or secondary olfactory cortices [51.15, 31, 33]. When extending the search including studies incorporating body odors of any sort [51.15, 34], only one of the two remaining experiments reveals activations at the level of the lateral orbitofrontal cortex. *Zhou* and *Chen* [51.32] recorded increased neural activity in that structure when participants were smelling the body odors sampled from donors watching erotic videos. Because the activation of the lateral orbitofrontal cortex is determined in *Zhou* and *Chen* [51.34] by contrasting body odors with a common odor (phenylethyl alcohol (PEA)) but a similar activation is also obtained when discriminating odorants within a mixture [51.228], whether the lateral orbitofrontal cortex activity is a clear demonstration of differential body odor processing still remains to be determined. What can be safely concluded though is that five neuroimaging studies, which have included different types of body odors and were performed by independent laboratories, have not been able to report activations located within the olfactory areas even though participants are presented with clearly perceivable odors. This phenomenon can have a series of explanations, for which direct experimental proof is still lacking. It is possible that the conscious perception of body odors is too transient to be detected at the level of the olfactory cortices, possibly due to the high susceptibility to habituation of such areas [51.237, 238] and activity is therefore only demonstrated in less habituating areas of the social brain [51.239]. If this were to be true, non-endogenous control odors (i. e., the fake body odors), should have not generated clear activations in olfactory cortices [51.15, 18]. One might also claim that the lack of olfactory activations depends on the neuroimaging analyses applied, which only correct for false positive errors but do not account for false negative errors. Therefore, lack of significant activity in a neuroimaging study cannot be taken as evidence for the hypothesis that a specific area is not involved in a task. However, if the lack of activation in olfactory cortices is due to cognitive processing, no differences should be found between real and fake body odors [51.131]. Finally, it is unlikely that five independent neuroimaging studies would produce five sets of results with similar false negative errors.

51.8 A Clinical Perspective on Human Chemosignals

The research on how a social message is transmitted from a sender to a receiver through body odors has mostly been conducted on healthy human participants. However, health and mental issues modify brain processing and experience [51.240] (Chap. 50). Given the social relevance of body odor communication, differences in the processing of such signals according to baseline social skills are expected. Only a few studies have empirically tested this assumption and demonstrated that socially skilled versus socially unfit individuals use body odor information differently. For instance, emotional competence facilitates the identification of familiar body odors [51.32] and highly sociable and assertive individuals process body odors within the central nervous reward system, as well as in structures involved in social cognition and in synchronization processes (inferior frontal gyrus) [51.241]. In other words, higher social emotional competence facilitates the transmission of socioemotional messages via chemosignals. This powerful feature of chemosensory stimuli, along with the automatic processing of these stimuli, might offer an opportunity for individuals with reduced social skills. This hypothesis has been tested in a group of children with autism spectrum disorders (ASD), a series of disorders known for their core deficits in social information processing [51.242]. When exposed to their mother's body odor, high-functioning children with ASD can readily start and conclude basic social actions [51.243, 244]. With specific reference to harm avoidance, individuals with high social anxiety quickly elaborate the

presence of body odors of anxiety, yet subsequent attentional processing is blocked, possibly reflecting a mechanism of perceptual defense [51.188]. Furthermore, individuals with high social anxiety show greater startle responses under the exposure of chemosignals of anxiety as compared to neutral chemosignals [51.245] and respond with heightened sensitivity to such chemosensory contextual information [51.229].

Recent studies in rodents indicate that a single receptor is involved in the transmission of warning chemosignals and, when blocked, the warning signal (predator odor) loses its threatening power [51.246]. Should a similar mechanism be proved in humans, it could be used as a therapeutic strategy for patients suffering from social anxiety.

Overall, these results show that socioemotional skills and human communication mediated via chemosignals are intrinsically interwoven. In light of the ability to sensitively detect, accurately process, and show appropriate responses based on relevant chemosensory social information, it is plausible that chemosignal communication critically contributes to the formation and maintenance of social groups [51.247], an essential condition for human evolution.

51.9 Conclusions

The evidence reviewed in this chapter suggests that humans do use chemosignals as a form of social communication [51.248]. A chemosensory signal conveying a social message is generated by the coactivation of the systems maintaining the homeostasis in the sending individual. In healthy individuals, genetics, the immune system, metabolism, hormones, and the autonomic and central nervous systems work in concert to determine the chemical composition of body secretions. With respect to the axillary sweat, such systems affect the apocrine and apoeccrine secretions. These secretions will be converted by the resident flora into a joint result. Such a chemosignal, conveyed from the sender to the receiver via airborne molecules, carries a variety of information, among which is that promoting survival. Suggested to date are chemosignals can communicate self-other information (e.g., kin recognition), emotional messages (e.g., chemosignals of anxiety), and those that inform the receiver about the presence of potential threats in the environment (presence of an unknown or sick individual). As noted by *Lübke* and *Pause* [51.104], the receiver's responses constitute the only unspecific step in the social chemosignal communication process, as outlined here. This would be in line with the need of receivers to adaptively respond to the presence of situations challenging survival. In the case of danger perceived through different sensory modalities, the same type of fight, flight, or tend-and-befriend responses would be required to handle the threat. Importantly, receivers can implement those responses without conscious awareness of having smelled the chemosignal [51.131].

With respect to methodology, researchers have not yet agreed upon guidelines for body odor collection, reducing the comparability across databases. In this respect, it is worth noting that the choice of the experimental control conditions is of primary importance. To avoid confounders in the perceivers' responses, secretions from the same glands should be sampled across conditions. Stringent control conditions can separately account for several significant factors and it is advisable to include common odors to reveal further distinctions and similarities across olfactory functional subsystems. However, the extensive time required to collect usable body odors, as well as the fast central habituation to olfactory stimuli [51.237], constrains the number of olfactory conditions and trials possibly included in one experimental session.

Delivery techniques have also been one of the major causes limiting the exploration of the field. Indeed, olfactometers – dedicated computer-controlled delivery systems, with rapid onset-offset stimulus release – are machines not widely commercialized and relatively expensive. Nevertheless, they are necessary for the study of the temporal dynamics and the neural underpinnings of body odor communication.

With regards to the neural bases of social chemosignal communication, future efforts should focus on increasing our knowledge of the neural bases of social chemosignal communication and further our understanding of the functional separation between pathways processing common and body odors. Furthermore, the neural underpinnings of chemosignals with different emotional characterizations should be included to assess emotion-specific dissociations. This would open the discussion on the communication strategies used by emotional chemosignals.

Finally, a better understanding of how social aspects of life are transmitted through chemosensory stimuli can provide additional insights on clinical populations struggling with social information processing (e.g., ASD, schizophrenia, social phobia).

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52. Human Chemosensory Communication

Bettina M. Pause

Social communication refers to a basic human need, and findings accumulate that show humans communicate numerous kinds of social information via chemosignals. Briefly, it will be introduced which chemicals are conveyed through body fluids and which sensory systems are considered to process social chemosignals. Then, it will be shown that pheromones in humans have not yet been discovered. Studies on putative pheromones in humans often are performed disregarding the biological underpinnings of chemical communication and seem randomly to investigate volatile substances without any theoretical background. However, evidence will be provided that human chemosensory communication has been well demonstrated, using natural body fluids (e.g., sweat) as the source of chemosignals. Humans can decode information about the immunogenetic profile and the level of sexual hormones from volatiles released from the sweat of other individuals. These chemical signals are considered to affect mate choice. However, the signal extraction also depends on the sexual orientation of the perceiver. Furthermore, the recognition of kin and mother-infant communication comprise the release and decoding of chemosignals. Both phenomena are important prerequisites for the formation of social bonding and harm protection. Finally, the communication of stress and anxiety in humans will be presented as an example of a chemical transmission of emotional states. At the end of the chapter it will be questioned whether chemosensory communication is a skill, protective for certain mental disorders.

52.1 Interindividual Communication

52.1.1 Social Communication

Like most mammals, humans are inherently social in nature. Social isolation and loneliness are major risk factors for mortality in humans [52.1, 2]. The impact of social isolation on health in humans is comparable to the effects of high blood pressure, obesity or smoking [52.3, p. 5]. Social exclusion leads to a decline in cognitive performance and decrements in intelligent thought [52.4] and is processed in the human brain similarly to physical pain [52.5–7]. In socially isolated adult mice, myelination of oligodendrocytes in the prefrontal cortex (PFC) is impaired [52.8]. Therefore, avoiding loneliness seems to be a basic drive in humans and group living might have been mandatory for phylogenetic survival. In fact, the social brain

hypothesis postulates that predation risk (through outgroup members or predator animals) was the ultimate cause for group living in primates [52.9]. Social complexity, in turn (e.g., group size, grooming clique size, the frequency of coalitions, and the prevalence of social play) might have been the main promoter of the neocortex size of anthropoid primates [52.10]. Social enrichment requires the development of behavioral flexibility, which in turn is essential for the formation of stable social bonds. In anthropoid primates, relationships involve a form of bonding that is found elsewhere in reproductive pair bonds only. Thus, the formation of long-term friendships is unique to humans and other anthropoid primates and inherently linked to our neocortex size.

In line with the consideration that the processing of complex social information requires larger brains is the discovery of specialized neural networks responsible for the processing of social information [52.11]. It has been demonstrated that the perception of other humans' facial expressions is different from the perception of common visual objects (fusiform face area, [52.12, 13]), and that memory for faces is specialized, holistic, and hippocampus-independent [52.14]. Moreover, social touch is probably also processed as a unique social signal [52.15, 16]. Finally, social feelings like empathy require the processing in specialized neuronal relays, different from common nonsocial feelings [52.17].

52.1.2 Chemosensory Communication

Successful social communication is based upon the release and processing of sensory signals, including speech, touch, biological motion, facial expressions and chemosensory signals. Among these, chemosensory communication is the least understood, which is usually justified by referring to the fact that some other species might have a keener olfactory sense than humans. However, just recently, it could be demonstrated that within the human species the olfactory sense might have a higher resolution than the visual or auditory sense [52.18]. Humans are able to distinguish up to seven million colors and about 340 000 tonnes, however, they are able to discriminate at least one trillion (10^{12}) odors. Thus, the chemical sense in humans is by far keener than the visual or auditory sense.

As compared to the other senses, chemosensory communication has several advantages [52.19]. Chemosensory signals can be used in darkness, and can easily cross barriers. Depending on the volatility of the molecules, chemosensory signals can be transmitted across very long distances. In contrast, low volatile molecules can keep the information for conspecifics at the place of release, while the sender has already disappeared [52.20]. Furthermore, as social chemical signals are usually based on a specific mixture of molecules, the potential number of social signals is extremely high.

Due to these advantages in communicating via chemosignals, high-resolution olfaction might have developed in mammals about 200 million years ago. Especially, the use of the olfactory sense in darkness might have been of special importance to early mammals, which were probably nocturnal animals in order to avoid to be eaten by early carnivores (dinosaurs) [52.21]. The improved ability to analyze and process the complex olfactory environment is considered the ultimate force for driving the encephalization of the unique mammalian brain (as assessed by X-ray computed tomography of fossil mammalian skulls) [52.22, p. 957]:

> *... at its start, the brain in the ancestral mammal differed from even its closest extinct relatives specifically in its degree of high-resolution olfaction, as it exploited a world of information dominated to an unprecedented degree by odors and scents.*

Surprisingly, the final encephalization phase in *Homo sapiens* can be explained in a similar way: Based on three-dimensional geometric morphometric analyses of the endobasicranial shape, it was reported that as compared to *H. neanderthalensis*, modern humans show larger olfactory bulbs, a relatively wider orbitofrontal cortex and increased temporal lobe poles [52.23]. According to the authors, evolution may have favored olfaction-related neuronal circuits in *H. sapiens* because chemosensory skills were of special importance for the development of optimum social strategies (e.g., in communicating emotions or messages related to reproduction), which in turn supported the survival of the modern human. Thus, in human chemosensory communication, the two main promoters of brain size seem to be included (social and chemosensory signals).

In the following, findings related to human chemosensory communication will be reviewed. The term *pheromone* will be avoided, as there is no general consensus on its meaning [52.19, 24]. Instead, the rather neutral term *body odor signal*, or *chemosignal* will be preferred. A signal can be defined as a stimulus that is produced and released by a sender and carries a message to a receiver. In contrast to a pure stimulus, which may or may not carry relevant information, a social signal always conveys specific information between two individuals.

52.2 Release of Chemical Substances in Humans

Since a number of excellent reviews have already summarized the potential sources of social chemosignals in humans (Chap. 51; [52.19, 24, 25]), here the current state of knowledge will be summarized briefly only.

Social chemosignals in humans are generally considered to be volatile, however exceptions might be possible [52.26]. The source of volatile molecules is body fluid of any kind ([52.27]; e.g., sweat, urine, vaginal secretions, sperm, and lacrimal fluid). In humans, most research focused on the secretions of apocrine and apoeccrine sweat glands in the axillae [52.28]. Four kinds of substances contribute significantly to the typical armpit smell. The first class comprises unsaturated or hydroxylated branched fatty acids, such as (*E*)-3-methyl-2-hexenoic acid (3M2H), which constitute the dominant, characteristic axillary odor [52.29]. 3M2H is carried to the skin surface by apocrine secretion odor-binding proteins and then liberated by axillary bacteria [52.30]. The second class of major odor constituents are thio-alcohols (sulfanylalkanols), such as 3-methyl-3-sulfanylhexan-1-ol (3M3SH), molecules with a high volatility and very low olfactory thresholds [52.31–33]. The third class of substances includes short-chain fatty acids, such as propionic, butanoic acid, and isovaleric acid [52.34]. Isovaleric acid is liberated by micrococci and gram-negative bacteria and might contribute to a sweat-like odor [52.35].

The fourth group of compounds are androgen steroids representing a musk or urine-like odor [52.34], such as 5α-androst-16-en-3-one (androstenone), 5$\alpha(\beta)$-androst-16-en-3α-ol (androstenol), and 4,16-androstadien-3-one (androstadienone). In the human testis as well as in the human axillae, androstenol is metabolized from androstenone, which in turn is metabolized from androstadienone [52.36]. As odorless testosterone occurs in apocrine secretions, androgen steroids (16-androstenes) might originate from testosterone metabolism. However, axillary bacteria, such as aerobic coryneform, convert testosterone directly only to dihydrotestosterone, androstanedione and androstenedione, but not to 16-androstenes. Therefore, the metabolism of odorous androgen steroids is considered to be rather complex and to involve numerous interconversions [52.37]. In females, the source of 16-androstenes are probably the adrenals and ovaries [52.34]. The amount of 16-androstenes in apocrine sweat varies considerably between individuals [52.38]. Nevertheless, androstenone and androstenol occur in larger concentrations in males than in females [52.34, 39].

In summary, the individual mixture of compounds within axillary sweat is determined by a) the amount of single molecules that are released by the apocrine and apoeccrine glands, which in turn partly depends on the formation of hormones (e.g., in the gonads and adrenals) as precursors, b) the composition of the axillary flora, responsible for metabolizing odorless precursor molecules to odorous molecules (e.g., 16-androstenes), c) the availability of carrier proteins for the transport of long chain fatty acids (e.g., 3M2H), and d) a specific variant in the ABCC11 gene [52.40]. Individuals who are homozygous for the single-nucleotide polymorphism (SNP) 538G→A of gene ABCC11 have significantly less of the acidic axillary odor [52.41]. The ABCC11 protein is expressed and localized in apocrine sweat glands and seems to be involved in the formation of glutamine precursors to axillary odors.

52.3 Perception of Social Chemosignals in Humans

As several chapters of this handbook are describing the perceptual systems in humans for environmental chemicals in detail, here I will briefly focus on whether social chemical signals are likely to be processed via a non-olfactory system.

Four different systems should be considered when looking for a chemosensory organ responsible for the transduction of social chemosignals: the primary olfactory system, the trigeminal system, the grueneberg ganglion (GG) cells, and the trace amine-associated receptors (TAARs). Whereas the former two systems are rather nonspecific systems, processing volatile chemicals either as smelling (olfactory system) or as cool, pungent or irritant (trigeminal system), the latter two systems might specifically process social chemosignals. The vomeronasal organ (VNO) has no proven function in adult humans.

Even though sweat contains a number of substances that could activate the trigeminal system (fatty acids, lactic acids, and uric acids), complex social chemosignals (body odor) seem not to be processed predominantly by the trigeminal system [52.42, 43].

However, the olfactory system is most probably involved in the processing of body odors. The main olfactory system can be subdivided into the level of the sensory neuron, the olfactory bulb and the olfactory

cortex. Each olfactory sensory neuron (OSN) is considered to express only one type of olfactory receptor that is highly specific for certain molecular features, but also tolerant of others [52.44]. The main olfactory bulb is the first relay station in the olfactory pathway, where axons of the sensory neurons form synapses with the dendrites of secondary neurons (mitral and tufted cells) and interneurons (periglomerular cells) within structures called glomeruli. From the olfactory bulb olfactory information is transmitted to the structures of the primary olfactory cortex (anterior olfactory nucleus, piriform cortex, olfactory tubercle, entorhinal cortex, amygdala) [52.45–47]. Neurogenesis is a central feature of the olfactory system [52.48–50]. The OSNs are in constant turnover through local progenitor cells. Also, neurogenesis occurs continuously in two brain regions of adult mammals, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus. Neurons born in the SVZ migrate into the olfactory bulb, whereas neurons born in the SGZ migrate into the dentate gyrus.

Specific OSNs and bulbar glomeruli mediate innate responses to biologically significant odors (spoiled food and predator odor) in mice [52.51]. In addition, the avoidance response to predator (fox) odor in mice requires constant neurogenesis of SGZ and SVZ neurons [52.52]. This neurogenesis seems also to be required for intraspecies chemical communication, since sex-specific responses that are olfaction-dependent and innately programmed (e.g., male-to-male aggression, female pregnancy and nursing behavior) are severely impaired in mice lacking neurogenesis [52.52].

Another chemosensory system, the GG, has been observed in a variety of mammals (including rodents, carnivores, and humans) and might specifically be involved in social chemosensory perception. In mice, the GG is located at the tip of the nose, close to the entry of the naris. The sensory neurons' axons project to a special set of glomeruli – the necklace glomeruli – in the main olfactory bulb [52.53], which are distinct from the glomeruli typically innervated by OSNs. In mice, GG receptors are sensitive to alarm pheromones, released by stressed conspecifics [52.54]. Just recently, it could be shown that mice GGs also respond to predator odor, an odor representing similar chemical features as the mouse alarm pheromone [52.54]. The authors conclude that the GGs might represent a chemosensory subsystem for the processing of danger.

TAARs have been detected within the mouse [52.55] and human olfactory epithelium [52.56, 57], but have also been described to be expressed on GG cells [52.58]. In mice, TAARs respond to predator odor and to volatile amines, released from urine of conspecifics and trigger avoidance-related motor behavior [52.59]. Therefore, TAARs might also process significant social signals in humans.

In a number of vertebrates, including mammals, the VNO is involved in the detection of social chemosignals [52.60]. In the 1990s one working group reported activation of the human VNO through a number of so-called *vomeropherins* [52.61–63]. These substances (e.g., androstadienone, estratetraenol, estratetraenyl acetate, and pregnadienedione) were reported to change mood as well as the endocrine and physiological status in a sex-specific manner. Due to these publications it was stated that pheromones do exist in humans [52.64], and numerous researchers started to investigate possible pheromonal effects of these substances in humans. However, it could not be confirmed by others that the human VNO is a functional sensory organ. The search for a VNO in humans has been revealed that there are no sensory cells, no functioning receptors, no connections to the brain, and no accessory olfactory bulb, at which a vomeronasal nerve would terminate [52.19, 25, 65, 66]. One single VNO receptor gene has been discovered to be expressed within the human olfactory epithelium [52.67], but its functional significance is strongly questioned [52.19]. However, the VNO is not necessary for social communication in mammals and some effects of social chemosignals on the behavior and physiology of conspecifics have been shown to rely on the main olfactory system instead [52.68, 69]. Moreover, as the VNO also processes nonsocial odors [52.70], its function still remains to be understood [52.71]. The main problem remaining until today is the fact that *vomeropherins* are still investigated, even though they have never been proven to stimulate a pheromone-detecting organ.

In summary, it seems likely that social chemosignals are at least partly processed in specialized neuronal systems, similar to social signals of other modalities. Promising candidates for the processing of social chemosignals are GG cells and TAARs.

52.4 Chemosensory Communication via Single Molecules

In 1959 the term *pheromone* was introduced to increase the understanding of insect communication [52.72]. Since then, pheromones were often considered to be monomolecular substances, and to release a stereotyped behavior or physiology in conspecifics. However, intensive research has meanwhile demonstrated that across phyla, including insects, pheromones are usually molecular mixtures and rather seldom a single substance [52.19]. Therefore, it is difficult to comprehend that just in humans single molecules should act as pheromones. Nevertheless, numerous papers on putative human pheromones were published. Below, those studies will be summarized, which are exemplary for the enormous inconsistency in results and conclusions.

52.4.1 Substances Related to Male Body Fluids

In males, the substances that have been intensively studied for their pheromonal effects are 16-androstenes, especially androstenone, androstenol and their precursor androstadienone. The search for male pheromones can be divided into two phases. During the 1970s and 1980s, research focused on androstenone and androstenol. Since the end of the 1990s most studies investigated whether androstadienone exerted pheromone-like effects.

Androstenone and Androstenol

Androstenone is a powerful chemical signal in the pig. When a boar is sexually aroused or aggressive it produces odorous saliva, including a mixture of steroid compounds. Androstenone is a main component of this mixture and its odor is attractive to sows and induces the mating stance (lordosis) in estrous female pigs [52.73, 74]. As androstenone was also detected in body fluids of human males [52.34], quite suddenly, numerous studies investigated whether androstenone has also pheromone-like effects in humans. Maybe the most popular study, made known to the public by the yellow press, investigated whether females prefer androstenone sprayed seats in a dentist's waiting room [52.75]. In this study, androstenone was applied to a specific chair in three different concentrations, and it was observed whether males or females preferred the treated chair in comparison to an untreated chair. According to the study, females preferred the seat sprayed with the high and the low androstenone concentration, and men avoided the seat treated with the high concentration. Despite its popularity in the public, many aspects of the study's methods have been criticized [52.76]. Especially, the appearance of the behavioral effect in association with the low and the high concentration but not with the intermediate concentration, strongly argues against a biological effect. Furthermore, it was suggested [52.76] that possible effects of androstenone on overt behavior might rather be due to a common odor effect than to a pheromone effect. In fact, the authors of the original study themselves questioned a direct effect of social chemical stimuli on behavior [52.77]. Instead, they declared that behavioral effects of odors always depend on the context of the odor exposure as well as on the odor meaning, which is acquired through the individual learning history.

Apart from behavioral observations, the mood during androstenone exposure was investigated. However, contrary to the expectations, androstenone exposure led females to describe themselves [52.78] and others (males and females; [52.79]) as less sexy, and led males to describe themselves and other males as less sexy [52.79]. The odor of androstenone itself is perceived as unattractive and unpleasant in women, however, during midcycle, around ovulation, the smell of androstenone becomes more neutral, but is not perceived as emotionally positive [52.80]. In the human brain, androstenone is processed like nonsocial odors (rose) in the (orbito-) frontal cortex [52.81].

Two studies investigated whether androstenol had an effect on social behavior. The first study [52.82] indicated that men avoid restroom stalls that are sprayed with androstenol. However, in this study, women's choices were not affected by androstenol. In the other study [52.83], the participants were asked to wear an androstenol sprayed necklace for 17 h, and were later on asked to recall their social contacts. Here, the female participants reported to have experienced intensified contacts with males (according to number of contacts, the emotional depth of the contact, and the contact duration). However, males' social contacts were not affected by androstenol exposure.

While the two studies on androstenol effects on human behavior revealed mutually incompatible results, similar incongruities cast a cloud over androstenol-related mood studies. One study suggests that females exposed to androstenol feel more submissive during the ovulatory phase of their cycle (the effect was significant for the beginning and end of ovulation time, but not for the core of ovulation time, as compared to 12 measurements during the menstrual cycle; [52.84]). However, the ratings on four other mood scales (like sexy/unsexy) were not affected by androstenol. Later on, and even though androstenol was presented to females within an erotic context (reading of an erotic text passage), this effect could not be confirmed [52.85]. In this study, an-

drostenol exposure had no effect on any of nine mood measures. This result is in accordance with a more recent study, which also could not confirm any effect of androstenol on mood (positive and negative mood, and alertness) either in women or in men [52.86].

Apart from behavioral and mood measurements, it was investigated whether androstenol has an impact on the perception of other males and females [52.79]. Smelling androstenol, men rated other males, but not females, as more sexy, and themselves as less sexy, and females rated other males as less sexually attractive.

Even though the reported findings on androstenol are highly inconsistent, a recent study [52.87] reformulated possible pheromonal effects of androstenol, as it is supposed to activate hypothalamic structures in females. In this study, androstenol was presented in crystalline form, thus its concentration exceeded the physiological concentration of androstenol in sweat by far.

In sum, both, androstenone and androstenol do not show any consistent effects on human behavior or mood. The study results are extremely contradictory, suggesting that significant findings appear by chance. As most studies suffer from an insufficient correction of the statistical alpha error, it is to be expected that from time to time some of the measured variables show statistically significant results (see the section on methodological suggestions). The accidental nature of the studies' results becomes obvious, integrating their outcome: Females dislike the odor of androstenone, and while exposed to it, they describe themselves and others as less sexy. However, in very low or very high concentrations, they seem to (unconsciously) approach this odor. Exposed to androstenol, females seem to describe males to be less sexy but are considered to get in contact with men more often. Men, on the other hand, are supposed to describe other males as more sexually attractive while smelling androstenol, but seem to avoid restroom stalls smelling like androstenol.

Accordingly, studies on single molecules like androstenone and androstenol, which are claimed to exert a pheromone-like effect in humans, have strongly been criticized [52.25, 88, 89]. Importantly, it has been stated that chemical communication in humans is affected by experience, culture, and the social and nonsocial context. In 1995 *Kirk-Smith* stated that [52.90]:

> *The experiments [on androstenone and androstenol] form an inconsistent pattern and thus provide little basis for concluding that human males produce odors directly analogous to the sexual attractants of other species.*

In fact, during the 1990s, the interest in possible pheromonal properties of androstenone and androstenol strongly disappeared. However, the run on human pheromones started again, since it was reported that single molecules activate the human VNO [52.61]. These molecules are androstadienone (putative male pheromone), estratetraenol and estratetraenyl acetate (putative female pheromones).

Androstadienone

Aiming to find a sexual attractant in humans, the effects of androstadienone on mood, physiology, and behavior have been studied extensively. However, as with androstenone and androstenol, the effects of androstadienone are highly contradictory across studies.

A number of studies investigated possible mood effects of androstadienone. According to *Jacob* and *McClintock* [52.91], androstadienone increased positive mood in females, but did not change negative mood or alertness. However, according to *Lundström* et al. [52.92] females felt more focused after androstadienone exposure, but did not show general mood changes. Several other studies could not find any mood effects in women after the mere exposure to androstadienone [52.93–97, 97, 98]. In males, only one study suggested that androstadienone perception decreases positive mood [52.91]; most studies, however, observed no effects of androstadienone exposure in men [52.94, 96, 97].

According to the aforementioned studies it seems obvious that simple androstadienone exposure exerts no (predictable) mood effect, either in men or in women. Therefore, some authors suggested that an appropriate context might be necessary for valid mood effects to occur. Thus, it was suggested that androstadienone should affect mood whenever presented by a tester of the opposite sex (in heterosexual participants). According to these studies, androstadienone increased positive mood in females, but only in the presence of a male tester [52.94, 95]. However, whereas some authors found these effects to only occur directly after odor exposure (6 min, [52.91, 94]), other authors report such effects even about 30 min after odor exposure [52.92, 95]. In men, no effects of androstadienone on mood were observable, either in the presence of a male or a female tester [52.94].

In another experimental context, participants were asked to view an erotic movie, while smelling androstadienone [52.98]. Here, it was reported that after androstadienone exposure, females' mood became more positive and females felt more sexually aroused. In a similar study with a comparable context movie, androstadienone increased the feeling of being sexually aroused in female participants [52.97]. However, positive and negative mood was not changed by androstadionone. Instead, viewing the same erotic movie,

the perception of androstadione also increased sexual arousal in male participants [52.97].

Summarizing the studies on mood effects of androstadienone, no consistent result emerges from the different studies. It remains unclear whether in females, androstadienone exerts effects on its own or only in a social or in an erotic context. It further remains unclear whether it has any effects at all, or effects on positive or on negative mood or on alertness and arousal. Finally, it is not clear whether androstadienone decreases positive mood or increases sexual arousal in males.

In addition to mood, a number of studies investigated whether androstadienone affects physiological arousal (assessed by measuring the activity of the autonomic nervous system) in men and women. Unfortunately, similar to the mood studies, the indicator of the autonomic arousal strongly varied between studies. Accordingly, some studies suggested that the exposure to androstadienone in the presence of an opposite sex tester affects physiological arousal, whereas others suggested that a specific erotic context is necessary for these effects to occur. According to *Jacob* et al. [52.94], androstadienone increased the activity of the autonomic nervous system in women (reduced the skin temperature and increased the skin conductance) in the presence of a male tester. Similar results were reported by *Lundström* and *Olsson* ([52.95], autonomic arousal as measured by finger pulse and skin temperature) and by *Bensafi* et al. ([52.96], combining eight physiological parameters to a physiological arousal index: skin conductance response, electrocardiogram, finger pulse, ear pulse, blood pressure, skin temperature, abdominal respiration, and thoracic respiration). However, another study reported null effects of androstadienone exposure, even when presented by an opposite sex tester ([52.97], separate testing of seven autonomic nervous system parameters). In this study a decrease in the physiological arousal level (increase in skin temperature) in women was observed, when administering androstadienone in an erotic context (erotic movie).

In addition, some studies tested whether the autonomic nervous system of males is affected by androstadienone exposure: Androstadienone decreased the activity of the autonomic nervous system in men (increased the skin temperature), independent of the tester's sex [52.94]. Similar results were reported by *Bensafi* et al. [52.96], calculating a physiological arousal index. However, again, in a third study, effects on the physiology of males did only occur when androstadienone was presented in an erotic context [52.97].

Only a few studies investigated whether androstadienone affects the endocrine system of the perceiver. *Wyart* et al. [52.98] presented androstadienone to females by a male tester and in the context of erotic video clips. These authors could show that the increase in activity of the autonomic nervous system after stimulus exposure was associated with an increase of the stress hormone cortisol. However, this effect was not confirmed in males by a more recent study [52.99]. Here, androstadienone, presented during a social interaction, did not change the cortisol level in men.

In summary, some studies suggest an increase while some suggest a decrease in physiological arousal in women after exposure to androstadienone. In addition, one study indicates the release of stress hormones (cortisol) in females smelling androstadienone. These effects sometimes occur in the presence of a male tester, and sometimes they can only be observed in an erotic context. In males, androstadienone exposure seems to decrease physiological arousal, however, the necessary context conditions for this effect are not specified.

Another area of research on androstadienone is related to possible effects on social perception. It was observed that androstadienone did not affect attractiveness ratings of male faces (on pictures) in females [52.95], and furthermore that androstadienone did not alter attention to male and female faces in male and female participants [52.93]. Albeit androstadienone seems not to change face perception, its perception might inherently be linked to the perception of males (and not of females). Accordingly, dynamic point-light displays reflecting walkers of ambiguous sex are categorized as more male-like by females exposed to androstadienone as compared to females smelling a control solution [52.100].

To the knowledge of the author, two studies assessed whether androstadienone directly affects social behavior in humans. *Saxton* et al. [52.101] reported that females chose males more often during a speed-dating event in order to meet again, when exposed to androstadienone as compared to water exposure. However, the selection rate was increased only in one out of three studies, and did not exceed the selection rate in females exposed to clove oil. In an economic exchange context, males who were exposed to androstadienone offered more and asked for less money than males exposed to a control substance (dry yeast), thus behaving more generously while being exposed to androstadienone [52.99].

Multiple brain clusters have been observed to be activated by androstadienone. Brain areas related to emotion processing (prefrontal cortex, cingulate cortex, amygdala and hypothalamus), to attentional systems (visual cortex, parietal cortex, thalamus, basal ganglia, premotor cortex, cerebellum), and to the olfactory system (inferior temporal lobe, hippocampus, amyg-

dala, thalamus) seem to process androstadienone in females [52.102]. However, as androstadienone was diluted in clove oil and smelled similar to the control (clove oil without androstadienone), it is rather puzzling that the test solution activated attentional and olfactory systems in a different way than the control solution. In the same year, another working group presented the same substance in much higher concentrations (in crystalline form) and found far less areas to be involved in androstadienone processing in females (hypothalamus), and no significant activations in males [52.103]. As androstadienone perception was compared to the perception of pure air, it is surprising that no other brain regions, responsible for attentional feature processing, were significantly activated. Later on, a study from the same working group compared androstadienone activations with brain activations evoked by neutral, positive, and negative odors [52.104]. Here, two large brain clusters (within the lateral prefrontal cortex and superior temporal cortex) discussed to be related to social cognition were observed to process androstadienone in females. Summarizing, the results revealed by functional brain imaging studies are again highly inconsistent. Furthermore, the validity of these studies is highly questioned, comparing the effects of androstadienone, as a substance that cannot be perceived chemosensorily but activates large brain areas [52.102] and as a substance that has a distinct smell on its own but activates either very small subcortical, or large neocortical brain areas [52.103, 104].

52.4.2 Substances Related to Female Body Fluids

Female Copulins

Since the late 1960s molecules deriving from female body fluids, functioning as chemical attractants, have been searched for. A possible candidate was the so-called *copulin*, which seemed to derive from vaginal secretions from female rhesus monkeys. It was reported that these secretions stimulate male sexual interest and behavior (mounting and ejaculation, [52.105]) and consist out of five acids: acetic, propionic, isobutyric, butyric, isovaleric and isocaproic acid [52.106]. Similar mixtures of these short-chain fatty acids have later been reported to occur also in human vaginal secretions [52.107]. Consequently, it has been speculated whether these copulins have an impact on human sexual intercourse [52.108]. However, it became evident later on that all experiments demonstrating behavioral effects of copulins were based on the data obtained from only six rhesus monkeys, which were preselected as positive responders. In addition, two of these monkeys contributed to more than 50% of the data [52.109]. Accordingly, in a series of well-controlled experiments conducted in an independent laboratory, neither the odor of vaginal secretions of rhesus monkeys nor the supposed mixture of fatty acids had any effects on the behavior of male rhesus monkeys [52.110]. The critique on the experiments on copulins in primates and humans was overtly stated in the early reviews on pheromones [52.88, 111, 112] and the interest of the scientific community in these female chemical attractants strongly declined during the 1970s. Only once, some time later, a single study examined chemosensory effects of short-chain fatty acids on social behavior in men and women and found no effect [52.83].

Estratetraenol and Estratetraenyl Acetate

The second wave of publications on single molecules released by female body fluids that should act as chemical attractants on males was related to the estrogen-related molecules estratetraenol and estratetraenyl acetate, both of which were considered to activate the human VNO and to be pure pheromones since the appearance of the publications of *Monti-Bloch* and *Grosser* in the early 1990s [52.61, 62]. However, only estratetraenol has been reported to be detectable in human body fluids, namely in the urine of pregnant females in the third trimester [52.113].

In a first study [52.91], mood was assessed on 14 different scales, which were combined into three mood dimensions via factor analysis (positive-stimulated mood, alertness, negative-confused mood). Estratetraenol affected mood only immediately after exposure (6 min), but not 2, 4, and 9 h after initial exposure. During the first mood measurement, females experienced a stronger positive mood than males, while exposed to estratetraenol. However, negative mood and alertness were not affected by estratetraenol. In the second study [52.94] mood was assessed on 17 different scales, which were reduced to three main mood dimension through factor analysis (alertness, bipolar mood, relaxed/open). The ratings were obtained prior to and after (6 min) exposure to estratetraenol, masked by clove oil. In addition, it was analyzed whether the sex of the tester modulates the effects of estratetraenol. However, estratetraenol did not affect mood in either condition. In the same study [52.94], it was also investigated whether estratetraenol has an impact on the activity of the autonomic nervous system. In men, estratetraenol increased palmar skin temperature and the skin conductance level; however, this was independent of the sex of the tester. In women, the skin conductance level increased through estratetraenol exposure in the presence of a male tester. Women's hand temperature was not affected by estratetraenol exposure in either context.

In a later study [52.96] two mood dimensions (positive mood, negative mood) were extracted from 16 emotional descriptors and mood was measured four times after estratetraenol exposure (10, 20, 30, 40 min after exposure). However, estratetraenol did not affect positive or negative mood or sexual arousal, neither in men or in women, regardless of the time point of measurement. In addition, the activity of the autonomic nervous system was measured via eight parameters (skin conductance response, electrocardiogram, finger pulse, ear pulse, blood pressure, skin temperature, abdominal respiration, and thoracic respiration) and the digitized data were separated into four measurements, paralleling the mood ratings. Perception of estratetraenol did not affect any of the physiological measures, at either time-point, neither in men or in women. A second study of the same working group [52.97] used the same methods in stimulus administration and data recording (except for not measuring blood pressure), but presented estratetraenol in three different contexts (during the presentation of either a happy, or a sad, or a sexually arousing movie). Estratetraenol did not affect any of the physiological parameters in either context, neither in males or females, and did also not affect positive or negative mood, but increased sexual arousal in males and females in the erotic context.

A subsequent study [52.114] used a design similar to *Jacob* et al. [52.94], but focused on the effects of estratetraenol in males and increased the number of study participants ($N = 21$ males in: [52.94]; $N = 80$ males in: [52.114]). In this study, mood (positive and negative mood, extracted by factor analysis from eight descriptors) and physiological arousal (skin conductance level, heart rate) were tested in the presence of a male or a female tester, 20 min after stimulus exposure. Estratetraenol had no effect on the physiological parameters but increased negative mood in the presence of a female tester and decreased negative mood in the presence of a male tester.

Brain activations through estratetraenol exposure were investigated in a PET experiment [52.103]. Pure, unsolved, estratetraenol in crystalline form was presented and its perceptual qualities were rated to be similar to those of androstadienone, with no rating differences between the sexes. Estratetraenol activated parts of the olfactory cortex in both sexes (amygdala, piriform and insular cortex, significantly in females and as a trend in males). In addition, only in males, the hypothalamus was activated through the smelling of estratetraenol. As the hypothalamus releases hormones that activate the development of sexual hormones, it was concluded that estratetraenol has *pheromone*-like effects in males.

One study investigated the brain response to estratetraenyl acetate in eight male volunteers by means of fMRI analysis [52.115]. Estratetraenyl acetate was diluted in mineral oil and presented in a high and a low concentration. As the low concentration was detected at chance level, it was concluded that it was perceived below the threshold level. However, largely independent of the concentration, estratetraenyl acetate induced activation within the anterior medial thalamus and the inferior frontal gyrus.

Summarizing the studies on estrogen-related compounds, there are no consistent effects among studies. Mood effects of estratetraenol were observed in two studies [52.91, 114], but could not be confirmed by three other studies [52.94, 96, 97]. In addition, the observed mood effects are not consistent with a hypothesis of estrateraenol being attractive for males: the observation that estratetraenol induces a decrease of positive mood in males, relative to females [52.91] would rather indicate that males avoid the signal. Furthermore, the finding that estratetraenol increases negative mood in males, especially when a female (tester) is present [52.114] indicates that stimulus avoidance in males will be even stronger if females are around.

Furthermore, it is not clear whether estratetraenol has an effect on arousal and alertness. Two studies could not find effects of estratetraenol on self-reported arousal [52.91, 94], similar to three studies that could not find effects of estratetraenol on physiological indicators of arousal [52.96, 97, 114]. However, one study [52.94] indicated an increase in sympathetic activity in males and females.

One study reported that estratetraenol exposure increased the self-reported sexual arousal [52.97]. However, as this effect was similar in male and female perceivers, its ecological relevance is strongly questionable. According to this study males and females were equally attracted by female *pheromones*.

The brain imaging studies do not illuminate a straight summary of the findings either. In the PET study [52.103], estratetraenol was presented in very high (pharmacological) concentrations, exceeding the physiological concentration range by far. The fMRI study on estratetraenyl acetate [52.115] found effects of subliminal (unconscious) perception on the attentional gating system (thalamus). However, attentional systems, per definition, depend on conscious stimulus processing and are inherently related to alertness, response selection and limited processing capacity [52.116, 117], and the thalamus is the main relay for these attention-related processes [52.118]. Thus, it seems to be impossible for subliminal stimuli to require neuronal capacity that is usually associated with conscious stimulus processing.

52.4.3 Methodological Suggestions

The studies on androstenes as male pheromones and estratetraenol as a female pheromone did not reveal any consistent results. This is due to a number of methodological flaws, which are related to:

1. A misunderstanding of the biological underpinnings of chemical communications in humans and animals
2. A misunderstanding of experimental and statistical methods, and
3. Missing theories that could explain the results.

These three areas of deficient research strategies will be outlined in detail.

Biological Underpinnings of Chemical Communication

The message conveyed through chemical signals always depends on the concentration of the chemical substance(s), and is usually valid only within a certain time frame. Moreover, in general, molecule mixtures, but not single substances, build up the signal and it is to be expected that the message conveyed by the chemical(s) varies with the internal and the external context.

Concentration of the Chemical Signal. Going back to the time of Paracelsus (1493–1541), a general law was stated that the effect of all substances always depends on their concentration (*dosis sola facit venenum*, the dose [alone] makes the poison). Thus, it has been known for 500 years that the effect of environmental substances on human body (receptor) cells is inherently contingent on their concentration. In this sense, all kinds of chemical communication in animals discovered so far are considered to be concentration dependent [52.19]. As an example, ants respond to almost all volatile compounds released by other ants with an alarm response, if the concentration of these compounds is high enough [52.119]. In the nematode and in the termite, the meaning of the chemical signal produced by conspecifics changes with the concentration [52.19, p. 54]. In mammals, the concentration range of the efficiency of the rabbit mammary pheromone has been found to be very narrow [52.120]: newborn rabbits only respond with head searching and oral seizing behavior to the mammary pheromone in a very tight concentration range. However, despite this basic principle, which seems to be valid across all physiological systems, the research on the efficacy of single substances in humans seems not at all to care about the definition of an efficient concentration range. Axillary hair of men contains on average about 200 pmol androstadienone and human plasma androstadienone has been found in concentrations of about 50 ng/100 ml [52.34, 38]. Studies investigating pheromonal effects of androstadienone in humans often used either 250 µmol [52.91–95, 101, 102], 500 µmol [52.100], 6250 µmol [52.121], or used androstadienone in its crystal form (200 mg in [52.103]; 50 mg in [52.96, 97]; 30 mg in [52.98, 99]). Thus, across studies, the concentrations investigated exceed the concentrations in human body fluids by about one million (10^6) and more. Therefore, it is highly unlikely that in any of these studies ecologically valid pheromone effects were described.

Duration of Signal Effectiveness. The longevity of chemical signals mainly depends on their volatility and molecular weight. The higher the volatility of a single molecule within a mixture used as a signal, the faster the diffusion rate and the faster the decay of the signal. Depending on the meaning of the message, signal molecules have different effect durations. Alarm pheromones, used to inform conspecifics about potential harm, are often highly volatile in order to spread the message immediately across long distances. Sex pheromones are often composed of larger molecules, allowing a higher specificity. Marking or trail pheromones often show a very low volatility and can last for months or years [52.19]. Furthermore, in complex body fluids (e.g., human urine), small molecules (< 250 D) responsible for signal formation (e.g., related to identity), are constantly rebuild from larger molecules [52.20]. Thus, a chemical signal can change its message over time. However, a possible effect duration time of androstadienone is not known and different effect times are reported (examples of effect peak latency: within a few seconds [52.103], within less than 20 min [52.91, 94], within 20 to 40 min [52.95, 96, 121], within one to two hours [52.101, 102]). As long as there is no agreement about the time frame of the expected effects of androstadienone, it is highly unlikely that reliable effects will be confirmed.

Single Molecules or Molecule Mixtures. *Doty* [52.24] and *Wyatt* [52.19] point to the fact that most (mammalian) pheromones are multicomponent mixtures. Signals based on mixtures of volatile molecules can be extremely diverse and can be specific for certain messages and species. However, signals based on only one molecule may have the strong disadvantage of being redundant as they could be used for different messages and different species. Androstenone, androstenol and androstadienone are produced in the boar testis [52.122], and all induce the mating stance in female pigs in estrus [52.123]. If these substances were equally effective in female pigs as in female humans,

female pigs should also be attracted by human males and female humans should be attracted by male pigs. These preposterous conclusions are critically put to the tip of the iceberg by *Doty* [52.24], concluding that human birth rates should be higher in areas with pig farms, if androstenes equally act as pheromones in pigs and humans [52.24, p. 141].

External and Internal Context. For a while now, there seems to be mutual agreement in pheromone research that the meaning of a social signal varies with its context. Accordingly, in various studies the putative pheromones were presented by an opposite-sex experimenter, e.g., [52.94, 95, 98, 121]. Furthermore, erotic movies [52.97] and speed-dating encounters [52.101] were introduced as context conditions for pheromone effects. However, in accordance with *Pause* [52.76] not only the external, but also the internal context of the signal perceiver should be considered when investigating chemical communication in humans.

The internal context is related to the motivational and endocrine status of the perceiver. It is well known that the motivational status of the perceiver affects the perception of food cues [52.124] and of social signals [52.125]. More specifically, in females the neuronal processing of erotic stimuli varies with the level of sexual hormones [52.126, 127] and it has also been suggested that the sensitivity to androstadienone might vary with the reproductive state in females [52.128]. Thus, an appropriate context should increase the likelihood of evoking an appropriate response in the perceiver. This is possible whenever the physiological system of the perceiver is primed for optimal stimulus processing and stimulus response.

Finally, it should be considered that the response to 16-androstenes could be affected by the amount of experience with the substance [52.129, 130]. After repeated exposure to androstenone or androstadienone, formerly anosmic subjects become sensitized and are able to smell the substance. The sensitization process is due to peripheral [52.131] as well as to central neuronal plasticity [52.132]. After being sensitized, the hedonic profile of the odor changes [52.133]. Therefore, it is assumed that an altered behavioral response could follow the changed emotional valence of the odor.

One Receptor for Different Androstenes. 4,16-androstadienone is the precursor of 5α-androstenone, and both appear in different human body fluids, including sweat [52.38]. Humans have about 400 functional olfactory receptor genes [52.134], each of them expressing receptors highly sensitive to a single or very few ligands. It has been shown that one single human olfactory receptor (OR7D4, as compared to 334 other human odor receptors) is highly sensitive to androstenone and androstadienone [52.135]. Perception of androstenone and androstadienone varies with two allelic variants (RT and WM) at the OR7D4 locus. Individuals homozygously carrying the RT allele are more sensitive to androstenone and androstadienone and judge the odors as rather unpleasant, whereas individuals homozygously encoding the WM allele are much less sensitive to both substances and find them less unpleasant. As both substances are processed via the same receptor and as the perceptional qualities of both substances depend on the same allelic variations, it remains a mystery why only one of these substances is called to be a putative pheromone (androstadienone), while the other (androstenone) is not [52.136].

Experimental and Statistical Methods

Most pheromone studies are conducted on the basis of an experimental design. In experimental designs the number of independent variables is not restricted, however, as long as the experimenter choses to use univariate statistical tests (such as t-tests, or ANOVAs), the number of dependend variables should not exceed one. Using more than one dependend variable will lead to an inflation of the alpha error. For example, if it is investigated whether a putative pheromone affects mood through four different and independent mood scales the empirical alpha error increases from 0.05 to 0.19; if 40 independent tests were carried out, the experimentwise error rate would even exceed to an alpha equaling 0.87. Thus, as long as the dependend variables are not introduced with a specific hypothesis each, a so-called Bonferoni correction is necessary. However, a number of pheromone studies did not follow this advice and thus the reported results reached the significance level more or less accidentally (androstenol: [52.83, 84]; androstenone: [52.75, 78, 79]; androstadienone: [52.92, 97]; estratetraenol: [52.97]).

In addition to the methodological problems regarding the alpha-error inflation, the mood studies on adrostadienone suffer from an inherent methodological flaw regarding the mood scales. Instead of using reliable and validated measurements to assess mood (e.g., by questionnaires or mood scales), in almost all studies a different number of items of different scales and questionnaires were merged and regrouped with different statistical analyses (e.g., factor analysis or cluster analysis). Hereby, studies investigated different mood dimensions that only occurred in single studies (e.g., [52.91]: positive-stimulated mood, alertness, negative-confused mood, extracted from 14 mood items by factor analysis; [52.94]: alertness, bipolar mood, relaxed/open extracted from 17 mood items by factor analysis; [52.96]: two mood dimensions – pos-

itive mood and negative mood – extracted from 16 emotional descriptors by principal component analysis; [52.121]: three mood dimensions – positive mood, high aroused negative mood and low aroused negative mood – extracted from 16 emotional descriptors by principal component analysis; [52.95]: two mood dimensions – positive mood and negative mood extracted from ten emotional descriptors by cluster analysis). The validity and reliability of these mood dimensions were never assessed, and studies are not comparable.

Theoretical Background

Studies on putative pheromones in humans were never based on a theory that could account for the reason why single substances (such as androstenes and estratetraenol) should affect mood, physiology and behavior in humans. As outlined above (Sect. 52.4.1, *Androstenone and Androstenol*) the interest in androstenone obviously results from the observation that it was effective as a sex pheromone in female pigs. It took 20 to 30 years until it became obvious that androstenone does not induce the mating stance in women [52.25, 76, 77, 89, 90, 137]. Due to the never-validated results that androstadienone and estratetraenol activate the (nonexistent) VNO, a second wave of studies on human pheromones was published. However, even though it became evident that adult humans do not possess a functional VNO [52.25, 65, 66], pheromone research remained active. However, 16-androstenes could have either no function in human chemosensory communication or a very different function from being a sex pheromone. For example, 16-androstenes could convey information about the sex of the sender or about the testosterone level of the sender.

In the search for a theory that could explain how and why androstene-related molecules should affect human behavior, it is necessary to specify the conditions of androstene production. In animals, the production and secretion of androstenone are tightly linked to the level of circulating testosterone [52.138–140]. Similarly, in humans, axillary androstenone is detected in larger quantities in men than in women [52.141], and its source seems to be mainly located in the testis [52.34, 36]. Thus, a link between androstenone and testosterone in humans seems as likely as it is in animals. High testosterone levels in men seem to be related to certain personality styles (traits), like aggression, dominance and competitiveness [52.142–145]. Thus, if 16-androstenes vary with endogenous testosterone, they could function as social warning signals [52.76], and activate withdrawal instead of approach behavior. It has recently been shown that men with a higher testosterone level are more sensitive to androstenone and dislike its odor [52.146]. The authors relate this finding to the possibility that androstenone might signal the readiness for competition in men. Moreover, the odor of androstenone is also disliked by women with higher estradiol levels. Thus, also in fertile women androstenone seems to induce escape-related behavior. However, this theory would comprise the assumption that specific messages can be conveyed via single molecules.

As stated above (Sect. 52.4.3 *Biological Underpinnings of Chemical Communication*), specific social chemosignals usually require multicomponent signals. If androstenes carry significant social information, it could be expected that these substances carry relatively redundant, nonspecific information, for example about the sex of the substance releaser. Two publications point to the possibility that androstenes might carry information specific for the male sex. In the first study [52.147], females with a specific anosmia to androstenone were successfully sensitized to androstenone and brain potentials (chemosensory event-related potentials, CSERPs) in response to male and female (self-related) sweat samples were recorded before and after sensitization. The CSERPs showed a general decrease in amplitude from the first to the second session, except for the sensitized females in response to male sweat samples. It was concluded that androstenone might carry specific information about the person's sex [52.147, p. 136]. These conclusions are similar to those of a recent study on androstadienone [52.100]. In this study, dynamic point-light displays reflecting walkers of ambiguous sex were to categorize. They were judged as more male-like by females exposed androstadienone as compared to females smelling a control solution. Thus, being part of a molecule mixture, 16-androstenes could add the information about the sex of the sender (maybe indirectly via being associated with the testosterone level) to more specific information, conveyed by other substances.

52.5 Chemosensory Communication via Complex Body Fluids

In the following, studies will be reviewed that used natural, complex body fluids as stimulus. Most often, axillary sweat was presented above or below the olfactory sensory threshold. In these studies, the characteristics of stimulus production were systematically varied and thereby the transmitted information. Thus,

instead of focusing on chemical characteristics of the signal, these studies focused on the specific kind of information, chemically transmitted and the related effects in the perceiver. In accordance with *Lübke* and *Pause* [52.148] and *Stevenson* [52.149], the two main areas of chemical communication in humans investigated so far can be divided into one area related to reproduction and one area related to harm avoidance.

52.5.1 Chemical Communication Related to Reproduction

Human Mate Choice and Sexuality

The major histocompatibility complex (MHC) is a highly polymorphic gene complex, encoding cell-bound glycoproteins that regulate T cell activity. Thereby the MHC creates histocompatibility or self-identification for the immune system. It has been shown in several vertebrate species that the individual MHC-type is associated to an individual body odor profile, which can be used to chemosensorily discriminate between conspecifics. Fertile individuals prefer the body odors of partners with a relatively dissimilar MHC type to their own [52.150, 151]. It has been proposed that this differential mating helps to maintain the high MHC polymorphism within species. A high MHC polymorphism enables the species members to resist a broader array of pathogens, which is crucial for survival.

In reference to the immunological function of the MHC in humans, it is called human leucocyte antigen (HLA). Humans exert body odor preferences for HLA-dissimilar individuals, but also preferentially select partners who possess a relatively different HLA type [52.152, 153]. CSERP analyses revealed that body odors of donors with a similar HLA type to the perceiver are processed faster and activate more neuronal resources than body odors of donors with a dissimilar HLA type to the perceiver [52.154]. This result suggests that the behavioral impact of chemosensory signals related to HLA similarity might be stronger than of signals related to HLA dissimilarity. Hereby, inbreeding avoidance could be successfully achieved if MHC similarity is transmitted as a signal activating avoidance behavior. Therefore, in humans, HLA-related signals seem to be associated with a negative selection bias in mating behavior [52.155]. Recently, fMRI indicated that peptides ligands that selectively bind to allelic variants of the perceiver's HLA system are processed within the right middle frontal cortex [52.156–158].

Endocrine Status of Sexual Hormones

In general, the body odor of women changes with the endocrine status of sexual hormones. Men judge female axillary and vaginal odors as most pleasant and most sexy around ovulation [52.159, 160]. Some authors discuss these results as pointing toward human female ovulation not being completely concealed, and thus body odors contributing to successful reproduction.

One study indirectly investigated whether an increase in the level of sexual hormones in men can be detected chemosensorily by the female brain [52.161]. Axillary sweat was collected from men while watching erotic videos. Brain imaging in female participants revealed that the sex-related sweat, as compared to axillary sweat collected during an emotionally neutral situation, was primarily processed within the orbitofrontal and the fusiform cortex and the hypothalamus. The activation of the hypothalamus and the orbitofrontal cortex could be related to the processing of the emotional significance of the stimuli, whereas activation of the fusiform cortex could be related to the social nature of the stimuli.

Another area of research in human chemosensory communication is related to the phenomenon of menstrual synchrony. Women living or spending time together show a synchronized menstrual cycle [52.162]. This phenomenon seems to be due to the communication of menstrual cycle-related chemosignals [52.163]: Whereas odorless axillary sweat samples of women in the follicular cycle phase shorten the menstrual cycle of the female recipients, chemical signals derived from sweat samples of women in the ovulatory cycle phase lengthen the menstrual cycle phase of the signal receivers. This study was the first to show that the endocrine status in humans is prone to effects of human chemosignals. However, the evolutionary significance of synchronized menstrual cycles in women is still debated [52.164, 165].

Sexual Orientation

Sexual orientation is conveyed between individuals through visual [52.166–168] and auditory social signals [52.169], and sexual orientation affects the response to these signals, as has been shown with visual social signals [52.170]. It is well known that the sex of individuals can be transmitted chemosensorily [52.171, 172]. In addition, chemosensory communication of sex varies with the sexual orientation of sender and perceiver: depending on their sexual orientation, men and women display specific patterns of preferences for body odors obtained from homosexual and heterosexual men and women [52.173, 174]. These preferences may be based on divergent central nervous responses to these body odors. According to CSERP analyses [52.175], individuals showed comparably faster processing of body odors derived from individuals constituting potential partners (e.g., gay male body odor presented to other gay men). Moreover, especially homosexual in-

dividuals' responses to body odors of individuals not constituting potential partners (heterosexual male body odor presented to gay men) showed a striking similarity to responses to body odors from HLA-similar individuals [52.154]. Thus, body odors from *incompatible* individuals in regard to sexual orientation might also function as social warning signals.

52.5.2 Chemical Communication Related to Harm Avoidance

Kin Recognition

Kin recognition is most important for structuring social relations in many diverse species [52.176]. In order to promote inclusive fitness, which can be understood as the successful transmission of one's own and relatives' genes to the next generation, pro-social behavior is favored among family members [52.177]. However, unrelated individuals, like out-group members, are more easily perceived as aversive [52.178] and more easily attacked [52.179]. In primates, being around related individuals (and friends) as in-group members is probably the most important strategy in order to avoid predation from out-group members or non-primates [52.9].

Numerous studies have shown that kin can be communicated chemosensorily: Newborns are able to chemosensorily identify their mothers, mothers and fathers are able to chemosensorily identify their children, siblings recognize each other by smell, and unrelated individuals are able to match family members by smell [52.180, 181]. The chemosensory transmission of kinship can be observed in noncohabiting individuals [52.182], and rats are even able to differentiate the degree of human relatedness through olfactory cues [52.183]. So far, one brain imaging study investigated whether body odor of kin is processed differently from body odor of non-kin [52.184]. The regional cerebral blood flow was higher in the frontotemporal junction, the insula and the dorsomedial prefrontal cortex during kin recognition. The activation of the dorsomedial prefrontal cortex is discussed to be related to self-referent stimulus processing during kin recognition [52.184, 185].

In line with the ability to recognize kin through olfactory cues, humans can also differentiate self from non-self via chemosignals [52.186]. Chemosensory information about the self facilitates self-face recognition in a reaction time task [52.187] and is processed faster than non-self information by the human brain [52.147]. According to the coemergence hypothesis, self-recognition and advanced expressions of empathy appear together in both individual development and phylogeny [52.188].

Mother–Infant Communication

The survival of a newborn is fully dependent on protection through adults, usually its mother. The protection covers all needs of the newborn, especially food (milk) delivery. Through a number of studies it has been demonstrated that newborns are able to detect their mother's odor in breast skin, milk odor, and axillary odor [52.181, 189]. Infants show preferential orienting towards their mother's breast odor as early as ten minutes after birth [52.190].

Importantly, smelling their mother's odor, newborns adjust their behavior. Two-week old sleeping infants exhibit sucking movements when presented with breast odors of lactating females [52.191]. Moreover, maternal breast odors elicit directional crawling in newborns [52.192]. Breast odors of lactating women further modulate breast- and bottle-fed newborn infants' arousal state. Crying babies calm down when they are presented with night gowns worn by their own mother or unfamiliar mothers [52.193]. The maternal body odor also affects social perception in infants: In the context of their mother's body odor, four-month-old infants look significantly longer at human eyes than in the context of a nonsocial odor [52.194].

Mothers, on the other hand, are equally able to recognize their offspring's unique body odor [52.195]. Even women who had only limited contact with their infant prior to testing recognize their infants' body odor [52.196, 197]. Moreover, infant chemosensory signals seem to prime affection in adult women in order to motivate them to care for infants: When exposed to body odors of unfamiliar newborns, primiparous and nulliparous women likewise respond with neuronal activity within the reward system [52.198]. However, only mothers, in contrast to nulliparous women, show activity of the orbitofrontal cortex in response to infant odors [52.199]. This result might reflect the attraction of mothers to infant odors.

Communication of Stress and Anxiety

Stress refers to a disturbance of the homeostasis of body systems [52.200] and thus to an imbalance of physiological messenger systems (e.g., neurotransmitters, neuropeptides, hormones, cytokines, etc.). The consequences of chronic stress will include the collapse of organ functions and in the long run inevitably cause the organism's death [52.201]. Thus, stress protection is among the most important prerequisites for ontogenetic survival. One highly efficient stress protection mechanism is a fast spread of information regarding a potential harm among all potentially affected group members. The stress signal should be distributed in a fast manner, should inform conspecifics about the

specific features of the potential danger, should be efficient during day and night time, and at best, stay at the place of potential danger until all group members are informed. Chemosensory signals fulfill all of these criteria regarding powerful warning signals. Thus, it is not surprising that chemosensory alarm signals evolved in the vast majority of species of the animal kingdom [52.19]. Resembling the originally stressed animals, the perceivers show increased motor activity and immediately escape from areas with high signal strength [52.202–204]. In addition, the perception of chemosensory alarm signals leads to the activation of physiological stress systems in mammals [52.205, 206].

Recently, a number of studies confirmed the phenomenon of chemosensory stress communication in humans. In these studies, axillary sweat was sampled from individuals experiencing stress-related emotions and afterwards the effects of sweat perception were measured. In some studies [52.207, 208] the sweat donors were exposed to extreme stress conditions (e.g., first-time skydiving or exercising on a high rope course). Such conditions induce strong physiological arousal in the sweat donors and activate a diverse set of physiological systems related to a mixture of different positive (e.g., surprise, joy) and negative emotions (e.g., disgust, fear). In other studies, emotion-inducing movies (related to happiness, anxiety, or disgust) were presented and thus the induced feelings were relatively weak but rather emotion-specific [52.209–211]. Another approach was realized by sampling sweat from university students while waiting for their final examination in order to obtain their academic degree [52.212, 213]. According to self-ratings, the sweat donors experienced anxiety, but no other emotion, and their physiological stress level (cortisol) was increased. This procedure induces a specific and relatively strong emotion. In the following, the nonspecific term *stress* will be used to describe effects observed in different studies. The more specific term *anxiety* will only be applied to studies that investigated this specific emotion.

Humans have difficulties in identifying the emotions of sweat donors [52.207, 209, 210, 214] and it has been reported that the chemosensory sweat stimuli were hard to detect in comparison to room air [52.212, 213, 215]. Therefore, the effects of stress related chemosignals seem to occur predominately without conscious experience.

Three effector systems have been described consistently across studies: First, in the context of chemosensory stress signals social perception is sensitively tuned to detect signals related to harm or danger. In detail, the perceptual acuity for social safety signals (happy faces) is reduced [52.208, 212] and the perceptual acuity and attention allocation for social threat signals (fearful and angry faces) is increased [52.207, 214, 216]. Moreover, alertness to indifferent social signals (ambiguous and neutral facial expressions) with no clear meaning is enhanced [52.217].

The second system that changes in the context of chemosensory stress signals is the motor system. Humans respond with an augmented startle reflex to sudden loud tones, in the context of chemosensory anxiety signals [52.218, 219]. This resembles the motor response of rats to startling noise in the context of chemosensory alarm signals of stressed conspecifics [52.220, 221]. As the startle reflex is an indicator of the activation of motor systems related to withdrawal behavior, it can be concluded that motor systems related to signal avoidance are automatically primed through the perception of stress-related chemosignals of conspecifics.

The third system that changes its activity in the context of chemosensory stress signals is the human brain: A study based on chemosensory event-related potentials (CSERPs) showed that the processing of almost odorless chemosensory anxiety signals requires enhanced neuronal energy (P3 amplitude) originating from medial frontal brain areas [52.213]. In a first brain imaging study it was shown that sweat samples collected during the experience of anxiety activate brain areas involved in the processing of social emotional stimuli (fusiform gyrus), and in the regulation of empathic feelings (insula, precuneus, cingulate cortex; [52.215]). It was concluded that the physiological adjustments in response to chemosensory anxiety signals seem to be mainly related to an automatic contagion of the feeling. The emotional contagion hypothesis is confirmed by the findings that fear chemosignals generate fearful facial expressions [52.211] and induce increased state anxiety [52.222] in the perceivers. In contrast to the anxiety study, stress-related sweat (from first-time skydivers) was mainly processed within the amygdala [52.207]. It is reasonable to assume that the perception of stress-related chemosignals does not activate emotion- and empathy-specific neuronal networks, but only less specific structures (like the amygdala), which prime nonspecific autonomic adjustments.

Thus, in summary, emotional states that are caused by potential threat or danger and associated with feelings of stress, fear or anxiety activate the release of chemosensory signals. These signals are processed in brain areas related to social fear perception and emotional contagion, prime withdrawal behavior and tune visual social perception to sensitively detect harm.

52.6 Outlook

The studies on human chemosensory communication using natural body fluids as stimulus reveal that many different kinds of information are transmitted via chemosignals. It has been outlined that the domains of chemosensory communication are related to phylogenetic (reproduction-related behavior) and ontogenetic (harm avoidance) survival. Therefore, chemosensory communication in humans touches social behavior with a high biological significance. Here, it is proposed that we are just beginning to understand human chemosensory communication, and that many areas of chemical communication are still to be discovered. For example, besides stress and anxiety, other emotions might be transmitted between individuals. The communication of such emotional states should support the fitness of the species. First results show that social dominance is communicated between humans [52.11]. Like in other mammals, the transmission of the signals might help to maintain group hierarchy and reduce the likelihood of status conflicts between group members. Another area of information transmitted via chemical signals is related to health and age. So far it has been shown that body odor contains information about an individual's diet [52.223] and age [52.224]. In rats, body odors transmit information about illness-associated states [52.225]. As dogs are able to detect particular diseases in humans by smell [52.226] it is to be expected that humans also chemosensorily communicate their health states.

Furthermore, the science of chemical communication in humans may help to understand and even cure mental diseases that are related to disturbances in social communication. It has been shown that patients with panic disorder show an intensified processing of chemosensory stress signals in the inferior frontal gyrus [52.227] and that individuals with social anxiety show an enhanced processing of and response to chemosensory anxiety signals [52.213, 216, 218]. As social phobia is a powerful risk factor for subsequent depressive illness and substance abuse [52.228], the explanation of its pathogenesis is of special importance. For the behavioral therapy of social anxiety it may be crucial to know that social anxiety is associated with an increased sensitivity to chemosensory threat signals. Autism is another disorder with severe impairment of social communication. Children with autism spectrum disorder show reduced motor imitation skills, which might be an indicator of a dysfunctional mirror neuron system. Just recently it could be demonstrated that the imitation of the action of others is strongly improved in autistic children exposed to their mother's odor [52.229, 230]. Thus, the implementation of body odors into psychotherapy will enhance social behavior among autistic individuals.

On the other hand, individuals with a high social intelligence might use social chemosignals highly effectively in order to adapt successfully to their social environment. In line with this consideration, individuals scoring high in social openness (being highly sociable and assertive, being able to initiate and maintain social contacts) process body odors within their brain reward systems (caudate nucleus, [52.231]). Furthermore, emotionally highly competent individuals, presumably also tending to be socially skilled, outperform less emotionally adept individuals in identifying familiar persons by their body odor [52.214]. In the beginning of this chapter it was highlighted that the development of long-term friendships between same- and different-sex anthropoid primates (including humans) was crucial for the increase of the neocortex. First data are published, indicating that chemosensation might in fact play a role in human friendship bonds, as friends share more similar olfactory receptor genes than expected by chance [52.232]. On average, two individuals have functional differences at over 30% of their odorant receptor alleles, resulting in a unique olfactory percept in each individual [52.233]. Thus, friends might experience the smelling (social) environment more alike than strangers.

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Part G Odors in

Part G Odors in Language, Culture and Design

Mankind learned to communicate immediate hedonic ratings of smells early on in the evolutionary process, using one of the oldest communication tools in the animal kingdom, i. e., facial expressions and species-specific mimics. Detection and discrimination of smells was an important strategy in survival, often with a binary differentiation of smells into *good* or *bad*, and these primary attributions could be clearly expressed, for example, by cringing. Over the course of evolution a more detailed communication of smells developed, for example, with humans pointing out to each other that they recognized the smell of specific food such as mushrooms. They maybe wanted to talk about spices, or they traced the path of specific animals not only via their footprints but also via their smell signatures. Nowadays, people might appear to be losing the competence of talking about smells, as they are no longer required to trace objects, living organisms, or food via their sense of smell. Verbal differentiation of smells, e.g., of spices, tends to be lost with less active involvement in food preparation and increasing consumption of ready-made meals, although global travel has expanded the diversified exposure to 'exotic' foods like never before. On the other hand, our modern world encounters an ever growing number of new materials and products that are, at times, associated with odorous substances that have never been encountered by human mankind before. Why these new substances are able to activate receptors, if only due to cross-reactions with established receptors, and false triggering of smell responses that were originally not created for the purpose of detecting these modern molecules, is a mere academic discussion. In any case, it may happen that smell impressions are generated in the course of such interactions and that they can barely be named, as natural analogs with which they may be associated seldom exist. In view of this, one might even consider inventing novel expressions for describing such smells, keeping in mind that the evolution of language is, in any case, a process that was only adapted to preexisting smell impressions.

Apart from the creation of language in relation to smell, creative processes can also relate to the generation of novel smell compositions, as is constantly undertaken in perfumery. This creative process is related to cultural premises, streamlines of fashion, and the zeitgeist in general. Humans use smells as an additional dimension to express themselves and their intentions in specific situations. Accordingly, it is a logical consequence that people try to induce specific attitudes using smells, to create expectations and feelings, and to leverage specific behavior, e.g., of consumers by the use of smell. Current research is, therefore, striving to monitor and predict such influencing effects on humans in specific scenarios, environments, and situations of life. In any case, the awareness of smell as another important sensory dimension in our everyday life is constantly increasing. Humans no longer only strive to optimize and control the temperature, humidity, sound and light, or visual appearance of their living, working, and leisure environments, but to also adapt the smell properties in a way that has a beneficial impact on their well-being and their attitude to life.

53. Odor Descriptions from a Language Perspective

Jeannette Nuessli Guth, Maren Runte

Talking about food is essential in our life. We love certain products, others are disgusting. While eating and drinking, we might express spontaneously our perceptions that foods have triggered. The range of reactions include nonverbal behavior, changing our facial expressions and bodily movements, verbal statements of liking and disliking including emotional and evaluative judgements, as well as analytical and neutral or objective descriptions. Our evaluation starts off with the perception of visual stimuli and volatile compounds eliciting an olfactory impression to the perception of various sensory modalities during breakdown of the food including taste, retronasal aroma and texture, as well as trigeminal impressions. As human beings, we use our senses to judge the quality of food. However, it is not always straightforward to verbalize what is perceived for all sensory impressions. Olfactory impressions are known to be difficult to describe. This chapter elaborates on language for odor descriptions from a language perspective with focus on the German and English or translated vocabularies. Culture and language are closely connected and shape the way we talk about olfactory impressions. Insights are given into vocabularies of people who are trained in describing food products and in everyday language, principally, what we experience in daily life when we are confronted with sensory stimuli.

Ein Tisch ist ein Tisch/A Table is a Table
Short story by Peter Bichsel, Swiss Writer, 1995

Naming objects helps us in daily life to communicate with the people around us. We understand each other when talking about a table, for example. The short story of Peter Bichsel illustrates how an old man has lost his connection to the outside world, when he started to name objects differently; *Bed* he called *picture*, *table carpet*, or *newspaper bed*. It is evident that he was not understood anymore nor could he follow any conversation in the world around him. What seems so obvious for these objects might not be so clear for odor perception. How do we name the odor of many objects in our world around us? How do we verbalize our perceptions? What kind of strategies does language have to support communication? Is there a universally valid concept of how we describe olfactory impressions?

Description of odors is not a trivial issue. In everyday situations or even in an experimental setting with untrained subjects many individuals feel speechless. They know what they smell, it is familiar, but they cannot name it verbally. Giving the solution is like a relief. Verbalization of sensory perceptions, especially taste and odor, is therefore a challenging process.

From a physiological perspective, perception of various signals like the different single components of foods ingested, the smell of perfumes, or visual cues of objects can be attributed to the reaction on specific receptor cells; clearly distinguishing the perception of

taste from other sensory modalities. However, the way of processing of all these signals in the brain is not fully clear. The integration of sensory signals and the subsequent verbalization in sensory analysis as well as in everyday life might need an interdisciplinary approach to better understand the perception of different sensory modalities and how we create meaning of terms in talking about our perceptions as human beings.

The difficulty of verbalization of sensory perceptions, especially odor descriptions is a common assumption as it matches everyday experience within the field of sensory and consumer science. As we often do not distinguish when talking about our perception in the mouth, flavor is used as an overall description for what we perceive while eating and drinking. According to *Small* [53.1], it is a multimodal sensory experience including the gustatory, retronasal olfactory, and oral-somatosensory signals. *Shepard* [53.2] introduced *retronasal smell* in his paper as term to avoid the double meaning of taste and a part of flavor. In sensory analysis, the definition of flavor is important as sensory panelists are trained in recognizing as well as verbalizing their perception of different sensory signals. However, this might only be true for communities speaking English or other Western languages for which lexica of sensory terms exist. Crossing cultural and language borders and investigating less known communities might reveal a totally different picture. In Jahai, a language spoken on the Malay Peninsula, people could name smell impressions as easily as colors whereas English speakers had difficulties naming odors [53.3]. A similar investigation was conducted with Maniq speakers (hunter-gatherers in Southern Thailand). The study showed that in Maniq odors can be encoded [53.4]. This underlines the fact that the chosen language plays a crucial role in how we verbalize our perceptions.

In our approach to investigate the verbalization of odors, we focus on how language deals with the descriptions of perceptions and what strategies might be used in communication to verbalize perceptions. Our experience focuses on the German language. Comparisons are mostly with terms in English as most publications list mainly English terms or terms translated into English. However, comparing Western languages, some of the concepts might be similar.

From a language perspective, the vocabulary of odor belongs to the subvocabularies of (the German) language, the size and specifics of which are hitherto little known. Although, odor is, in everyday life, in perfumeries, wine or coffee, valued and also described. However, untrained subjects find it difficult to describe odors with appropriate words: How is it possible to adequately capture in words the odor of commonplace smells like strawberries, coffee, or vanilla? And what is actually odor in understanding of untrained people? As the term *taste* in language comprises more than only the mere physiological understanding of taste limited to basic tastes, what about odor? Is the same true for this sensory modality? To address these questions focus is on German and English.

To actually learn about flavor terms in language, a linguistic perspective might add another piece to the understanding of flavor perception and in particular to odor descriptions. The objective of our language data survey for the compilation and description of the German odor vocabulary was to reveal an everyday concept of *odor*. In everyday life, we clearly understand more by odor; in everyday odor descriptions we do not confine ourselves to purely physiologically perceptible impressions, when we speak about odor, we frequently say something about the intensity – neutral, intensive or obtrusive, or of the pleasantness of the odor, good or bad.

But it is not only the extended concept of odor that leads to the everyday vocabulary of odor being relatively extensive and complex – smelling is frequently connected to emotional experiences and memories – but also appraisals, good or bad, and emotional descriptions must be counted among the odor terms.

With the example of the German language, we will elaborate on odor terms. We would like to illustrate the differences between the concept of odor in sensory science and the everyday concept. Furthermore, it is of interest to look at spoken versus written language. In spoken language, we might have more strength in describing our perceptions as nonverbal gestures or the exchange with communication partners might help picturing our efforts of verbalization. Also, the connection to the expert or specialized language in sensory analysis is made. In the following, the terms *specialized language* or *expert language* are both used when referring to the language of people trained in sensory analysis, i. e., in describing sensory perceptions professionally. Profiling of food for product development purposes is a standard procedure whereby the development of the appropriate vocabulary is a crucial step. An extended vocabulary taking into account all aspects of flavor perception helps to build a bridge to the consumer, for example, in advertisement.

The interdisciplinary approach to combine language and sensory science started off with a project on sensory semantics with focus on the consumer aspects of freshness [53.5]. A thorough investigation of taste and specific products was conducted in collaboration with different universities in Switzerland from disciplines in food and sensory science and linguistics.

53.1 Comparison of Odor Terms Used by Experts and in Everyday Language

Descriptions of sensory perception are used in both professional environment and in everyday life. As there are substantial differences of the objectives and in the way how language is used for these two groups, this chapter covers different aspects of expert and everyday language.

53.1.1 Sensory Vocabulary of the Specialized Language

In sensory science, well-established methods exist for the description of food products as well as for the measurement of intensities for the various descriptors, words used to analytically describe perceptions in sensory analysis. Trained people use their senses to evaluate and describe food products in an analytical way, without naming of hedonic, emotional, or evaluative judgements.

The typical approach in sensory analysis is to recruit people based on their sensory acuity as well as their communication skills and ability to describe sensory sensation besides availability and personality. An example of such a procedure is given in the document 8586 of the International Organization for Standardization (ISO). A part of the screening procedure is the description of odors. The ISO norms differentiate between ortho- and retronasal sample presentation. In the first example, samples are presented as smelling strips or small bottles that can be sniffed. In the latter case, aqueous solutions are ingested and evaluated. Olfactory materials include substances like benzaldehyde, menthol, and eugenol and are presented with the most common name associated with the odor being in the present case bitter almond or cherry, peppermint, and clove. Selected assessors are then trained in detection and recognition as well as in using scales for measuring the sensory sensations of interest. In principle, this is the procedure for all sensory sensations, not only for odor. However, based on the speciality of the panel, the focus can vary. It can be of interest to train assessors for a certain product category. From this perspective, they become experts in describing and measuring the sensory sensations of this specific category.

A continuous monitoring process on the performance of single assessors during an evaluation session by including replicate samples or in the long term over a period of several weeks or months guarantees the quality of the results. Within this process of generating profiling data, sensory descriptions and intensities of the sensory sensations measured by the assessors, the development of a vocabulary or lexicon is an important step. In most cases, the panel starts from a pre-existing list of terms for the description. For many products or product categories, separate lexica as a source of descriptors or terms for describing sensory sensations are published, as apparently, no universally valid lexicon exists. Selected examples of recently published sensory lexica on a broad variety of food are given in the references [53.6–17] For all lexica is common that panelists are trained extensively and are confronted with a large set of products for term generation. In many cases, the initial list of descriptors usually embraces a larger variety of terms, which are reduced in panel discussions to a list that includes terms that are nonredundant and are considered to be discriminative for the purpose of product description.

It is not surprising that lexica principally exist for specific products only as the terms for descriptions need a context to be fully understood by the assessors. Definitions of terms and reference substances might only be appropriate for the product chosen and not universally valid with the exception of taste references for sweet, sour, or salty. Thus, the expert assessors are trained in describing and measuring their perceptions with the help of definitions and references in a reproducible and consistent way.

It is indeed an artificial situation in which human beings are trained in using a custom-made language for the specific purpose within sensory science. In most instances, this custom-made language is valid and understood within the community that has developed the language, mostly the panel, that is, the group of assessors.

To illustrate what type of terms and the versatility of odor descriptions are present in the domain of sensory science, the above cited references of 12 publications and 13 product categories for sensory lexica [53.6–17] were taken as the basis for odor term evaluation. All the publications chosen are in English whether or not the original language of descriptor development was English or another language except for the study on Kimchi [53.8]. Translation of descriptors is a critical issue, which is however not addressed in the evaluation of the terms.

In total, over 200 terms were extracted, including words listed multiple times. Overall, from the terms the following observations can be made:

- $\approx$ 170 Different terms or word combinations were listed.
- $\approx$ 140 Terms were single words like fruity, caramel. rancid, in contrast to word combinations, colloca-

tions (cooked lemon juice, cut grass, canned tomato, fresh citrus etc.).
- About 60% of the terms name an object like peach, tobacco, chocolate, bacon, dried plum, spiced tea, or thyme.
- Twelve terms mentioned chemical substances like diacetyl, sulfur dioxide, carvone, eugenol, or ethyl acetate.

Table 53.1 shows the terms found multiple times with their definition and references if available. Some authors present only the descriptors [53.1, 15]. The list includes only terms that were identical and they were *citrus, fruity, fermented, green, molasses*, and *woody*. For citrus, molasses and woody and to a lesser degree for green the definitions are comparable. Citrus is always related to citrus fruits and green to grass or vegetables whereas in the case of fruity and fermented the definitions vary more. References strongly depend on the product category investigated and/or the cultural background. This becomes evident if a panel group is investigating an unfamiliar product and needs to gain experience first. This shows the example of a US panel that started with the sensory evaluation of Kimchi. In the process of lexicon development, the panel traveled to South Korea to refine the lexicon for this product category and to test actual samples [53.8]. As Kimchi might gain more global attention according to the authors, the awareness for the product outside Asia might increase and as a consequence increase the experience of consumers with the product.

It has to be noted that the examples are more or less common products and include results of the classical sensory science approach. In other domains like wine, expert language might be linked to less accurate evaluation and thus, the word lists would be more extensive including not only sensory descriptors. *Lehrer* [53.18], for example, differentiates between two types of experts for wine evaluation, the ones with scientific ambition and those who are in wine trade, sommeliers, or wine writers.

What can be extracted from these observations for expert language in general and with respect to odor terms:

- Sensory scientists elaborate comprehensive lists of terms for a specific product category that differentiate between products when the terms are used for intensity evaluation.
- Panel discussions make sure that all members evaluating the products know what the terms mean. In order to facilitate this process, definitions are elaborated and references are presented to the panel members so that they can taste or smell this reference and match their perception with the term given.
- The definitions alone often advise of associations. The reader of the definition has to know the object of the association by either experience during daily life or by evaluation of the reference.
- Considering odor terms, in particular, most of the terms name an object and not the sensory impression involved. Occasionally, names of chemical structures are given. Especially for experts in the aroma analytics domain, people are trained in linking a sensory perception to a distinct chemical substance and naming it which as such is not the description of the sensory perception either.
- Single terms naming objects are often not sufficient to precisely describe a perception. Fruits change their aromatics during ripening and during processing (cooking), resulting in terms like cooked plum, dried tomato, overripe fruit or processed berry juice. Other terms relating to a certain state of the products are rancid butter, spiced tea or dark chocolate.
- Word creations with the expression – *like* were hardly listed (hay-like, wine-like). This is more common for everyday language when talking about sensory perceptions (Sect. 53.2.2).

A curiosity is the term *other* that was mentioned in reference [53.16] as the *aromatic associated with noncitrus fruit*. The word has no relationship to sensory perceptions and can only be understood by the panel using the term.

Overall, in expert language, pure odor terms are limited in the (English) language. Most of the terms with its definition and references are applicable for the product category investigated. *Miller* et al. [53.19] made an attempt to characterize the descriptor *nutty* as an example for different food categories like nuts, grains, or beans. They could reveal five concepts for the term nutty as there are nutty-beany, nutty-buttery, nutty-grain-like, nutty-woody, and overall nutty including definitions and references for different intensities for measuring on a scale for sensory evaluation. The references, however, are quite specific for the United States and might not be so easily transferable to other countries. Although there might not be a fully developed vocabulary for expressing olfactory perception in English or German and other languages, people are still able to exchange their perceptions by the help of naming objects or physical references. Interestingly, there are cultures having limits in their language to express another sensory perception, and that is color. In languages, such as Umpila, spoken in Cape York Peninsula, Australia, or Kilivila, spoken in the Trobriand Islands of Papua New Guinea, color terms are limited in the range black–white–red. Similarly as to odor descriptions, the speakers of these languages have to find other strategies by naming objects, e.g. it is like a banana or a flower [53.20].

Table 53.1 Examples of sensory descriptors and their descriptions and references

Odor term	Definition	Reference	Literature
Citrus	Aromatic associated with general impression of citrus fruits	–	*Lawless* et al. [53.6]
	Aromatics associated with freshly squeezed orange or lemon juice	Freshly squeezed orange and lemon juice	*Bett-Garber* and *Lea* [53.9]
	Aroma associated to citrus as lemon, orange, mandarin	Natural products placed in sensory tasting glasses	*Monteiro* et al. [53.11]
Fruity	The aromatic associated with a mixture of nonspecific fruits: Berries, apples/Pears, tropical, melons, but usually not citrus fruits	No reference	*Lawless* et al. [53.6]
	–	1″ slice each of chopped Granny Smith and Fuji apples	*Haug* et al. [53.7]
	Aroma associated to tree fruits like peach, apple, apricot, plum	Natural products placed in sensory tasting glasses	*Monteiro* et al. [53.11]
	Aromatic blend which is sweet and/or sour reminiscent of a variety of different fruits	Welch white grape juice	*Cherdchu* et al. [53.12]
	An aroma blend which is sweet and reminiscent of a variety of different fruits. When possible, specific fruits were described.	–	*Suwonsichon* et al. [53.15]
	Odor/aroma characteristic of fresh olives, either ripe or unripe	Extra virgin olive oil from Aloreña variety	*Galán-Soldevilla* et al. [53.13]
	Aromatic associated with Fruity Pebbles cereals	Post-brand Fruity Pebbles	*Leksrisomong* et al. [53.16]
Fermented	The aroma of over-ripe fruit, slightly fermenting sugarcane juice	Mango juice fermented with yeast	*Smyth* et al. [53.8]
	Aromatics associated with fermented fruits, vegetables	Juice of sauerkraut, 1 part juice to 2 parts water	*Bett-Garber* and *Lea* [53.9]
	Sweet, overripe, rotten, musty. Sweet, slightly brown, overripe aromatics assoicated with fermented fruits, vegetables, or grains; can have a yeasty note	Great lakes sun dried tomato	*Cherdchu* et al. [53.12]
	A combination of aromatics that are sweet, slightly brown, overripe and somewhat sour	Blackberry WONF 3RA654	*Suwonsichon* et al. [53.15]
	Combination of sour aromatics associated with somewhat fermented diary/cheesy notes that may include green vegetaton, such as sauerkraut, soured hay or composed grass	Frank's Quality Kraut	*Leksrisomong* et al. [53.16]
Green	Aromatic characteristic of freshly cut leaves, grass, or green vegetables		*Lawless* et al. [53.6]
	A green aroma associated with fresh stems, also pear kins or watermelon rind	*cis*-3-Hexen-1-ol, floral stems, tomato stems, watermelond rind	*Bett-Garber* and *Lea* [53.9]
	Sharp slightly pungent aromatics associated with green plant/vegetable matter, such as asparagus, Brussels sprout, celery, spinach, etc.	Dried seaweed (kelp)	*Cherdchu* et al. [53.12]
	Odor/aroma characteristic of newly cut grass	1 drop of *cis*-3-hexen-1-ol in 50 ml of water or newly cut grass	*Galán-Soldevilla* et al. [53.13]
	Slightly sour aromatics, commonly assoicated with underripe fruit (astingent)	Green Granny Smith (without peel)	*Suwonsichon* et al. [53.15]

Table 53.1 (continued)

Odor term	Definition	Reference	Literature
Molasses	–	1/2 tsp. unsulfured molasses (exact product given)	*Haug* et al. [53.7]
	Dark caramel top notes, which may include slightly sharp, acrid, sulfur like of molasses notes characters	Grandma's molasses	*Cherdchu* et al. [53.12]
Woody	The flat, dark, dry, musty aromatics associated with the bark of a tree.	Oil of cedar wood	*Suwonsichon* et al. [53.15]
	Aroma associated to barrels, wood	Maceration of 1.0 g/l of french oak chips, medium toast, in ethanol (19% v/v)	*Monteiro* et al. [53.11]
	Flat, dark dry aromatics associated with the bark of a tree or wood by-products	Popsicle stick 4-Ethylguaiacol	
	Odor/aroma characteristic of wood	Wood shaving in 60 ml flask	*Galán-Soldevilla* et al. [53.13]

Looking at the sensory science literature, various methods for measuring the intensity of predefined descriptors exists. *Lawless* and *Civille* [53.21] give a summary of the most important methods and outlined in a review on how to develop lexica, that is, standardized vocabularies for the purpose of sensory analyses, and as they write to *facilitate communication across diverse audiences*. According to their view published, lexica might help to standardize the sensory language across panels, companies, or even countries. Consistency in sensory descriptions of products therefore seems to be a need as business is more and more globalized. The authors outline the procedure for collecting terms, generating definitions as well as to find the appropriate references and validation of the lexicon. From a language perspective, this approach is somehow an imposition that works well in small groups like panels. For expert language sensory lexicon development might foster communication; however, it is actually an open question whether this would be the case for nonexperts.

Giboreau and coworkers [53.22] concentrated on how to define descriptors for sensory evaluation using an integrative approach. The principal goal of the descriptor definitions, as it is the common approach in sensory science, is to minimize any ambivalence in the meaning for descriptors within a panel, but it additionally would also help to support users of the results or translations of descriptors according to the authors. Also, more subject instead of object centered definitions as it is the case for many sensory lexica might be advantageous.

It will be interesting whether within the discipline of sensory science, it will be possible to find a language that measures sensory sensations across geographical and cultural borders. As published for commonly unknown languages, simple translation of terms is not possible [53.23]. Concepts of perceptions might not be congruent as culture, type of language, and experiences in a society influence the importance of naming specific olfactory impressions. An example of frequent odor terms in Jahai, which must be classified as verbs, is given in reference [53.23]. The verbs listed cannot be translated with simple English terms. They are related mostly to unpleasant odor impressions, such as urine, blood, feces, and rotten meat. Pleasant terms are linked to sweets, cooked food, or flowers. This might reflect that cultural imprint to a certain extent is the case for all cultures and languages.

53.1.2 Comparison to Terms in Everyday Language

Language of experts or standardized language is somehow an artificial world. As outlined in Sect. 53.1.1, all precautions are taken to standardize which terms are taken for a specific sensory perception. The objective is to be able to measure the properties of food or nonfood products sensorially with a small number of people which makes it necessary to train them extensively for such a task.

This is in contrast to what we experience in everyday life. Depending on our personal experiences, our cultural background and language we might express our perceptions in a not necessarily comparable way. However, language in the written and spoken form allows elucidating our perceptions.

Urdapilleta et al. [53.24] conducted a study for the collection of odor descriptions for floral scents. They asked university students with no experience in sensory descriptions to describe the odors, which were

presented to the participants on paper strips. The participants were given the task to describe the odors as well as what they made them think of and what terms would characterize them. As a result, the terms were classified whether they were sensory terms, personal memories, intensity, hedonic expressions, or other characteristics. Not surprisingly, only 18.6% of the terms referred to sensory perception. Most of the terms, 52.8%, referred to objects. That is actually comparable to expert language. Hedonic expressions with 17.1% are common for novices in sensory descriptions as perception of food or perfume, for example, are strongly linked to liking and disliking in a first step and not especially a neutral and analytical description of the sensory stimulus.

In the domain of food, a speciality in everyday language are conversations about wine; although nowadays products like coffee and chocolate, for example, are also promoted with an elaborated language. The domain of wine offers even a more versatile language that might not only be a sensory description of the product. *Lehrer*, for example, has investigated the American language for wine [53.18]. She noted that wine language only has few pure odor terms including, for example, fragrant and perfumed. Most of the odor related terms include the name of objects like blackberry or asparagus as is the case also for the sensory lexica. Nonsensory descriptions of wine comprise evaluative terms as *balanced* or *complex* or body language as *muscular*, *brawny*, *lean* or *sleek*, words not necessarily related to odor perception in wine.

Odor descriptions of nonexperts for sensory descriptions focus also mainly on naming objects. However, the vocabulary is enriched with hedonic expressions, metaphors, intensity description and to a lesser extent with personal experiences.

The method of data collection influences the outcome, the terms listed. From a semantic perspective, two approaches are possible. The first one is an *onomasiological* approach by starting off with tasting products and then collecting terms for descriptions as well as discussing them. The second approach is called *semasiological* whereby the starting point are words, and discussions should elucidate their meaning [53.25]. To provide insights into this methodology, experimental data is presented for the German language in the following section. Furthermore, the investigation of written and spoken language is of interest as conversations allow us to better understand the strategies on how we create meaning in the exchange between partners whereas in written language creating meaning is limited to a single person.

53.2 Odor Terms in Everyday Language

The basic principles of odor terms are built up on the vocabulary of what is called taste in the German language. Since taste and odor are closely related in language as the physiological differentiation for human beings in everyday life are not of importance some insights are given on taste terms (Sect. 53.2.1). In the literature, this is described as taste–smell confusion [53.26]. Also, the duality of the olfactory sense as *Rozin* [53.26] describes it might impact what we consider as olfactory impression. Volatile compounds are either perceived orthonasally with the object being at a distance or retronasally, the situation where the object is actually ingested in the mouth.

The principles were elaborated for the German language; however, they might be applicable similarly to the English or other Western languages, but definitely not universally.

A critical note is added here to the issue of translation. Attempts have been made to publish multilanguage vocabularies. Examples are the ISO norm 5492, listing sensory vocabulary with definitions, or the special multilingual lexicon with focus on textural terms which was published as polyglot list [53.27]. The one-to-one listing of terms is critical as not for all words translation is simple without giving a context. Concepts in different languages are not necessarily congruent, especially for polysemous words, terms with several different meanings. This has been shown for *fresh* as an example [53.5].

53.2.1 Basic Taste Terms

The German taste vocabulary was collected in collaboration between linguists and sensory scientists. Different procedures (Sect. 53.2.2) were combined to collect and to confirm the terms by which taste perceptions in everyday language can be described. The result was a collection of 1000 taste terms, but also other strategies for describing taste were identified and analyzed [53.25]. Figure 53.1 shows the most important taste terms of the German language (results in English translation).

53.2.2 The Odor Terms in German Language: Data Collection

To show a concrete example of how data on language data can be compiled, our approach to investigate the

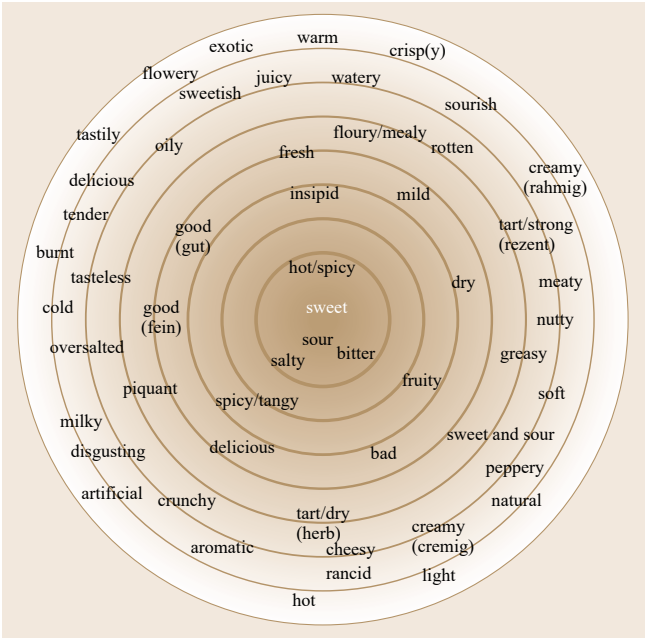

Fig. 53.1 The central taste terms of the German language translated into English

German odor vocabulary which combines three procedures is presented here:

1. The first part included the extraction of odor terms in different general monolingual dictionaries of German: Different dictionaries were screened to find all adjectives which were used to describe odor. The critical part of the extraction was that the words were used in at least one version in connection with *Geruch* or *riechen* (*odor* or *smell*, noun, and verb). These words had to be explicitly mentioned within the description of meaning or the examples.
2. The second part comprised the evaluation of the largest written language corpus of the German language called the *German Reference Corpus* (DeReKo), developed and maintained by the Institut für Deutsche Sprache (IDS, Institute for the German Language) in Mannheim. It was searched with identical criteria for odor terms.
   By using current research tools such as COSMAS II, it was also possible to detect and verify odor terms which at first glance were rather untypical, such as *grün* (green) usually mentioned in connection with oil, a descriptor quite common in sensory lexica (Sect. 53.1.1, Table 53.1), *neu* (*new*), usually in connection with new machines and cars or *warm* (*warm*), usually in connection with animals, especially horses. Additionally, it is possible to state in which way and with what frequency odor terms are used in connection with the words *odor* or *smell*.
3. The third part included the collection of odor terms actively listed by test persons using the so-called *Basic Odor Term Test* (BOTT) similar to the *Basic Taste Term Test* presented in Sect. 53.2.3.

Based on the results of the three procedures, the odor vocabulary of the German language was compiled.

The evaluation of different dictionaries resulted in (only) 44 odor terms and by searching the corpus the odor vocabulary could be completed with additional 290 terms. Finally, the BOTT revealed 451 odor terms which were actively listed by test persons. After revision of the results of the combined approach about 600 different terms were identified, which are actually used in the German language to describe odor.

It is worthwhile noting that only 125 terms were mentioned in both corpus research and in the BOTT, and therefore, the terms are used with a certain frequency. This rather limited intersection could be an indication that the actual use is either not documented in dictionaries or that the use of specific terms is reduced to certain situations, in written communication.

Furthermore, the third part with the BOTT provided illustrative information about the structure and inward lexical relationships of the odor vocabulary.

53.2.3 The Basic Odor Term Test (BOTT)

The number of terms is only partial information for a comprehensive understanding of the odor vocabulary. Structural characteristics can provide important insights about a certain sub-vocabulary, the information on how central or peripheral in terms of usage a certain word is. In order to identify those words which form the central odor vocabulary of the German language, the method of the BOTT is presented to differentiate between central and less central odor terms.

The Method of the Basic Odor Term Test

In accordance with the color term test by *Morgan* and *Corbett* [53.28] and the *Basic Taste Term Test* (BTTT), 213 students of ETH Zurich, Switzerland, were invited to write down as many words as possible with which the odor of foodstuffs or beverages can be described within a limited time frame. In order to determine the number of terms listed per minute they were instructed to draw a line under the already written words after each minute.

Additionally, data on age, gender, and the region in which they have grown up was collected. Both, number of mentions and time of mention played a decisive role in the evaluation.

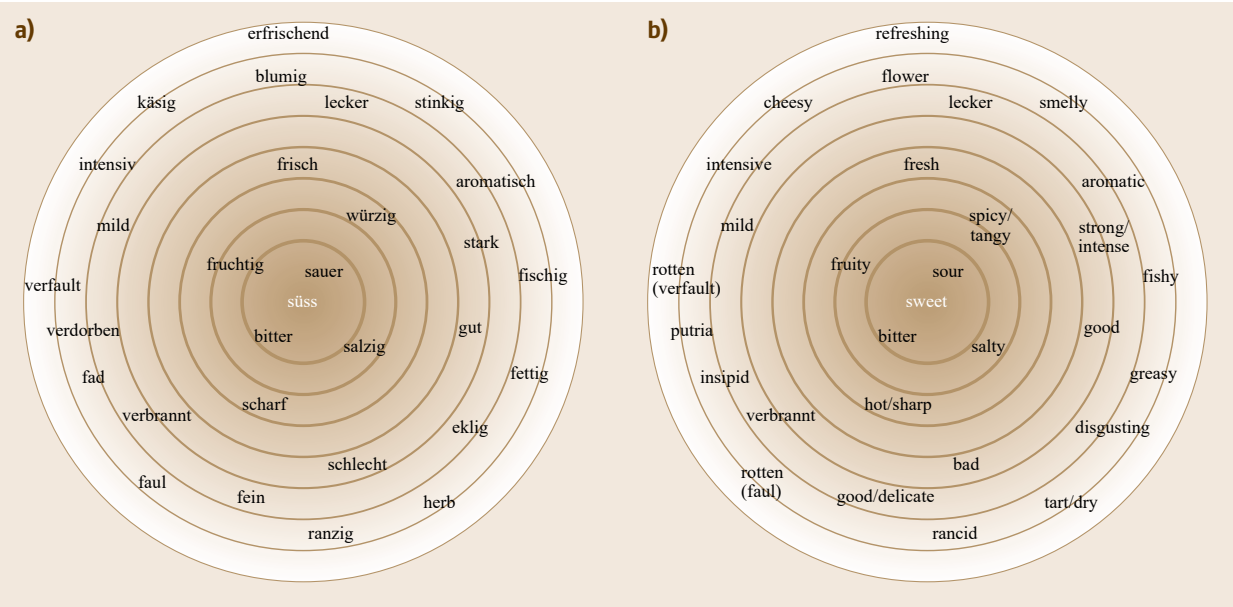

Fig. 53.2a,b The 30 most central odor terms of the German language **(a)** and their approximate translation into English **(b)**

Results of the BOTT

The evaluation of the frequency of each term and the time when a term was listed during the BOTT helped to sort the odor terms collected by the dictionary and corpus research approach according to their relevance. In total, 451 terms were listed. These results for the German odor terms are visualized in Fig. 53.2. Figure 53.2a shows the original results in German and Fig. 53.2b the approximate translation into English. Again, it has to be noted that translation of terms is critical. Especially word-by-word translations without context have to be handled with care as the meaning might not be fully captured. Furthermore, the results are valid for the German language and might be only partially applicable to the English language.

In Fig. 53.2, the position of the odor terms is indicated within the circles according to their frequency of mention with the central terms in the graph being the ones listed as the 30 most frequent odor terms.

Based on this evaluation, the following observations are especially interesting:

- *Süss* (*sweet*) is mentioned very often as well as at the very beginning of the BOTT – normally in the first minute – while all other central odor terms are mentioned later (Table 53.2). *Süss* must therefore be considered as the central odor term of the German language although it is a taste descriptor from a physiological point of view.
  The finding that *süss* seems to be the central odor (Fig. 53.2) and the central taste term (Fig. 53.1) of the German language, demonstrates the proximity between odor and taste. It also supports the fact that in everyday life no clear distinction is made between taste and odor perception [53.26].
- Therefore, it is not surprising that also other taste terms are found to be central to the odor vocabulary in the German language. They include basic taste terms in the following order: *Süss* (*sweet*), *sauer* (*sour*), *bitter* (*bitter*), and *salzig* (*salty*) (Table 53.3). The term *umami* for the fifth basic taste is not mentioned. This supports the confusion of taste and odor. Apparently, it is not important how human beings perceive a sensory stimulus, but the quality is, whether it is sweet or sour for example.
- Another interesting point is the evaluation of the perception. Positive and negative evaluation seem to be very important for the description: *Gut* (*good*), *schlecht* (*bad*), *fein* (*good*, *delicious*), and *lecker* (*delicious*) are found among the terms most frequently mentioned.
- Negative terms for the description of unpleasant odors are listed very early. Examples include *verbrannt* (*burned*), *ranzig* (*rancid*), *stinkig* (*smelly*), or *verfault* (*rotten*).
- Furthermore, intensity of odor perception is important. Terms like *fad* (*flavorless*, *insipid*), *mild* (*mild*), *intensiv* (*intensive*) or *stark* (*strong*) show the significance of intensity descriptions.
- 10.2% of the odor terms indicate the end of a – positive or negative – food processing step. They

Table 53.2 Listing of the 10 most important odor terms during the first minute of the BOTT

Odor term	Translation	Frequency of mention (%)
Süss	Sweet	78.9
Sauer	Sour	58.2
Bitter	Bitter	41.8
Salzig	Salty	37.6
Fruchtig	Fruity	26.8
Scharf	Hot/sharp	26.3
Würzig	Spicy/tangy	22.5
Frisch	Fresh	17.4
Gut	Good	13.6
Fein	Good/delicate	12.7

Table 53.3 The 20 most central odor terms of the German language

Odor term (original word)	Translation	Frequency of mention (%)
Süss	Sweet	85.0
Sauer	Sour	69.5
Bitter	Bitter	61.5
Salzig	Salty	57.3
Fruchtig	Fruity	54.9
Würzig	Spicy/tangy	51.2
Frisch	Fresh	46.0
Scharf	Hot/sharp	44.6
Gut	Good	30.5
Schlecht	Bad	27.2
Mild	Mild	23.9
Verbrannt	Burnt	22.5
Stark	Strong/intense	21.1
Fein	Good/delicate	20.2
Lecker	Delicious	20.2
Verdorben	Rotten	18.8
Fettig	Greasy/oily	18.3
Blumig	Flowery	17.4
Faul	Rotten	16.9
Herb	Tart/dry	16.9

include terms like *verbrannt* (*burned*) or *gepökelt* (*cured*, *salted*).

- A few terms make reference to different times of the year like *weihnachtlich* (*christmas(s)y*), *herbstlich* (*autumnal*), *sommerlich* (*summerly*). In this context, specific events (Christmas) seem to be inextricably linked to different odors (*zimtig*, *like cinnamon*).
- As the food we eat is more and more globalized, it is not surprising that reference is made to specific food experiences. This becomes evident with terms like *thailändisch* (*Thai*), *orientalisch* (*oriental*), or *amerikanisch* (*American*).
- The German language has various means to mark the lexical origin (e.g., the suffixes *-ig* and – *artig*, for example *zitronig*, *zitronenartig* (like lemon) or intensity of odors (the suffix *-lich*, which expresses a lower intensity). An example is *süsslich* (sweetish). It is therefore possible to adapt the term of an object into an adjective and by this actually generating a sensory odor descriptor. This is the case for 20.8% of the odor terms.

The evaluation of these results gives insight into the size and structure of the German odor vocabulary, which apparently is rather small with 451 terms in comparison to the vocabulary of taste terms as shown in the BTTT (Sect. 53.2.3) with 836 terms. Although the number of different odor perceptions from a physiological perspective is less limited than the perception of taste, the number of adjectives used for describing odors in language seems indeed to be limited.

Two explanations are conceivable for this finding. First, there are many other ways to describe odor (Sect. 53.3), the use of adjectives is not the only option. For example, phrases can be used that express a comparison including an object as *der Geruch erinnert mich an* (*The smell reminds me of*) or *das/es riecht wie* (*it smells like*). The latter example was found 300 times in the IDS corpus.

On the other hand, the BOTT reveals that many adjectives are listed that can be derived from a noun indicating a reference or an object, such as *beerig* (like berry) or *knoblauchig* (like garlic). These references can thus be described in German by ad hoc formations which are not frequently used.

As mentioned at the beginning of this chapter, the 600 odor terms collected by various procedures have only a small intersection of 125 terms. A possible explanation could be that the terms are used in different language varieties like active versus passive or written versus oral language use.

The terms found by corpus search are used in written communication. In contrast, the terms collected with the BOTT include many ad hoc formations and taste terms. The latter could be an indication that the test persons in such an analytical experimental situation do not distinguish between taste and odor. Furthermore, it might not be of relevance in daily life to do that.

As for the study of *Urdapilleta* et al. [53.24], especially naming objects occurred when collecting odor descriptors. However, the language approach has been carried out without real tasting of samples. Interestingly, the absence of a product for tasting during the task limits the variety of terms listed.

53.2.4 Odor Terms in Written Language

Evaluation of corpus data yields information on written language. This is in contrast to listing terms as in the BOTT. Consequently, differences in the list of terms

are found. Interestingly, they include terms that are often not used anymore or they belong to the language of a special group. At first, exalted terms are mentioned. An exalted word only found in the corpus data is *balsamisch* (*balmy*). Another interesting observation is that terms are used by special groups, such as educated people or experts. An example of a term used by educated people is *ominös* (*ominous*). Even terms from expert language, such as *campherartig* (*like camphor*) are included in dictionaries and used in written language, but they are rare in everyday life.

53.2.5 Odor Terms in Spoken Language

Spoken language has the advantage of being more flexible and to adapt to specific situations [53.29]. The BOTT has shown that not only lexicalized odor terms, but also terms that show a correspondent definition in dictionaries, are used. In order to be able to capture the complexity of odor perceptions and describe them to others, words, which are used in a quite different context as, for example, *hässlich* (ugly) or *grün* (green), as well as numerous ad-hoc formations, adjectives that are derivated from nouns by the suffix *-ig* like *holzig* (like wood), *erdig* (earthy), *zitronig* (like lemon), and *schokoladig* (chocolatey).

As another way of capturing spoken language data, focus group discussions are an interesting method. In our research, we could observe that in focus groups discussions the participants often use ad hoc formations to describe a specific taste. Likewise, the participants often agree after a longer discussion on a term, which includes what has been described previously. This is an analogy to the sensory panel discussions with trained assessors, in which the development of lexica is conducted in a similar way.

53.3 Strategies to Describe Odor Perceptions in Everyday Language

Based on vocabularies of people trained in describing sensory perceptions and of those being untrained, it becomes clear that pure odor description terms are not numerous. It is therefore of interest to look at the strategies language offers to express our perceptions. From corpus data and evaluation of dictionaries, some aspects were already mentioned. They include naming objects, hedonic, and intensity descriptions. In daily life conversations communication partners seldom describe an odor with sensory descriptors. It is therefore necessary to investigate the strategies that are used in discussions or conversations to describe individual perceptions to better understand the means in the German language for the description of odor perception.

In the following subsections, we want to show that the description of sensory perceptions may vary between giving a single word, discussing a term in contrast to others (word fields) or to narrative sequences.

Language data was collected by conducting focus groups discussions. Several product categories were chosen and participants were selected based on gender, age, and mother tongue (native speakers of German). Focus groups are group discussions with a limited number of participants; the moderation is limited to the activation of the conversation, so that a free discussion can develop in a relatively relaxed and informal atmosphere. In contrast to focus groups in market research, all conversations were fully transcribed based on the video- and audio-recordings including detailed information on how people were talking. This includes also nonverbal reactions and interjections like *pfui* (ugh) or *igitt* (yuck). Therefore, the results of focus group discussions can help to reveal underlying strategies in communication.

In the following, we illustrate findings with concrete language examples. They are presented in the original transcript as well as in a translated version. Emphases are highlighted by capitalization, breaks in different lengths by brackets, a short break by (.), (–) and (–) indicate medium breaks.

53.3.1 Word Fields

In most cases, a simple term is not sufficient for odor description; more than one (odor) term is needed to convey the individual perception of a person talking to the others. This is demonstrated by the delimitation of a word (and semantic clarification) within a word field (Table 53.4).

Table 53.4 is an extract of a focus group discussion with women about yoghurt. The participant Mia first attempts to describe the odor of a yoghurt sample by the term *faulig* (rotten), but is unsure herself, if this term is correct. Zoe agrees, but uses for clarification the semantically similar term *sour*, which is finally adopted by Mia as appropriate – *sour* is *okay* in conjunction with milk products.

53.3.2 Images/Scenarios

A further strategy can be observed in the focus group conversations. The participants frequently rea-

Table 53.4 Linguistic transcript for illustrating the concept of a word field based on focus group data with women and yoghurt as product example

German	English
Mia: ich glaub er RIECHT auch nicht beSONders; ich find wenn man (.) wenn man JOghurt aufmacht- und er RIECHT irgendwie stark nach- ((bewegt Hände mit gespreizten Fingern))- was: weiss nicht so (—)- ja nicht FAUlig IST ja es nicht;	Mia: I think it SMELLS not particularly well I think when (.) when you open a yoghurt cup and it has a strong SMELL like ((moving hands with fingers spread)) what: I don't know (—)- well it is not really ROTTen
Zoe: Ja EINmal dieses SÄUerliche	Zoe: Well for a start this is kind of SOUrish
Mia: Ja (–) ja- aber so SAUer ist ja mmh in verbindung mit MILCH oKEY weil es gibt ja auch so verschiedene KÄse die ziemlich heftig RIEchen; und aber das ist dann so diese EIgenart das ist oKEE	Mia: Yes (–) yes- but so well in connection with MILK sour is okay because there are also so many different CHEEses that smell pretty intensive and but that is then this peculiarity that is okAY

Table 53.5 Linguistic transcript for illustrating the concept of a scenario based on focus group data with women and bread as a product example

German	English
Ida: Aber wie das (.) um das BROT irgendwie den geSCHMACK zu beschreiben; und dann sind mir so (—)- eben so BAUMpilze in den sinn gekommen also wenn du in den WALD gehst- Ida: Und dann ((mit den Händen Pilze anzeigend)) überall- die PILze an den BÄUmen hängen so riecht (.) also so wie das RIECHT und so SCHMECKT auch das brot. also (–) vielleicht auch ein bisschen so- wie TRÜffel oder so (—); aber irgendwas das aus dem BOden kommt; und aus dem wald-	Ida: But how to do it (.) the BREAD somehow to describe the TASTE and then what occurred to me (—)- was TREE fungi. like when you go into the FOREST Ida: [and then ((demonstrating with her hands)) everywhere the FUNgi hanging on the TREES. smells like this I mean, it SMELLS like this and that's how the bread TASTES. well maybe also a bit like like TRUffles or something. but something that comes out of the GROUND and out of the forest.
Joy: ERdig;	Joy: EArthy.
Ida: mhm (.) also ähm GANZ genau	Ida: mhm (.) well, umh, PREcisely.
Ada: ERdig ist gut	Ada: EAarthy is good

Table 53.6 Linguistic transcript for illustrating the concept of a description with the help of prototypical references based on focus group data with men and natural/artificial as examples for important sensory descriptors

German	English
Tina: Und WIE SCHMECKT es dann (.) wenn es KÜNSTLICH schmeckt (–)? was macht dieser (.) KÜNSTLICHE GESCHMACK aus (2.45)	Tina: And HOW does it TASTE like (.) if it tastes ARTIFICIAL (–)? what makes this (.)TASTE ARTIFICIAL (2.45)
Rolf: Hat auch VIEL mit GERUCH zu tun und wenn der GESCHMACK so n bisschen is wie DAS (—), wenn man ne TÜTE aufmacht, und es riecht nach PLASTIK, dann (—) hat man oft SCHON- den GESCHMACK so: Auch auf der ZUNGE-	Rolf: It has a LOT to do with ODOR and if the TASTE is a little bit like THAT (—), when you open a BAG, and it smells like PLASTIC, then (—) one often has ALREADY- the TASTE on the TONGUE-

son using references to concrete situations or describe possible or actual experiences with different products. For these verbalization strategies the participants sketch action scenarios, pictures and scenes by means of which they contextualize odor terms and thereby concretize their vague meanings at the same time.

Table 53.5 is an extract of a focus group discussion with women about bread. The participant Ida uses the image of fungi in the forest to describe the

odor of a particular type of bread, because an appropriate word for the odor is missing. Joy finally tries to put the description in one word – *erdig* (earthy). It has to be especially noted that Ida illustrates not only by using a picture but supports her description by gestures.

53.3.3 Prototypical References

Prototypical references are of great relevance for the description of odor terms. They can specify the meaning of odor terms and give an indication of the specific contexts in which the terms occur in everyday language use.

It was observed that prototypical references are an important strategy for the clarification of odor terms, as also generally for the verbalization of taste [53.25] and odor perceptions.

Table 53.6 is an extract of a focus group discussion with men about the taste terms *artificial* and *natural*. To describe what constitutes an artificial flavor, the participant Rolf first makes a comment on artificial odor, at which he gives the scent of a plastic bag as reference for artificial odor.

53.4 Conclusion

Investigations on how we talk about odor perceptions reveal interesting features of language and by this, our ability to verbalize them even if language is limited to specific terms. It is clear that the type of method used for this kind of investigation influences the outcome. Our focus was on different language-based approaches.

Depending on the language investigated, the number of terms to precisely describe or other strategies to capture our perception can vary. Furthermore, there are differences between people. Training, for example, in verbalizing perceptions as it is done in sensory analysis might influence the way terms or strategies in language are used. Research on the German language demonstrated that for this specific language, the odor vocabulary is limited. There are differences in vocabularies between different groups of people whether they are trained or not. However, the lack of clearly defined terms is evident for all groups. The most prominent feature for describing odor is probably the fact that objects are named or ad-hoc formations occur.

In comparison to the taste vocabulary comprising approximately 1000 terms, the German odor vocabulary which consists of 600 terms is rather small. For this fact, two explanations can be given. First, the taste vocabulary also includes other perceptions as taste in physiological sense (Fig. 53.3). Texture terms (crunchy, creamy), description of mouth feel (dry), indication of temperature (warm, cold) or hedonic descriptions are all taste terms from a language perspective. A second reason could be that other constructions are used like the phrase *that smells like*, as the naming of a reference is more conventional than the use of an odor adjective.

Figure 53.3 shows an illustration of the type of terms used for odor as well as for taste perception in the German language and how they are connected in everyday language. It is clear that there is a certain overlap as we often do not distinguish between the perceptions of different sensory modalities as it is known for the confusion of taste and smell [53.26].

Overall, it can be concluded that language offers versatile strategies to express our sensory perception even though we might not have precise terms for them as the clear description of basic taste like salty or sweet. Furthermore, in communication we can understand each other although we might use vague descriptions. Verbalizing sensory perceptions, something very personal happening in our body, might not be a major issue in daily life. However, understanding how language is used to verbalize sensory perceptions supports the professional approach to describe sensory perceptions.

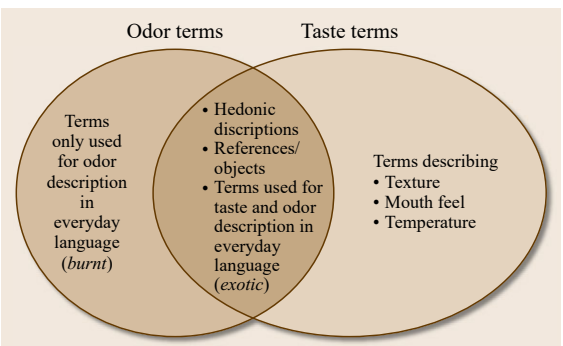

Fig. 53.3 Odor and taste terms identified in everyday language: similarities and differences

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54. The Scent Creation Process

Elise Sarrazin

The scent creation process consists in creating an original and attractive combination of fragrance ingredients. As the great perfumer Jean Carles said in 1961 *perfumery is an art, not a science*. Perfume creation indeed is far from easy and results from an extensive work of olfactory training and memorizing. Moreover, various parameters now have to be taken into account to develop a new fragrance. Technical aspects, such as performance, stability, as well as regulation and toxicology induce new issues that perfumers have to consider during the creation process. Perfume creation is thus at the crossroads of creativity and technology, art and science.

54.1 The Role of Perfumer, Between Craftsman and Artist

54.1.1 A Brief History of Perfumery

Ancient Perfumery

The word perfume derives from the Latin *per fumum*, meaning *through smoke*. Perfume is a mixture of fragrant essential oils or aroma compounds, fixatives, and solvents used to bring a pleasant scent. The art of making perfumes began in ancient Egypt and was later developed by the Romans and the Persians (Fig. 54.1). In these civilizations, perfume was rare and mainly used during religious rituals, as attested by ancient texts and archeologicals digs. Then, between the eighth and the fourteenth centuries, the Arabs significantly improved perfumery by developing steam distillation for plant extraction.

Perfumery was introduced in Europe during the fourteenth century by Crusaders who came back from the Holy Land. The use of perfume then spread throughout Europe among the aristocracy and the first modern perfume, consisting of scented oils blended in an alcohol solution, was created in Hungary in 1370, at a command of Queen Elizabeth of Hungary. During Italian Renaissance, the art of perfumery prospered in Europe and Venice became the capital of perfumes. Later on in the sixteenth century, Catherine de' Medici introduced Italian refinements in France. France was already known for its fragrant extracts made from flowers cultivated in the region of Grasse, and gradually became the European center of perfume and cosmetic manufacture.

Modern Perfumery

At the end of the nineteenth century, the various limitations of natural ingredients, as well as the need of a broader palette encouraged perfumers to introduce synthetic compounds already identified in nature, such as vanillin or coumarin. Since then, modern perfumery began. During the twentieth century, the increasing use of synthetic compounds in fragrance compositions made chemical industry essential for perfumery creation. As the perfumer's palette was significantly extended, the use of perfumes was popularized and lost its elitism as explained by *Elisabeth de Feydeau* [54.1]. Perfumery became a global industry, and numerous small local companies merged or were absorbed by bigger corporations. As a result, between 2008 and 2012, five fragrance manufacturers held 60% of the fragrance market: Givaudan (Switzerland), Firmenich (Switzerland), IFF (USA), Symrise (Germany), and Takasago (Japan), according to Leffingwell and Associates [54.2]. In this globalized context, only three luxury houses have been preserving their own perfume creations internally: Chanel, Guerlain, and the Patou-Rochas group.

New Trends

These days, to reinforce the status of perfume as a luxury product, several brands have chosen to hire a master perfumer as observed by *Elisabeth de Feydeau* [54.1]. In 2004, Hermès hired the perfumer Jean-Claude Ellena as director of perfume design and Christine Nagel joined him in 2014. Following the same strategy, LVMH Group recruited François Demachy in 2006 as head of the Fragrance Creation of Christian Dior, and in 2011 Jacques Cavallier-Belletrud was chosen to create the first perfume for Louis Vuitton.

Another trend recently appeared: the alternative perfumery or *niche* perfumery [54.1]. This tendency is drifted by discreet brands, such as L'Artisan Parfumeur, Diptyque, Serge Lutens, or The Different Company, and aims at repositioning perfume as a luxury product through highly selective advertising and retailing.

Fig. 54.1 Fragrance flasks from the Gallo-Roman Era (Daoud – Fotolia.com)

Due to historical know-how, Europe, and the United States to a lesser extent, preserves a major role in perfume design and trade. According to the International Fragrance Association (IFRA), today there are globally less than 900 perfumers and 60–70% of the perfumers reside in Europe. Moreover, perfume creation is mainly localized in two cities: Paris and New York.

54.1.2 What is a Perfumer?

A perfumer is an expert in scents, who composes fragrant creations. He aims at bringing not only pleasure and well-being, but also at achieving the desired effects that suit the application use. When perfumers are asked about their sources of inspiration, answers are countless. Depending on their own experience and their personal feelings during the creation process, perfumers do not always follow the same approach to develop a new fragrance. Some perfumers are interested in specific materials they want to glorify, or are inspired by olfactory families they are fond of. Others try to translate intimate olfactory feelings into fragrances. Music, literature, or painting can also inspire perfumers. However, first and foremost, the creation of a scent takes place in the mind. *Yet this concept* is paradoxically *neither an idea, nor an image, but a smell*, as the perfumer Jacques Polge, director of the Chanel perfume laboratories since 1978 observed [54.3]. In the same manner, *Jean-Claude Ellena* said *I am the only person who can conjure the smell [I am creating] mentally* [54.4].

Therefore, perfume creation is first an intellectual approach. This idea may look surprising for newcomers, and a French research team recently studied perfumers' brain to get new insights into perfumer know-how. They demonstrated that intense olfactory training led to a reorganization of key olfactory and memory regions, and increased gray matter volumes in the corresponding brain areas [54.5]. As a consequence, the authors showed that perfumers are able to imagine an odor clearly enough to *smell* it, even though it is physically absent, which is hardly possible for the general population [54.6]. Moreover, the authors demonstrated that greater the level of expertise, less key brain regions (right primary piriform cortex, left orbitofrontal cortex, and left hippocampus) are activated. Olfactory expertise even appeared to counteract the effect of aging as gray matter volumes increased with years of practice, whereas these volumes decreased with age in naive subjects. These results demonstrated the astonishing plasticity of human brain and the essential role of olfactory training to become familiar with fragrance ingredients, and also to mentally learn odors. Taking into account these recent results, one can better appreciate

perfumer work and the need of a highly specific olfactory training.

54.1.3 How to Become a Perfumer?

Jean Carles said *the perfumer's only tool is his nose* [54.7], and it is commonly thought that perfumers are endowed with an exceptional sense of smell. A good nose indeed is a major criterion and aspiring perfumers have to pass various olfactory tests during their application program. However according to Jean Carles, *no "nose" can be said to be better than another* and *anyone may acquire a highly developed sense of smell, as this is merely a matter of practice* [54.7]. Olfactory training is thus the first step to become a perfumer. Olfactory training consists in studying and memorizing hundreds of natural and synthetic materials used in perfumery. This training also implies learning which odorants work well together to create harmonious combinations. Due to the extensive variety of fragrance materials, olfactory training must be rigorous and intense. For many perfumers, daily training is not only necessary, but indispensable [54.4, 7]. Odorant learning is thus based on a substantial personal work. According to IFRA, to qualify as a perfumer requires more than 7 years training.

Industry Schools

Since there are few official courses for perfumery in the world, perfumery can be a difficult industry to break into. Remembering his own start, when he felt lost in front of the hundreds of odoriferous materials, Jean Carles founded the first Perfumery School in 1946 in Grasse. This school was part of the Roure company and was aimed at training the future in-house perfumers according to the method he developed and which now bears his name. Later on, in 1992, when Roure merged with Givaudan, the Perfumery School became the Givaudan Perfumery School. Many great perfumers, such as Jacques Polge (from Chanel), Jean-Claude Ellena (from Hermès), or Thierry Wasser (from Guerlain) started in this industry school and this institute can claim to have trained the perfumers responsible for approximately one third of the fragrances on the market today [54.8].

Now, all the major fragrance companies possess their own in-house perfumery training programs. Internal training programs are generally planned for an average length of 4 years to allow future employees to learn the various raw materials used in perfumery, as well as the techniques of fragrance formulation, and technical aspects of the work of perfumer. Afterward, students become Junior Perfumers and complete their instruction under the mentorship of a Senior Perfumer. Industry in-house programs are highly selective: they attract hundreds of applicants from all over the world and from various backgrounds, but only a few aspiring perfumers are qualified each year and there is no guarantee of a position afterward. Jean Guichard, the director of the Givaudan Perfumery School, indicated that only three students out of 200 candidates were selected in 2011 [54.1]. He added that the applicant culture is now a major criterion as fragrance companies aim at suiting local olfactory tastes.

Independent Schools

Besides industry schools, several university programs have been created to train would-be perfumers. The most famous independent school of perfumery is based in Versailles (France) and is called ISIPCA (Institut Supérieur de la Parfumerie, de la Cosmétique et de l'Aromatique Alimentaire). This school was founded by perfumer Jean-Jacques Guerlain in 1970. ISIPCA is now internationally recognized for its expertise in the field of perfumery, as well as cosmetics and flavors, and proposes a wide range of education programs, from undergraduate to graduate degrees. Most of these programs are proposed in the context of day-release contracts with perfume companies. In addition, ICATS (International Centre for Aroma Trade Studies), which is part of the University of Plymouth (UK), offers flexible quality learning to professional and aspiring professionals in the Aroma Trades, Perfumery, and Flavor industries. This school provides distance learning on a global basis leading to the IFEAT (International Federation of Essential Oils and Aroma Trades) Diploma. Besides these well-known schools, the École Supérieure du Parfum which is based in Paris (France) has recently launched a new scholar program. It consists of four full years of teaching, interspersed with two internship periods, followed by a fifth year dedicated to work placements and final year projects. Courses in perfumery techniques are also offered by the Grasse Institute of Perfumery situated in Grasse (France) and the Perfumery Art School based in London (UK). Both programs are planned for one year and combine courses given by confirmed perfumers and practical work.

Like industry schools, independent schools are highly selective. Less than 20 students out of the hundreds of candidates are selected every year by each school. Finally, out of all the schools, less than a dozen students will become perfumers. The other graduates will find jobs as cosmeticians, evaluators, marketing assistants, quality controllers, production managers, and the like [54.8].

54.2 Perfumery is an Art

54.2.1 Perfumer's Palette

As *Jean-Claude Ellena* said, *The art of perfumery is closely associated with chemistry* [54.8]. Perfumer's palette indeed is composed of various materials and has been continuously evolving with scientific discoveries in the field of chemistry. Generally, the materials used by the perfumer are categorized in three families: natural ingredients, synthetic substances, and bases (Fig. 54.2).

Natural Ingredients

Natural ingredients are obtained from natural raw materials using various extraction techniques. They consist of essential oils, concretes, resinoids, absolutes, or butters, depending on the amount of waxes in the extracted product. The most frequently used techniques are steam distillation, solvent extraction (using generally ethanol or hexane), supercritical fluid extraction, fractionation, and expression. Natural raw materials are essentially plants and various parts of the plant are used to obtain fragrance ingredients: flowers, buds, fruits, leaves, barks, woods, resins, gums, seeds, roots, and lichens. As an example, rose and jasmine extracts result from the treatment of fresh petals, whereas iris butter is obtained from the distillation of dry matured rhizomes, and geranium essential oil comes from the distillation of fresh leaves (Figs. 54.3, and 54.4). The chemicals responsible for the typical odor of natural extracts are secondary metabolites of the plant, such as terpenes, norisoprenoids, or fatty acid derivatives. Natural extract composition can thus be deeply modified depending not only on the botanic variety used, but also on the origin of the plant. Moreover, different parts of a same plant can be extracted, leading to different olfactory materials. The steam distillation of cinnamon bark leads to an essential oil rich in cinnamic aldehyde, whereas the distillation of cinnamon leaves results in an essential oil rich in eugenol [54.9].

Fig. 54.2 Fragrant ingredients composing perfumer's palette (monropic – Fotolia.com)

Animalic fragrance materials, such as civet, castoreum, or ambergris, were traditionally used as fixatives. However, they all have been replaced by chemical reconstitutions. One exception is beeswax absolute that is still used for perfume creation.

Despite their historical use in perfumery and their unique olfactory complexity, natural ingredients present several major limitations. First, the cost of natural ingredients can be highly prohibitive. Indeed, due to poor extraction yields, some natural ingredients, such as rose or jasmine absolutes, are hardly reachable for daily applications. Table 54.1 illustrates this constraint show-

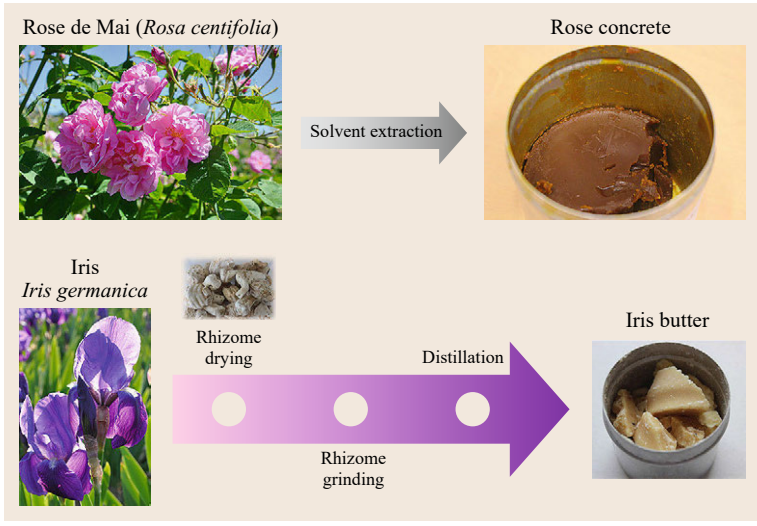


Fig. 54.3 From natural botanic sources to perfumer's ingredients. Two examples: rose de mai and iris (courtesy of Jean-François Vieille)

Fig. 54.4 Geranium leaves and flowers (*Pelargonium graveolens*). Geranium essential oil is obtained from hydrodistillation of fresh leaves (courtesy of Jean-François Vieille)

ing the quantities needed to obtain natural ingredients from various botanical sources [54.10, 11]. Moreover, even today, the scents of some plants, such as lily of the valley (*Convallaria majalis*), frangipani (*Plumeria acutifolia*), heliotrope (*Heliotropum arborescens*), or honeysuckle (*Lonicera caprifolium*), remain hardly extractible because of the lack of raw materials, or poor extraction yields. Finally, natural extracts are hardly reminiscent of the real natural scents as the use of heat and harsh solvents often distort the odor of the raw materials. All these constraints encourage the use of synthetic alternatives, especially for functional perfumery compositions.

Synthetic Ingredients

When modern perfumery began at the end of the nineteenth century, most of the synthetic ingredients used in perfumery were identical in structure to natural odorants. Later on, improvements in organic synthesis led to a significant extension of perfumer palette and offered new perspectives of creation. Table 54.2 lists the main synthetic ingredients introduced in perfumery since 1960 and gives examples of perfumes created with them [54.1]. As Ernest Beaux, the creator of Chanel N°5, used to say *a worthy creation must contain a new material* and *one has to rely on chemists to find new aroma chemicals creating new, original notes*. Therefore, scientists are continuously searching for new synthetic materials. According to IFRA, the fragrance industry invests up to 18% of its total annual revenues in research and development, especially to discover new molecules and patent them. These innovative ingredients are known as *captives* and are crucial to provide newness and originality to perfumers' palette. However, the search for new scent ingredients often comes as a result of serendipity. As an example, every year, over 2000 new molecules are developed by Givaudan researchers, but only three or four per year are selected for launch, after scent evaluation, synthesis studies, and testing [54.12]. Like the best natural ingredients, the best synthetics are extremely expensive. Consequently, captives are generally dedicated to fine fragrances in a first time, and their use is extended to a wider range of products in a second time.

Bases

The third class of fragrance ingredients corresponds to bases. Bases, also known as accords, are simple formulae that provide a premade fragrant blend that can be incorporated as a component on its own in the full perfume formula [54.13]. Bases are olfactory interpretations that are used to bring specific olfactory facettes, such as leathery, fruity, or flowery notes. Bases present several advantages for perfumers. First, they make easier the use of powerful ingredients that are difficult to directly add at very low levels in compositions. They also represent an alternative to the use of materials of animal origin that are no longer available. Moreover, bases widen perfumer palette with olfactory reconstitutions of flowers or fruits whose scent cannot be extracted. Bases can sometimes be better scent approximations of the flower scent than the corresponding natural extract, especially when perfumers aim at bringing the odor of fresh flowers.

Table 54.1 Quantities needed to obtain natural extracts from several botanical sources [54.10, 11]

Product	Extract	Origin	Botanic variety	Quantity needed to obtain 1 kg extract
Mandarin	Oil	Italy	*Citrus reticulata*	1350 kg fruits
Neroli	Oil	Tunisia	*Citrus aurantium*	1000 kg flowers
Jasmine	Absolute	Egypt	*Jasminum grandiflorum* L.	400 kg flowers
Rose	Oil	Bulgaria	*Rosa damascena*	4500 kg flowers
Rose	Absolute	Turkey	*Rosa damascena*	700 kg flowers
Rose de mai	Concrete	France	*Rosa centifolia*	400 kg flowers
Iris	Absolute	Italy	*Iris pallida*	2000 kg roots
Patchouli	Oil	Indonesia	*Pogostemon cablin* (Blanco)	50 kg leaves
Vetiver	Oil	Haiti	*Vetiveria zizanoides* L.	250 kg roots
Ylang ylang	Oil	Comores	*Cananga odorata*	50 kg flowers

Table 54.2 Main synthetic compounds introduced in the perfumers' palette during the last 50 years (after [54.1])

Year	Event	Attributed to	Applications in perfumery
1957–1962	Methyl dihydrojasmonate (MDJ) or Hedione	Firmenich	*Eau Sauvage* (Dior, 1966)
1961	Z11	Firmenich	*Bulgari pour homme* (Bulgari, 1995)
1962	Vertofix	IFF	*N° 19* (Chanel, 1970) *Silence* (Jacomo, 2004)
1964	Lyral	IFF	*Fidji* (Guy Laroche, 1966) *Parfum d'été* (Kenzo, 1992)
1965	Galaxolide	IFF	*Jovan Musk* (Jovan, 1974) *Trésor* (Lancôme, 1990)
1965	Trimofix	IFF	*Amarige* (Givenchy, 1990) *Allure* (Chanel, 1996) *Armani Code* (Armani, 2004)
1966	Dihydromyrcenol	IFF	*Cool Water* (Davidoff, 1988)
1966-74	Calone 1951	Pfizer	*New West for her* (Aramis, 1990) *Kenzo masculin* (Kenzo, 1991) *L'Eau d'Issey* (Issey Miyake, 1992)
1967	Damascenones alpha and beta	Firmenich	*Poison* (Dior, 1985)
1968	Cashmeran	IFF	*Ivoire* (Balmain, 1980) *Amarige* (Givenchy, 1990) *Jungle Elephant* (Kenzo, 1997) *Alien* (Thierry Mugler, 2005)
1969	Ethyl maltol	Pfizer	*Angel* (Thierry Mugler, 1992)
1970–1974	Damascones alpha and beta	Firmenich	*Nahema* (Guerlain, 1979)
1973	Mayol	Firmenich	Largely used in perfumery
1973	Neobutenone alpha	Fimenich	*Cool Water* (Davidoff, 1988)
1973	Canthoxal	IFF	*Vanderbilt* (Gloria Vanderbilt, 1982) *Amor Amor* (Cacharel, 2003)
1973	Helional	IFF	*Alliage* (Estée Lauder, 1972) *L'Eau d'Issey* (Issey Miyake, 1992)
1974	Dynascone	Firmenich	*Eternity for men* (Calvin Kelin, 1989) *Romance* (Ralph Lauren, 1998)
1974	Oxane	Firmenich	*In Love Again* (Yves Saint Laurent, 1998)
1975	Iso E Super	IFF	*Trésor* (Lancôme, 1990) *Light Blue* (Dolce & Gabbana, 2001) *Terre d'Hermès* (Hermès, 2006)
1977	Floralozone	IFF	*Acqua di Gio* (Armani, 1996) *Very Irrésistible* (Givenchy, 2003)
1979	Liffarome	IFF	*J'Adore* (Dior, 1999) *Pure Poison* (Dior, 2004)
1979	Bacdanol	IFF	*Eternity* (Calvin Klein, 1988) *Hervé Léger for women* (1999)
1982–1986	Norlimbanol and Norlimbanol dextro	Firmenich	*Tommy* (Tommy Hilfiger, 1995) *Light Blue* (Dolce & Gabbana, 2001)
1983	Polysantol	Firmenich	*Samsara* (Guerlain, 1989)
1984	Coranol	Firmenich	*L'Eau d'Issey pour homme* (Issey Miyake, 1994) *Romance* (Ralph Lauren, 1998)
1984	Florol	Firmenich	Largely used in perfumery
1984	Pomelene	IFF	*Roma* (Laura Biagiotti, 1988) *Pleasures for men* (Estée Lauder, 1997)
1984	Ambrox DL	Firmenich	*Drakkar Noir* (Guy Laroche, 1982)
1986	Kharismal	IFF	
1986	Violiff	IFF	*Unforgivable* (Sean John, 2006)
1988	Myrrhone	Firmenich	
1989–2008	Muscenone and muscenone dextro	Firmenich	*L'Eau d'Issey* (Issey Miyake, 1992) *XS* (Paco Rabanne, 1993) *Noa* (Cacharel, 1998)

Table 54.2 (continued)

Year	Event	Attributed to	Applications in perfumery
1991	Verdox	IFF	*Be delicious* (Donna Karan, 2004) *Boss Bottled* (Hugo Boss, 2006)
1991	Helvetolide	Firmenich	*Flower by Kenzo* (Kenzo, 2000) *Miracle* (Lancôme, 2000)
1992	Fructalate	Firmenich	*Light Blue* (Dolce & Gabbana, 2001)
1993	Unsaturated macrocyclic musks: Habanolide, Muscenone, Exaltenone	Firmenich	*Jean Paul Gaultier feminine* (1993) *Bulgari for men* (Bulgari, 1995) *Truth* (Calvin Klein, 2000) *Flower by Kenzo* (Kenzo, 2000)
1993	Habanolide	Firmenich	*Cologne* (Thierry Mugler, 2001)
1993	Cetalox	Firmenich	*L'Eau d'Issey pour homme* (Issey Miyake, 1994)
1993	Nirvanol	Firmenich	
1993	Paradisone	Firmenich	
1994	Cassiffix	IFF	
1994	Hivernal	Firmenich	
1995	Dartanol	Firmenich	
1996	Lilyflore	Firmenich	*Eau parfumée au thé blanc* (Bulgari, 2003)
1997	Romandolide	Firmenich	
1997	Peonile	Givaudan	
1997	Georgywood	Givaudan	*Artisan* (John Varvatos, 2009)
1998	Sclaréolate	Firmenich	
1998	Opalal	Givaudan	
1998	Exaltenone	Firmenich	*Truth* (Calvin Klein, 2000)
1998	Musk Z4	IFF	*Very Irrésistible* (Givenchy, 2003) *Pure Poison* (Dior, 2004) *Armani Code* (Armani, 2004)
1999	Dihydro Farnesal	Givaudan	
2000	Javanol	Givaudan	*Wonderwood* (Comme des garcons, 2010)

54.2.2 Ingredient Classification: Top, Middle, and Base Notes

In order to make the learning of fragrance materials easier, Jean Carles developed a method where ingredients are divided into three categories according to their volatility and tenacity [54.7, 8, 13]. These three categories are known as top notes, middle notes and base notes. Table 54.3 presents three categories of volatile notes and gives examples of odorous materials. Top notes (or head notes) are highly volatile products that lack tenacity. They represent the first 15 min or so of evaporation. Middle notes (or heart notes) present an intermediate volatility and tenacity and last for several hours. Base notes (or end bottom notes) are characterized by a low volatility and a high tenacity and can last for days on a blotter (Fig. 54.5). As explained by *Jean Carles* in 1962, *base note compounds generally give off a rather unpleasant smell when freshly deposited on a paper strip, but the scent given off after the subsequent stages of evaporation is excellent* [54.7]. Base notes thus need to be improved by materials with high and intermediate volatility to impart to the perfume composition an attractive odor on opening the bottle. Consequently, perfume is an optimized equilibrium between top, middle, and base notes. According to Calkin and Jellinek, the balance between the three categories of materials should be 15–25% top notes, 30–40% middle notes, and 45–55% base notes [54.13].

Nevertheless, the concept of top, middle, and base notes mainly aims at guiding students and cannot restrict fragrance creation. Indeed, odorant classification depends mostly on the ingredient blend. As an example, clove essential oil is generally listed as a middle note ingredient but has also some top note character. On the contrary, neroli essential oil, considered as top note material, can also impart some middle note character. In addition, the balance between top, middle and base notes can vary depending on the perfume application. A fine fragrance for instance is formulated to have a distinctive *sillage* to persist even hours after application, whereas a shower gel aims at bringing immediate freshness and blooming. Therefore, one should only remember that a well-constructed perfume must have notes that blend well and run into each other successfully as the perfume evaporates with the fragrance theme apparent at every stage [54.7, 13].

Table 54.3 Odorant classification based on volatility and substantivity [54.7, 8, 13]

Top notes	
Citrus	Orange essential oil, lemon essential oil, mandarin essential oil, grapefruit essential oil, bergamot essential oil, lime essential oil, petitgrain essential oil
Herbal	Pine essential oil, rosemary essential oil, basil essential oil, oregano essential oil, tarragon essential oil
Aldehydic	Decanal, undecanal, 2-methyl undecanal, dodecanal
Marine/ozone	Calone, Ozonal, Algol
Green	Galbanum essential oil, *cis*-3-hexenol, and its esters, coriander essential oil
Fruit	Black currant absolute, isoamyl acetate, ethyl caproate, ethyl butyrate
Middle notes	
Floral	Rose (rose essential oil, odors of hyacinth, lily of the valley): phenylethyl alcohol and geraniol White flowers (orange flower absolute, jasmine absolute, tuberose absolute): methyl anthranilate and indole Yellow flowers (osmanthus absolute, cassia absolute, odor of freesia): beta ionone Exotic or spiced flowers (ylang-ylang essential oil, odors of carnation, lilies): benzyl salicylate and eugenol Anise flowers (mimosa absolute, odors of lilac, wisteria): anisic aldehyde or heliotropin
Spice	Cool spices: pepper essential oil, cardamom essential oil, nutmeg essential oil, pink pepper essential oil Hot spices: cinnamon essential oil, clove essential oil, pimento essential oil
Base notes	
Wood	Cedarwood essential oil, sandalwood essential oil, patchouli essential oil, vetiver essential oil, agarwood absolute, oak moss absolute
Animal	Civet, synthetic musks, castoreum absolute, birch tree essential oil
Amber	Ambergris, labdanum absolute, myrrh, cistus essential oil
Vanillic	Tonka absolute, vanillin, ethylvanillin, ethylmaltol, benzoin resinoid

54.2.3 Perfume Classification – Olfactory Families

Just like in all artistic disciplines, students first learn raw materials and then pursue their apprenticeship with the copying of perfumery models. They learn olfactory families, as well as the perfumes that are particularly characteristic of their era. According to *Jean-Claude Ellena*, *all this imitation drives home the importance of the interactions between materials, the role of fragrance's total construction, and the detail* [54.8].

The most famous fragrance classification was developed in 1984 by the Société Française de Parfumerie (SFP) and is based on seven olfactory families [54.1, 8, 10]:

- *Citrus* or *Hesperides* defines fragrances reminiscent of citrus. This family consisted historically in Eaux de Cologne. Later on, around 1960, it was widened with the introduction of synthetic compounds reminiscent of citrus that present higher tenacity than naturals.
- *Floral* corresponds to fragrances dominated by a scent of a particular flower or combining several flower scents.
- *Fougère*, meaning fern in French, corresponds to compositions made from a combination of lavender, geranium, coumarin, and oak moss. This family emerged in 1884 with *Fougère Royale* by Houbigant.
- *Chypre* defines fragrances based on an olfactory accord of oak moss, cistus labdanum, patchouli, and bergamot. This class of fragrances was named after

Fig. 54.5 Fragrance flasks and paperstrips used for sensory evaluation (rdnzl – Fotolia.com)

a perfume created by François Coty in 1917, *Chypre* being the French translation of Cyprus.

- *Woody* fragrances are mainly dominated by woody scents. Some woody notes are opulent, such as sandalwood and patchouli, while other scents are dryer like cedarwood and vetiver.
- *Amber* or *Oriental* represents a large fragrance class structured around three main notes: amber notes, like vanilla, tonka bean, and balsams, spicy notes reminiscent of clove and cinnamon, and woody notes with patchouli and sandalwood.
- *Leather* fragrances are composed of scents of honey, tobacco, wood, and wood tars.

Each olfactory family is divided into several subfamilies depending on the additional accords of the compositions. Since new fragrance materials are introduced every year, fragrance subfamilies evolve continually. As an example, the introduction of Calone 1951 by Pfizer in 1966 resulted in the rising of the *Aquatic/Oceanic/ozone* subfamily, which defines fragrances reminiscent of the ocean. In the same manner, in the early 1990s the introduction of Ethylmaltol in perfumer's palette launched the trend of the *Gourmand* accord reminiscent of sweet delights.

Recently, the fragrance-dedicated website Osmoz proposed a new classification that considers the increasing number and diversity of men's fragrances. Indeed, as observed by Elisabeth de Feydeau, in 2007 men's fragrances represented one third of fragrance launches, whereas this market only started around 1930 [54.1]. Osmoz classification is based on eight major families: four families are dedicated to women's fragrances (*Citrus*, *Floral, Chypre*, and *Oriental*), and the four other correspond to men's fragrances (*Citrus*, *Aromatic*, *Woody*, and *Oriental*). *Aromatic* defines fragrance accords based on one or more aromatic herbs, such as clary sage or rosemary.

54.2.4 How do Perfumers Work on a Creation?

According to IFRA, fragrance is a blend of 50 to 250 raw materials drawn from a palette of up to 3000 available ingredients. In front of the hundreds of fragrance materials and compositions, perfumers are constantly seeking for new olfactory sensations. As already explained, scent creation is first an intellectual work, which is designed according to the perfumer's personality, in agreement with the context of the project, that is, the name of the future fragrance, the bottling, or the consumer target.

Once a perfumer has an idea – or a smell – in mind, creation process begins. Fragrance formula consists of a list of ingredients in specified quantities. The creation process is iterative: a first formula is made and evaluated on a smelling strip then the formula is adjusted and reevaluated, etc., [54.10]. For every trial, the overall evaporation profile is considered in order to assess gradual changes from the volatile top-notes to the most retained base-notes. Creation process can last from a couple of weeks to several years, so the perfumer needs to persevere to succeed in creating the best formula. Finally, as Jean-Jacques Guerlain said, *after much groping in the dark the perfume begins to resemble the image which I had forged abstractly in my mind* [54.3]. Christopher Sheldrake, Perfumer and Director R&D Perfumes, Chanel, added that *a fragrance is successfully completed when it smells obvious; when the perception of the fragrance is coherent with objectives researched; when the aesthetic appreciation of the fragrance replies convincingly to one's expectation* [54.14].

Fragrance is thus a personal work that depends on its designer and many perfumers consider scent creation as an art, such as painting, or music. The great perfumer Edmond Roudnitska, who created *Eau Sauvage* (Christian Dior), was the first to defend this idea. Besides his well-known creations, he collaborated with the philosopher Etienne Souriau to demonstrate the artistic nature of fragrance creation (Fig. 54.6) [54.15]. For Edmond Roudnitska, learning is as essential to appreciate perfume creation, as it is to appreciate music. He added that *taste evolves chiefly with the acquisition of learning, with the knowledge of facts and of aesthetic accomplishments, which makes it possible to analyze them and to provoke instructive comparisons*. Today, Christophe Laudamiel (from IFF) defends the same idea. According to him, perfume is an everlasting quest for beauty: perfume translates the intimate character of its creator,

Fig. 54.6 Perfume is an everlasting quest for beauty that can be seen as an artistic creation (ra2 studio – Fotolia.com)

and is designed to inspire not only sensory pleasure, but also emotions [54.16].

Nevertheless, as the fragrance industry evolves, the perfumer's work also changes. Many fragrances are now created by a perfumer team and no longer signed by only one nose as it used to be traditionally. Cosigning is recent and opinions disagree on this creation method. According to Olivier Cresp (from Firmenich), working on a creation with another perfumer is perfect [54.17]. For him, during the creation process, a perfumer sometimes needs help or advice and it is useful to share these issues with another perfumer. On the contrary, *Jean-Claude Ellena* declares that *even if the exchange is beneficial, the accumulation of ideas is an utter negation of any creative process* [54.4].

Moreover, despite their personal investment in perfume creation, perfumers generally work on several creation projects at the same time. Most of the designers need to move from one project to another to get new ideas and avoid creator's blocking [54.4, 14, 17].

54.3 Perfumery is a Science

Creating a fragrance requires a specific know-how which is based on an intensive olfactory training. However, the perfumer also has to take into account technical parameters, such as performance, stability, and regulation, to create the blend that best suits to the context.

54.3.1 Physicochemical Parameters Used to Define Fragrance Ingredients

Several physicochemical values are available to describe sensory performance of fragrance ingredients. These physicochemical data, and especially saturated vapor pressure and $\log P_{o/w}$, are useful for selecting ingredients [54.8, 10, 13]. Saturated vapor pressure refers to the equilibrium pressure exerted by a substance in a closed system at a specified temperature. As can be seen in Table 54.4, the vapor pressure of ambrettolide is below 0.01 hPa at 25 °C, making it a product with low volatility that lasts over time, whereas prenyl acetate has a high vapor pressure of 2.53 hPa (at 25 °C) and therefore fades in less than a minute [54.18].

In addition, as perfume composition aims at sticking to final application, it is important to consider the partition of the material between aqueous and organic phase. This property is given by $\log P_{o/w}$, also known as partition coefficient. Higher values of $\log P_{o/w}$ indicate higher hydrophobicity of the substance, and thus higher affinity for any alternative surface in comparison with water. As can be seen in Table 54.4, ambrettolide is much more hydrophobic than prenyl acetate and thus has a better affinity for skin, hair, or clothes than prenyl acetate [54.18].

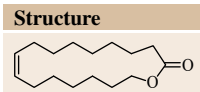
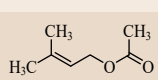
Actually, saturated vapor pressure, as well as $\log P_{o/w}$, express material physicochemical behavior that perfumers used to learn empirically. Since perfume is a mixture of various materials, these data cannot predict the behavior of the materials in the final product.

54.3.2 Volatility, Tenacity, and Substantivity

Fragrances must be volatile to be perceived. Volatility of an odorant is determined by two main factors: its molecular weight and its polarity. Generally, molecules with fewer than eight carbon atoms in their structure are too volatile to be used in perfume creation [54.13]. On the contrary, compounds that comprise more than 18 carbon atoms are usually not volatile enough to reach the olfactory receptors in the nose. Volatility is also affected by the ability of odorant to form nonbonded interactions, and thus by the nature of its functional groups. As explained by *Charles Sell*, *the more polar a molecule is, the more easily will it form electrostatic bonds, such as hydrogen bonds, to other molecules around it, whether they are other fragrance molecules, cellulose (paper strips), or proteins (skin, hair)* [54.13].

In addition to volatility, perfumers have to consider fragrance ingredient tenacity. Tenacity, also known as substantivity, defines perfume long lastingness, and is empirically measured as the duration the fragrance remains detectable on a blotter [54.8]. Tenacity depends mainly on the volatility of the fragrance components [54.13].

Table 54.4 Comparison of physicochemical values of two fragrance materials [54.18]

Compounds	Structure	Olfactory description	Saturated vapor pressure (hPa)	$\log P_{o/w}$	Substantivity
Ambrettolide		Musk	< 0.01	6.510	> 48 h
Prenyl acetate		Fruity, floral, pear	2.53	1.650	< 3 h

54.3.3 Sensory Thresholds

The olfactory perception threshold is defined as the lowest concentration at which a perfume ingredient can be perceived, whereas the olfactory recognition threshold represents the lowest level at which a perfume ingredient is identified in odor terms. Clearly, the olfactory perception threshold is important in perfumery since it gives information about the olfactory strength of odorants, and consequently of the acceptable level range in a composition. As an example, the olfactory perception threshold of vanillin is 0.02 ng/l, which means that vanillin is perceptible even in highly diluted form [54.8]. By contrast, the perception threshold of isoamyl acetate is 95 ng/l, so this compound becomes quickly undetectable once diluted [54.8].

54.3.4 Radiance, Bloom, and Sillage

Charles Sell defined *radiance* as *the ability of a perfume or perfume ingredient to fill space* [54.13]. One of the best examples of radiant materials is methyl dihydrojasmonate, also known as Hedione. When this material is smelt from a perfumer's blotter, it does not seem to have a high olfactory impact, but if the blotter is left in a room, its floral odor is easily perceptible by anyone entering the room. Two properties are essential in making a material radiant: volatility and sensory threshold. Indeed, the perfume material must be poorly volatile not to evaporate too quickly, and it must have a very low perception threshold to be immediately detected.

Another important parameter is *bloom* which is defined by *Charles Sell* as *the ability of a fragrance to perfume a room when the fragrance is introduced, not directly as an oil or aerosol, but in a product, such as a soap* [54.13]. One should distinguish *dry bloom* that corresponds to the fragrance effect of a dry bar of soap left in a room, from *wet bloom* that defines the fragrance effect in the room when the soap is in use. Bloom is a main feature for functional products, such as body care and home care products.

Finally, *sillage* is the phenomenon of a scent trail being left by a person. Sillage is a property that is highly evaluated in the creation of fine fragrances [54.14].

54.3.5 Influence of the Application Matrix

Fragrance results from the blend of various materials that can react together or with the matrix used [54.10, 13]. In the case of fine fragrance, the product base is aqueous ethanol that is relatively perfume friendly. However, some applications, such as soaps or deodorants, are more aggressive media. In any case, the fragrance has to subsist through manufacture, distribution and storage. Consequently, the perfumer must learn to design fragrances that will be stable in the product and not interfere with its active ingredients.

The most important parameter the perfumer has to take into account during the creation process is pH [54.10, 13]. Indeed, perfumers have to design fragrances for products with pH values ranging from one end of the scale to the other. Fine fragrances are characterized by neutral pH, whereas the composition of functional products generally induces acidic or alkaline conditions. In these harsher media, various chemical reactions are readily observed, such as acetalization, ester hydrolysis and aldolization, and restrain perfumer's palette. As explained by *Charles Sell*, *while all fragrance ingredients are stable enough to be used in neutral conditions, only* 65% *of the palette can be used by the perfumer creating a fragrance for an antiperspirant,* 45% *in a laundry powder,* 25% *in an acid lavatory cleaner, and mere* 5% *in a dish wash powder* [54.13].

Besides pH matrix, the perfumer has to consider the presence of oxidizing or reducing agents that can induce undesired chemical reactions. The designer has also to take into account the presence of surfactants, opacifiers, or dyes [54.10, 13]. These latter compounds induce loss of available fragrance ingredients because of adsorption onto or into the active compounds and thus lower fragrance intensity.

54.3.6 Sensory Performance

Fragrances are created in order to bring a pleasing odor, but the hedonic dimension of a perfume is highly subjective. To ensure success of fragrance creations, various sensory studies can be performed before launching a new product. Historically, sensory studies focused on the bipolar hedonic valence dimension, that is, the propensity of an odor to be liked, or on the contrary disliked. However, this methodology can only predict the success of a product and cannot explain hedonic responses. To get new insights, various researches have been performed during the last 20 years especially to measure emotional and behavioral responses to odor. Indeed, odors can elicit innumerable emotions because of direct connection between the brain limbic system, which is the center of emotions, and the olfactory system.

Generally, emotional response is categorized according to six basic emotions: anger, disgust, fear, happiness, sadness, and surprise [54.19]. However, this classification hardly fits the emotions linked to olfactory experience. Recently, researchers from the Swiss Centre of Affective Sciences demonstrated that feelings induced by odors are structured around a small group of dimensions that reflect the role of olfaction in well-

being, social interaction, danger prevention, arousal and relaxation sensations, and conscious recollection of emotional memories [54.20]. With this concept, these authors developed the Geneva Emotion and Odor Scale (GEOS), which is based on six dimensions: sensuality, relaxation, pleasant feeling, refreshment, sensory pleasure, and unpleasant feeling [54.21]. Through collaboration with Firmenich, the authors extended their study to other regions and developed a global verbalization tool called ScentMove, which is adapted to commercial and development needs and aimed at better discriminating fragrance products focusing on emotions [54.22].

Using a similar strategy, IFF focused its research on mood. Indeed, pleasant odors are likely to induce positive moods, whereas unpleasant odors tend to induce negative moods [54.23]. In order to measure both subjective and physiological effects of aromas and fragrances on emotions, IFF has developed a self-report method called Mood Mapping [54.24]. This method is based on eight mood categories: happy, relaxed, sensuous, stimulated, irritated, stressed, depressed, and apathic. For each sample, the panelists are asked to pick a mood category that best matches the aroma of the sample. In the same context, Takasago developed a method for measuring the emotional response to olfactory stimuli [54.25]. This method requires the use of magnetic resonance imaging to assess the ability of fragrance sample to elicit a reward through the dopaminergic pathway. Applying this method, they were able to assay the sedative or energizing effects of odors for various applications. More generally, all the leading fragrance companies now integrate the importance of emotions in the development of new fragrance products.

54.3.7 Stability

During storage, the quality of the final product evolves: the fragrance evolves and the matrix also changes. Therefore, the physical and chemical characteristics of the final product need to be checked to ensure the absence of significant changes over time before the product reaches the market place. Stability tests consist in monitoring any changes in the fragrance odor once in the product matrix and in the final packaging, as well as any changes in the product aspect, over a period of accelerated storage at different conditions. Accelerated test theory is largely based on the Arrhenius rate equation: for every 10 °C increase in temperature, the rate of the reaction doubles. Consequently, a 12 month-aging at 20 °C equals a 12 week aging at 40 °C, or a 6 week aging at 50 °C, or a 3 week aging at 60 °C. In practice, fragrance companies test all their products at 0–4 °C, 20 or 25 °C, and 37 °C, for 12 weeks as a minimum standard [54.13]. These conditions are usually more severe than real conditions to ensure good stability.

Moreover, depending on the product packaging, additional stability tests can be performed. First, humidity tests can be carried out for products that are likely to be packed in permeable materials, such as paper or cardboard. Humidity tests commonly consist in monitoring any changes over high-humidity conditions, that is, 37 °C/70% relative humidity or 40 °C/80% relative humidity [54.10].

Light testing is also commonly performed for products that will be exposed to daylight or strong sunlight. Light tests are carried out using a Xenon arc lamp that emits UV light in the 300–800 nm wavelength range, with a 400 or 1000 W burner fitted. In these conditions, a 6 h exposure to the 1000 W lamps is sufficient to monitor any changes that are likely to occur in about 3 months of daylight testing [54.13]. However, light testing presents a severe limitation. Indeed, the temperature inside the UV cabinet can be drastically high despite the presence of a cooling fan, resulting in a temperature artifact in testing conditions.

In practice, fragrance ingredients are storage tested before the introduction to the perfumer's palette and their stability performances are collected in in-house databases to guide perfumers in picking out the optimal odorants for the product application [54.13].

54.3.8 Safety and Toxicology Issues

Around 1960, the fragrance industry established a self-regulatory system that involves the two major international fragrance organizations: the Research Institute for Fragrance Materials (RIFM) and the International Fragrance Association (IFRA).

RIFM was formed as a nonprofit corporation in 1966 and represents the international scientific authority for the safe use of fragrance materials. The RIFM database of flavor and fragrance materials is the largest available worldwide, classifying more than 5000 materials. RIFM has settled several science programs to cover all the aspects of fragrance safety. Its research is supported by more than 60 companies that consist of fragrance manufacturers and consumer product manufacturers. Moreover, its activities are reviewed by an independent Expert Panel, which provides strategic guidance, determines scientific study design and interprets results for relevance to human health and environmental protection.

IFRA was established in 1973 and represents over 100 fragrance manufacturers in 15 countries. Its members account for 90% of the global production volume. IFRA is registered in Switzerland and has its operational center in Brussels, Belgium. IFRA board is

composed of six active members that are Firmenich, Givaudan, IFF, Robertet, Symrise, and Takasago. IFRA is responsible for issuing and up-dating the Code of Practice which sets standards for good manufacturing practice within the industry, quality control, labeling and advertising. This code also sets limits or even prohibits the use of certain ingredients. The Code of Practice is updated according to the conclusions of the RIFM Expert Panel. Currently, the IFRA safety program contains 186 Standards, which restrict, or prohibit, the use of selected fragrance materials.

In addition to these two international organizations, the European Commission acts as a major actor in fragrance regulation. Indeed, fragrance industry is now global and changes in European regulation directly impact international market by restricting the perfumer's palette. More precisely, the 7th amendment to the European Cosmetic Directive 76/768/EEC has highlighted 26 fragrance materials identified as potential skin sensitizers by the Scientific Committee for Consumer Safety (SCCS). As a consequence, since March 2005 the presence of these compounds has to be indicated in the list of ingredients on the cosmetic label in Europe. This labeling is triggered if there is equal or above 10 ppm of any one of these materials present in a leave-on cosmetic product, and if the level in a rinse off cosmetic product is equal or above 100 ppm [54.10]. As most of these ingredients were commonly used in perfumery before 2005, the 7th amendment to the European Cosmetic Directive 76/768/EEC induced numerous modifications of the ingredient list labeling or reformulations depending on the companies' strategy.

54.4 New Challenges in Scent Creation

Perfumery evolves in the course of scientific discoveries. In the same time, customer demand changes generating new challenges for fragrance creation. Several new trends are detailed in this paragraph.

54.4.1 Environmental Issues

During the last two decades, consumer interest for environmental issues has been growing, especially focusing on sustainability. Ecological and ethical awareness has resulted in modifications of perfumers' palette. First, there has been an increasing demand for natural ingredients with full traceability. IFF-LMR Naturals was one of the pioneers of this approach as it uses to contract directly with farmers from all over the world to guarantee full traceability and sustainability of its products. As an example, in 2010 IFF-LMR renewed decade-long contracts with Burgundy farmers to ensure the production of blackcurrant bud absolute. Now, this strategy has spread in line with fair trade, and several fragrance manufacturers have developed partnerships with local farmers to ensure sustainable sourcing of natural ingredients of strategic importance, such as vetiver, vanilla, ylang-ylang, tonka bean, and patchouli. Through these partnerships, farming communities benefit from a guaranteed minimum price and regular technical support in order to improve harvesting and manufacturing processes. In this context, a partnership was developed in 2009 between Chanel, Robertet, and Serei no Nengone (SNN) to ensure sustainable production of sandalwood oil in New Caledonia [54.14]. In addition, customer concern for organic crops has resulted in an increasing demand for organic-certified natural ingredients. This rising interest induced new challenges in analytical research, especially to assay pesticide residues in natural ingredients.

Sustainability issues also concern synthetic substances. Indeed, various researches are currently performed to get new insights into substance biodegradability, as well as to integrate green chemistry into manufacturing processes. Green chemistry designs products and processes that reduce waste, limit the use of solvents, promote biodegradability, and improve energy efficiency.

54.4.2 New Regulation Issues

In June 2012, the Scientific Committee on Consumer Safety of the European Commission adopted a new opinion on fragrance allergens in cosmetic products (SCCS/1459/11). This opinion updated the list of fragrance allergens that the consumer should be made aware of when they are present in cosmetic products (European Cosmetic Directive 76/768/CEE). It identified among fragrance ingredients some allergens established in humans for which a maximum limit of concentration in the cosmetic product should be fixed. It also indicated that three fragrance allergens (HICC, atranol, and chloroatranol) should not be used in cosmetics. After a public consultation of the different stakeholders, the Cosmetic regulation will be amended accordingly.

54.4.3 New Fragrance Applications

These days, the best perfumer's challenges consist in exploring new sensory territories. Some of them are detailed in this paragraph.

Scent Branding

Various companies now aim at strengthening their brand identity and enhancing customer well being. With this goal, several brands have already adopted an olfactory signature. Olfactory signature, also known as scent branding, defines an ambient fragrance exclusively designed for a brand and diffused in its establishments. Fashion and beauty brands were the first to adopt a fragrant ambiance. But, this trend is now followed by numerous hotels and spas, as well as spirits and food brands. First results have demonstrated that fragrant ambiance can improve customer well-being, resulting in an enhanced time spent in the establishment. Olfactory marketing thus opens up a wide range of new opportunities for fragrance creation.

Medical Support

According to *Jacques Polge*, *Olfaction is the most vital and the most animal of our five senses: if one loses the sense of smell, he loses taste for life* [54.1]. However, the sense of smell used to be poorly regarded by the medical staff. As olfaction is now recognized as a powerful elicitor of emotions, it also begins to be considered for medical support. Indeed, odors can evoke autobiographical memories that are emotionally intense and long forgotten, and even more effective than cues from other sensory modalities [54.26]. Since 2000s, several medical projects have been performed using olfaction as a re-education tool for patients with neurological disorders. As an example, a medical program was launched in 2001 by the team of Pr Brussel at Raymond-Poincaré Hospital (Garches, France) and IFF to help brain-injured patients to recover memory and improve their quality of life. In this context, IFF provided olfactory kits containing reconstitutions of daily odors in order to train patients and to increase their olfactory sensitivity [54.27]. This re-education experiment was a success, and since then the Raymond-Poincaré Hospital has launched several re-education programs in collaboration with speech therapists and nutritionists. *Hummel* et al. confirmed the effects of olfactory training on patients with olfactory loss, and verified the amazing plasticity of human brain even after severe injury [54.28]. More recently, patients with Parkinson's disease performed olfactory training and this training was demonstrated to significantly increase their olfactory capability, and thus their quality of life [54.29].

54.5 Conclusion

Today scent creation process requires not only creativity, but also technical understanding of molecular interactions in the final product, as well as in contact with skin, and environment. These constraints are challenging for perfumers and force them to develop ingeniousness to design original products. Besides these limitations, perfumers have to interpret customer desires. Indeed, perfumers have to be aware of all the emerging trends and tastes to propose creations that anticipate customer needs. In a sense, perfume reflects our civilization. Its use has been evolving throughout the centuries, from a religious gift, to a medicine and since the nineteenth century as an hygiene and beauty product. Even now, despite industry globalization, perfume preferences vary from one region to another one, and perfumers have to understand these cultural contrasts.

Perfumers generally agree that there is no key for success in perfume creation. However, some qualities seem to be essential to become a perfumer: creativity, curiosity, open-mind, perseverance, and obviously an excellent memory.

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55. Odor in Immersive Environments

Manfred Dangelmaier, Roland Blach

Immersive environments provide a computer-generated virtual reality for their users. They are applied in various fields such as engineering, marketing and sales, education and training, therapy or entertainment. Besides the dominant visual aspects or acoustic perception, olfactory perception can be one of the addressed senses in such a multimodal experience. The purpose is to immerse the user in a virtual scene in order to support decision making and design processes as well as learning. While odor can fulfill several functions in human–computer interaction there is currently no big market for computer-controlled scenting devices. Such devices should provide at least a subset of the functions of scent synthesis, dispensing, diffusion/ventilation, neutralization and exhaustion. Immersive environments in addition have to provide an interactive realtime experience and need to be controlled in synchronization with the other experiences and the scene context. This requires the integration of the olfactory simulation with the scene graph. Simple event-driven control models can be used as well as more complex fluid dynamics models to achieve a credible experience. Repeated attempts in previous decades to introduce scenting devices into mass markets for entertainment such as cinema and games were not successful. A more likely development path is the development in the aroma application industries, for example wearable devices for dispensing and dosing scent and aromas.

55.1 Defining Immersive Environments

Immersion means a sense of presence in a virtual environment. Virtual environments are generated in computers (Fig. 55.1). They appear for instance as computer games for entertainment, but are also used in serious applications such as education, decision making in product development, production planning or in purchasing decisions. The opposite of a virtual environment is a physical environment. An immersive environment is thus a virtual computer-generated environment, which allows the user to feel like they are there. This implies that immersive environments provide a realtime experience and that the user can interact with it.

Immersive environments go beyond simple windows as known from graphical user interfaces in daily work with workstations, mobiles and wearable computers. This is typically achieved by a selection from the following set of measures [55.1]:

- Spatial representations are used. A digital world in three dimensions is modelled.
- Stereoscopic displays allow for spatial vision.

Fig. 55.1 Virtual computer-generated environment (Victor Brigola)

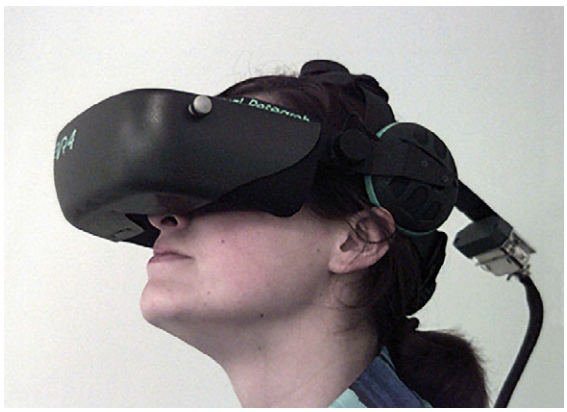

Fig. 55.2 Head-mounted display (courtesy of Fraunhofer IAO, University of Stuttgart IAT)

- The user is surrounded by displays, which can be either head-mounted (Fig. 55.2) or positioned in the environment (Figs. 55.3–55.4).
- Eye or head tracking is used to allow for natural motion in the virtual world.
- The user can navigate and manipulate the virtual environment with spatial interaction devices [55.2].
- A multimodal experience is provided. That means that additional senses are addressed. Sounds, motion, haptic clues (Fig. 55.5), odor or taste [55.3, 4] etc., are added to the virtual environment. This means the olfactory sense is one of the perceptual modalities used to create immersive environments.

Fig. 55.3 Stereoscopic projection wall (courtesy of Fraunhofer IAO, University of Stuttgart IAT)

Fig. 55.4 Immersive Projection room (courtesy of Fraunhofer IAO, University of Stuttgart IAT)

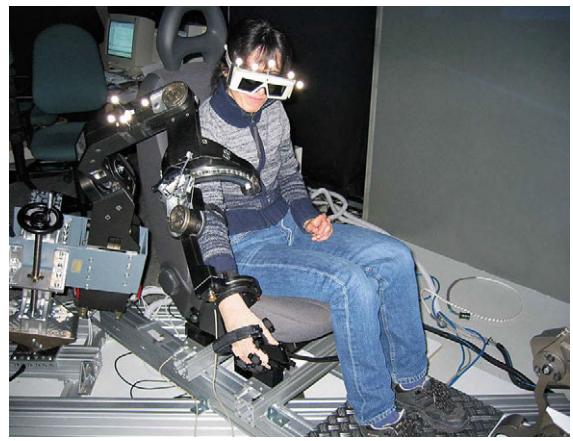

Fig. 55.5 Haptic feedback by an exoskeleton device (courtesy of Fraunhofer IAO, University of Stuttgart IAT)

55.2 Virtual Reality

An immersive environment provides virtual reality [55.5] to its user. It avoids the need for physical presence in the physical environment or even the need for an existing physical environment. Good reasons for using virtual environments and realities are seen in Table 55.1.

A virtual reality (VR) is never an exact reproduction of a physical reality. It always differs for several reasons:

- VR differs from reality in substance.
- VR provides models of a reality and contains modeling errors.
- VR is perceived through imperfect human–machine interfaces with restricted performance and reproduction acuity.
- VR is typically a simplification or idealization of a reality.

Therefore it is not helpful and inefficient in most cases to follow an ideal concept of VR as a perfect reproduction of a physical reality. It is better to understand VR as a sufficient representation of a reality for a specific purpose. Imperfections may be intended, for example in artwork [55.6]. Frequently they are not desired but accepted by users [55.7]. Furthermore a cognitive immersion or adaptation effect occurs in the course of an immersive experience – the virtual reality becomes the present reality for the user.

Following this paradigm it is helpful to add odor to VR where it is crucial for the purpose of the application. Designing a shop interior with VR requires fragrances in VR in case aromatization is part of the proposed shop experience. For example in case a museum wants to present a virtual medieval town to its visitors following an experiential archeology approach, it should consider odor as an option for the virtual environment.

Odor in VR requires an appropriate dispensing device (Chap. 58). In case scene-dependent change of odor is required, a computer-controllable dispenser is needed as well as an appropriate controllable evacuation or neutralization device.

Table 55.1 Reasons for using virtual environments

Reason	Example
Physical environment does not exist anymore	Archeology
The physical environment does not yet exist	Architectural planning, product development
The environment is not physical in its nature	Games, abstract information spaces
The physical environment is not accessible or physical presence is too risky	Expedition to Mars, training environments for hazards
The physical realization is too expensive or takes too long	Construction of prototypes

55.2.1 Augmented Reality

In contrast to VR, where a virtual model of a reality is in the focus of consideration, *augmented reality* (AR) denotes the introduction of virtual elements in a physical perception space (Fig. 55.6). Visual virtual elements can be models of physical objects, abstract spatial information, or two-dimensional graphical components.

Unlike VR, AR is always related to the present spatial situation of the user and depends therefore on the situation. It is a situational context-related technology. AR can use a stationary device in the rare cases that it is only related to one specific location. Mostly, however, AR has to rely on mobile or wearable devices.

Examples are sightseeing information virtually added to physical landmarks, navigation information superimposed in the field of vision, personalized location-dependent advertisements presented in a shop-window, furniture from a catalog virtually positioned in an existing living room, or a virtual temple superimposed over an ancient ruin.

AR uses either the natural perception of the human and adds information through a wearable device or it uses video camera images and augments those with additional information. One category of AR applications uses commodity smart phones or tablets to augment their camera live videos with additional information.

Like in VR spatial representation is crucial for AR. In addition spatial relation plays an important role. Augmenting a word processor screen with correction information is not considered as AR. Labeling a tower in a city skyline with its height is however accepted to be named AR. In order to place augmenting informa-

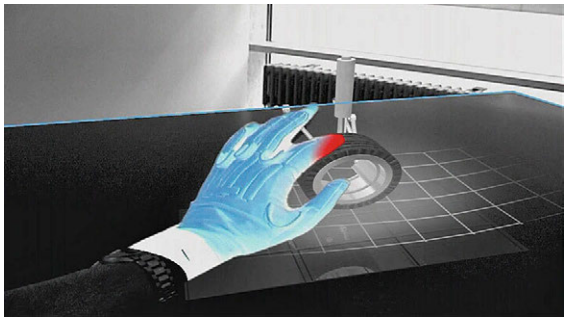

Fig. 55.6 Augmented reality in engineering workspaces (courtesy of Fraunhofer IAT, University of Stuttgart IAO)

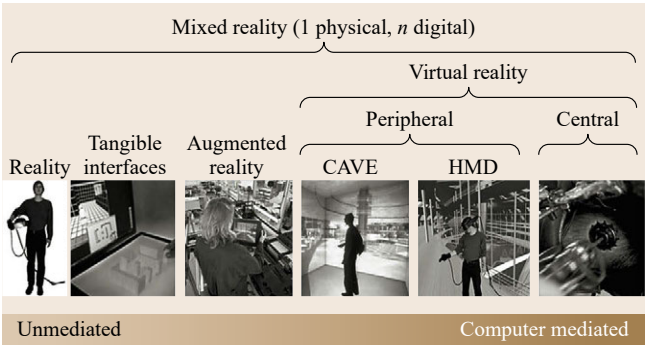

Fig. 55.7 Mixed reality – between unmediated physical reality and computer-mediated virtual reality (after [55.8], courtesy of Fraunhofer IAO, University of Stuttgart IAT)

tion correctly, positioning virtual objects correctly in an observed reality is a key technology for AR.

Using odor in AR is more difficult than in VR. AR is frequently applied in the public space. Released fragrances can be disturbing for persons in the environment and appear as pollution. Wearable devices with integrated dispensing devices could be an option to avoid this. Devices for that purpose are however not yet available. On the other hand situational perfuming using wearable devices could be considered as a contribution to the augmented reality of the future.

55.2.2 Mixed Reality

Mixed reality is an extension of the concept of virtual reality. It denotes a mixture of physical and virtual computer-generated elements in a perception space [55.9]. Augmented reality is hence mixed reality. But mixed reality is not necessarily augmented reality. Mixed reality (MR) is a more precise expression for many VR applications. As soon as the user perceives herself or himself as part of the scene, for example when experiencing architecture or playing games, MR is a more precise denomination than VR. When seated in a virtual scene the user needs a physical seat or chair and VR becomes MR. Perceptible hardware in a virtual scene always means mixed reality (Fig. 55.7).

Applying odor in immersive environments representing a marketplace by commercial devices as used in scent marketing would be considered as MR. In the case where the odor is computer-generated and provided by a universal computer-controlled scent output device it would be considered as VR.

55.3 Multimodality in Immersive Environments

Multimodality is one of the characteristics of immersive environments. Frequently the visual mode dominates the virtual experience and other modalities are neglected. In particular this is the case in many visually based decision support applications in design, architecture and engineering disciplines. In simulation and entertainment applications however, the auditory and haptic modes become important and are addressed. Games and simulations typically utilize sound and proprietary haptic interfaces to improve the experience. The senses to be addressed include the well-known five senses:

- Visual perception
- Auditory perception
- Olfactory perception
- Gustatory perception
- Tactile perception.

Four further modes of perception are relevant including:

- Thermoception
- Nociception
- Equilibroception
- Proprioception.

All modalities are relevant for and used in immersive environments with the exception of nociception, which is usually neglected in nonmedical applications due to ethical reasons.

Nambu et al. [55.10] showed in an experiment that modalities are not independent. They found that olfactory perception is changed by visual cues. They could even achieve olfactory perception by pure visual stimuli.

The multimodal nature of human perception may also lead to simulation sickness in immersive environments. Such a phenomenon occurs among users depending on predisposition as well as on the simulated motions. Simulation sickness arises by reason of conflicts between stimuli and user experience – in particular if perceived motion clues are not in line with previous user experience. A well-known example is missing or imperfect representation of acceleration cues in simulated driving. Impaired breathing air quality and disagreeable olfactory stimuli are supposed to contribute to simulation sickness.

55.4 Functional Aspects of Odor in Human–Computer Interaction

Odor is yet not widely used in human–computer interaction and in immersive environments [55.11]. This is due to the fact that odor is not a means of voluntary communication in humans. But humans perceive and respond to odor, so there are functional aspects of odor, which can be addressed in human–computer interaction as shown in Table 55.2.

As odor depends on chemical substances in the breathing air it is not suited for quick interaction tasks. The substance has to be dispensed and needs time to be transported through convection, diffusion and inhalation to the olfactory receptors. In case the odor needs to be changed the previous substance has to be removed from the inhalation zone or neutralized chemically and to be exchanged by another one. Furthermore the perception of odor has a spatial component, which is determined both by the presence of the aroma and the perception of the airflow [55.12, 13]. The following technical functions are relevant:

- Synthesis
- Dispensing
- Diffusion and ventilation
- Neutralization (chemical decomposition)
- Exhaustion.

A computer-controlled scenting device has to cover at least a subset of these functions and requires also a computer interface.

Table 55.2 Functions of odor in human–computer interaction

Function	Examples
Output of odor as a main purpose of the computer system	Smart wearable perfume dispensers Room ambience control
Feedback in working with odor	Computer-assisted synthesis of fragrances
Odor as part of a simulated experience	Training of firefighters
Improvement of the sense of presence in immersive environments	Virtual service scape engineering for a bakery
Triggering emotional responses	Affective computing
Support of memorization	Ambient assisted living for dementia patients
Nonurgent warnings and notification	Announcing coffee breaks

55.5 System Design

55.5.1 Hardware for Odor Exposure

Hardware for creating scents can be classified according to different variables. Important ones are:

- Affected space
- Fragrance creation
- Physical principle of dispensing (particle release to airflow, vaporization)
- Physical principle of removal (diffusion and ambient convection, exhaustion, neutralization)
- Control of fragrance release.

The affected space can be firstly the personal body space, which can be addressed by head-mounted or otherwise wearable devices. In this case the scent releasing system moves with the user. Secondly the same personal space can be addressed by stationary devices. This includes seat-mounted devices in a cinema or scent-generators mounted at a workstation. Thirdly the affected space can be of room size or partial room size as it is intended with most ambient scenting systems applied today and as it is needed for stereoscopic projection rooms (CAVEs) (Fig. 55.4).

Fragrance or scent creation can take place inside the scent generating system or outside. In the latter case fragrances and scents are prefabricated and only released by the scenting device. In the first case the fragrance is mixed from component substances inside the scenting device directly on site, which allows for much higher variety and flexibility but leads to less sensory fidelity.

The physical principles of dispensing the substances include vaporization from a liquid solution by heat or airflow, Venturi mixing and others. Heat might cause chemical reactions and impact the fragrance.

Overall, there are several ways to remove the scent. The easiest is to use dilution by diffusion and ambient convection. This does not require technical measures. Technical system-integrated solutions comprise exhaustion and chemical neutralization by decomposition of the molecules or by binding odorous molecules to others. The latter method requires neutralizing substances such as cyclodextrins as used in household odor eliminators.

The control of the scent can be achieved manually by switching on and off the dispenser. Closed-loop controllers would for instance control the concentration of

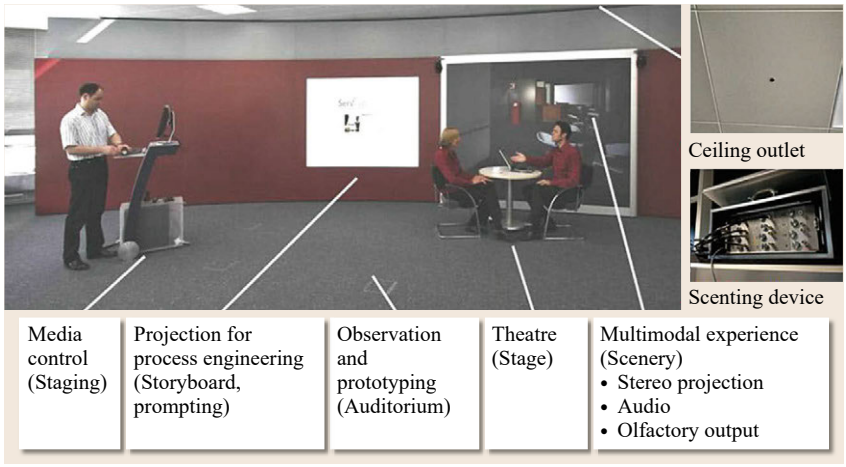

Fig. 55.8 Immersive system for service engineering with scenting device (courtesy of Fraunhofer IAO, University of Stuttgart IAT)

the fragrance in the air. Computer interfaces are the most flexible way to control scents and are targeted for universal use in virtual environments.

There is a limited range of scenting devices on the market. The mainstream can be characterized as single-scent dispensing devices for prefabricated scents. Some products on the market also support chemical neutralization by catalytic decomposition. This can either be implemented as an alternative to scenting or as an additional function.

Most of the devices on the market do not have computer interfaces to allow for software control, which makes them useless for computer-created and controlled immersive environments.

An example of a mixed reality system with a computer-controllable scent output device is shown in Fig. 55.8. The scenting device dispenses up to five different prefabricated scents. These are released from a ceiling outlet. The room is equipped with an air conditioning system to provide ventilation. Furthermore, neutralizing substances can be dispensed by a separate system.

Programmable scenting devices like Smell-O-Vision [55.14] were invented around 1960. The scent experience was designed for cinemas. The system used containers of prefabricated scents ordered in a belt in sequence of appearance. The release was controlled by signals encoded in the film roll. The scents were delivered through pipes at the seats of the audience. The film Scent of a Mystery was produced for this system.

This idea was recently taken up by a Japanese group of researchers from Tokyo applying it to computer or TV screens [55.15]. By controlling the airflow in front of the screen a two-dimensionally distributed localized scent experience is achieved. Most of their research in olfactory displays goes in the direction of supporting spatial perception of odor [55.12, 13, 15].

The first decade of the new millennium saw new start-up companies in the field of computer-controlled scent output trying to commercialize devices. Israel-based Scentcom [55.16] claimed to have developed a technology and proposed applications in games, home movies, toys, military simulation and training, mobile devices and for warnings in motor vehicles. Most of these initiatives and start-up companies failed. Like Scentcom some of them followed the approach of composing odors from a limited set of base aromas. AromaJet [55.17] planned to use 16 ingredients to synthesize scents. But to date commodity computer-controlled multi-scent devices for personal computing or entertainment are not available.

55.5.2 Software and Integration Aspects

Immersive environments have to provide an interactive realtime experience of a virtual, computer-generated reality for its user. This requires software corresponding to these needs. The software must deliver, together with the hardware, a consistent user experience over all modalities. In particular the sensory modalities have to be synchronized. Because olfactory perception is related with time lags due to the physical nature of diffusion and convection the synchronization of scents with the other modalities is not time critical. Acceptable time lags depend on the scene and are typically in the magnitude of seconds. Hardware pipe dead times have to be taken into account as well.

Immersive environments are frequently built on a scene graph representing the structure of the represented scene. Nodes in such a graph represent the objects in the scene. In case of an immersive environment with scent output olfactory properties could be assigned to those objects and will thus become part of the scene graph.

Objects associated with a certain odor could be spaces or rooms. When the user is inside a space or room, the corresponding odor could be released. When the user changes his space the odor of the new space has to be displayed. Another approach would be to assign smell to physical objects in the scene. When the user approaches the object in the virtual space, the corresponding odor will be released from the scent output system. When the user moves away from the object the scent output is reduced or stopped. A certain ventilation of the room is required in these cases to ensure vanishing odors when the user moves in the scene.

These models do not match physics very well. A more complex system would model fluid dynamics and odor transmission in the virtual world on the one hand and in the physical world of the immersive system on the other hand. It will try to match the physical world with the virtual world. The used fluid dynamic computation can vary from calculating simple one-dimensional transportation models up to solving full spatial fluid dynamics models. However, as complex fluid dynamics models cannot be solved in realtime a compromise is necessary.

The disadvantage of this forward control approach is the lack of feedbacks from the physical world. This could lead to a divergence between calculation and reality. Odor sensors with a control algorithm could be used to implement a closed control-loop to overcome this issue.

55.6 Applications

Immersive environments are widely in use in different fields like engineering, architecture, marketing and sales, eduction and training as well as entertainment and even in therapy. Many of them are multimodal. But only in rare cases are olfactory displays applied. The following paragraphs will focus on applications with odor. So it will rather show potentials for the future than productive settings of the present.

55.6.1 Computerized Scenting and Therapy

Immersive environments are usually expected to include computer graphics plus other sensory modalities. In this sense computerized scenting is a border case of a virtual environment or mixed reality. The scent does not originate from the natural physical environment and is not dispensed manually by its user but is generated by the computer and a scent output device thus augmenting the physical environment.

Wearable computer-controlled perfuming or ambient scenting devices belong to this class of application. They are expected to appear in the future either as an evolvement of the products of the traditional aroma industry or as an additional function of the increasing market in wearable devices for sports and lifestyle.

Chen proposes combining virtual reality therapy with aromatherapy approaches [55.11]. One specific suggestion is to apply scents as stimuli in VR-supported therapy of phobia.

55.6.2 Engineering

User-centered product development has become an important approach in product design and development. Human factor engineering includes more physically oriented ergonomics and hardware- and software-related usability aspects but also the aspect of user experience. Instead of focusing on the design of a physical product this approach aims at designing an experience for the user. This paradigm is supported by the view that products are not mainly hardware and software but provide services to their users. Products can also be pure services, where no physical product is provided at all.

This also means that product engineering becomes experience engineering. In digital or virtual engineering immersive environments are used to provide an experience of the product before a physical prototype or mock-up exists. This experience is used for evaluation purposes involving end users and other stakeholders in the product lifecycle.

An example of an immersive experience engineering system is shown in Fig. 55.8. A stereoscopic projection wall is used to provide a visual immersive scene of a service environment for service engineering purposes [55.18, 19]. The laboratory is also equipped with an audio system and an accenting device to allow for a multimodal experience.

Another example for the aroma industry is use-context-sensitive decision making for aroma products. The context of use is displayed visually or audio-visually and scents are presented in parallel. The decision makers experience the product in the use context and decide context-aware.

Odor is frequently considered as a component of the product and the user experience. This holds true not only for cosmetic products, food or coffee but also for more technical products like cars, where the odor is even used for branding. In these cases odor has to be an integral part of experience engineering. In consequence

it needs to be implemented in immersive environments for holistic experience engineering.

A special case of experience engineering is product customization. The customer is involved in the design of the product and has degrees of freedom in selecting among a finite number of options or even a range of product parameters. This results in a high number of combinations and requires production on demand.

This can also be applied to product-related aromas. In these cases the customer needs a feedback on his/her choice, which again results in the need for computer-generated aromas. The World Wide Web and a web browser are frequently used for mass customization. Due to a lack of commodity scent output devices for computers this is not possible for scent customization. Therefore special devices have to be provided at the point of sale.

55.6.3 Marketing and Sales

Scent marketing has gained importance during the last decades. Scent is used in supporting the sales process by:

- Providing a positive shopping experience
- Advertising the product with its own odor (billboard scenting)
- Establishing a brand (scent branding).

Frequently natural scents are used. A typical example is baking at the point of sales in the bakery. If this is inappropriate, for example for packed convenience food stores, scenting devices at the point of sale can be applied. They also allow for a better control of intensity of the odor. In cases where the experience varies over time a computer-controlled immersive environment could be applied. This can be the case if several user experiences are related to a product, or a product story needs to be told, or if several products or product variants shall be displayed in sequence. A typical and comparably simple setup would include a visual display presenting video sequences and a synchronized scenting device.

55.6.4 Education and Training

Immersive environments allow for the creation of a sense of presence for education and training purposes. In particular an immersive simulator is a valuable tool where highly automated decisions and behavior have to be acquired. This holds particularly true if the situations are dangerous or hard to reproduce.

In a more uncritical situation a programmable scenting device could be applied for training sensory analysis. A multimodal immersive system could be used to train scent recognition, design or selection in a sensory context.

In more complex situations scents are also relevant, for example in medical education and training or in chemical and fire hazard and military training.

Immersive environments with scent output can also be applied in virtual experimental history approaches to generate historical experiences for research and educational purposes.

55.6.5 Entertainment

Audio-visual entertainment is developing towards more immersive experiences. Stereoscopic devices are used in movie theaters as well as in home entertainment and games. While scent as an supplementing modality for audio-visual experiences in entertainment for the masses such as movies, TV and games has been a topic of research and start-up enterprises for decades, it has not yet been possible to establish such a technology for the masses.

Even custom-built multimodal theaters, presenting so-called four-dimensional (4-D) programs, rather focus on kinesthetic and tactile enhancements and use smell only sparingly.

55.6.6 Conclusion

In conclusion, there is a lack of immersive environments using odor. The application of odor suffers from the nonexistent market of computer-controllable scent output devices. Although there were several attempts during the last decades to introduce scenting devices into mass markets for entertainment purposes such as cinema, TV and games, there was no success. A more likely path to such devices might be the developments in the aroma application industries. Wearable devices for dispensing and dosing scent and aromas could be point in the right direction.

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56. Odor in Marketing

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Marketers are increasingly using scent for differentiating, enhancing, and promoting products, and services. The spreading commercial use of scent, however, stands in contrast to a limited and fragmented body of knowledge on how people as consumers perceive and respond to olfactory stimulation. To facilitate a better understanding of opportunities and limitations to the commercial use of scent, this chapter reviews the state of research in the psychology, consumer behavior, and marketing literature. Extant studies examine scent as a primary product attribute, a secondary product attribute, an agent for promotional efforts, and as an ambient cue. Organized in six major sections, the chapter starts with a discussion of effective characteristics and the human processing of scent. A comprehensive review of consumer responses to scent follows. Section 56.4 adopts a multimodal perspective to illustrate how scent interacts with other sensory modalities to influence consumers as multisensory beings. Section 56.5 highlights individual and situational factors that can enhance or mute olfactory effects. The chapter concludes with a discussion of ethical aspects and outlines avenues for future research that may prove beneficial to both researchers and practitioners.

56.1 Using Scent for Marketing

In a world where consumers encounter countless choices, marketers are trying to find new ways for their offers to stand out, appeal to, and ultimately persuade shoppers. One option that is attracting more and more attention is to deliver more complete and holistic sensory experiences. For example, engaging multiple senses aids brands in being perceived as more distinctive and successful [56.1], and getting better remembered [56.2]; it also increases the attractiveness of retail environments to visitors [56.3], and enhances the overall persuasiveness of marketing communication [56.4]. While vision can be considered the key modality in consumer perception and processing of offers [56.5, 6] – engaging additional senses can enhance the overall consumer response, leading to more favorable affect [56.7], cognition [56.8], behavior [56.9], and memory [56.2, 8–10]. This capacity for triggering more positive consumer response is particularly evident with the sense of smell due to its unique properties [56.11]. Scent marketing, defined as *using scents to set a mood, promote products or position a brand* [56.12], has thus attracted the attention of both researchers and practitioners.

Morrin [56.3] classifies the use of scent in marketing products and services into four categories:

1. Scent as a primary, product attribute
2. A secondary product attribute
3. Part of a promotional effort
4. Ambient scent.

Scent is considered a *primary product attribute* when it represents the major driver of consumer purchase, such as the fragrance in perfumes or deodor-

ants [56.13]. In those cases, scent provides key information about the product, such as a deodorant's freshness or an after-shave's soothing quality. In contrast, scent is considered a *secondary product attribute* when the product's primary attributes are not scent related. In those cases, scent is used for differentiation, distinguishing the product from alternative options. For example, some people can tell the Nivea crème apart from other brands even when blindfolded because they recognize the brand's unique scent [56.14]. In the third category, scent is being used in *advertisements and sales promotion*. For example, in 2006, advertisers attached scented paper stripes to *Got Milk* billboards promoting dairy products to commuters at various bus stations in San Francisco. The stripes emitted the scent of freshly baked cookies to entice consumers. Similarly, in 2012, McCain launched an integrated marketing communications campaign involving scent to promote a baked potato product to shoppers in Great Britain. After pressing a button on a bulging 3-D-baked potato, consumers could smell the product and receive a money-off coupon for their purchase [56.15]. McCain also used other measures including automatic scent dispensers that released the smell of baked potatoes in the frozen-food aisles of supermarket every time someone passed by and specially fitted taxis with huge three-dimensional (3-D) potatoes on their roof driving through cities in the United Kingdom. Perhaps the most common use of scent in marketing is its diffusion as an *ambient cue*, a scent that cannot readily be attributed to a particular object but is present in the environment. Ambient scent is widely used in many kinds of environments ranging from hotel lobbies to retail outlets, casinos, airlines, and hospitals [56.16]. Pioneering the commercial use of ambient scent in the 1990s, Singapore Airlines had an aroma specifically designed for its fit with the airline's corporate image to be used on the hot towels served before takeoff and landing as well as in the flight attendants' perfume [56.1].

Using scent for marketing to consumers in a psychologically sophisticated manner provides challenges that do not necessarily follow all the rules of other types of (sensory) marketing. Olfactory marketing has distinct combinations of properties and mechanisms that lead to unique patterns of psychological responses and therefore demand out-of-the-ordinary attention to a variety of issues. This chapter advances the understanding of scent in marketing by bringing olfaction, consumer psychology, and marketing communications together to further business practices and research. Readers will learn some of the latest insights in the areas of:

1. Effective properties of scent (specific drivers of consumer response)
2. Effects attributable to scent (the range of consumer reactions to scent)
3. Interactive effects with information obtained through other sensory modalities
4. The contingency of effects upon individual and situational factors.

This chapter further summarizes and refines what we know about marketing and consumer psychology in relation to scent; it includes a comprehensive review, innovative conceptual frameworks, and empirical studies. We trace the work of scholars who can be characterized as pioneers in the field. Our review is selective and not exhaustive. Our intention is to illustrate how the training, creativity, and motivation of notable researchers provided a significant part of the foundation of this field as we know it today and as it is reflected by the sections in this article. The chapter's sections are structured as follows. First, a discussion of effective characteristics and the human processing of smell is presented. Then a detailed review of odor effects on consumers follows. Given that in the marketplace consumers are usually not exposed to scent only but additionally to information obtained through other senses, the third section sheds light on the interaction of scent with other sensory input. The fourth section offers evidence for what individual and situational factors enhance or attenuate scent effects. Finally, ethical aspects and promising avenues for future research are discussed.

56.2 Effective Characteristics and Processing of Scent

56.2.1 Effective Properties

In psychology and the cognitive sciences, perception is the process of acquiring, interpreting, selecting, and organizing sensory information [56.17]. What, exactly, is it that makes people react to scent, specifically, what are its effective properties? Research points to four properties that trigger consumer response to scent: character, pleasantness, familiarity, and intensity.

Relying on six to ten million receptors located in the olfactory epithelium [56.18], the human nose does relatively well in detecting and discriminating the *character* of between 2000 and 4000 different scents [56.19]. Untrained people are not good, though, when it comes to

identifying and characterizing scents [56.20]. For example, when asked to name the scent of at least half of the scented household items used daily, consumers fail [56.21–23]. The rate of success improves when the familiarity of the scent increases [56.24], but, very commonly, people experience a feeling of recognition and familiarity while being unable to voice a verbal description or assign a semantic label (the *tip-of-the-nose* phenomenon) [56.24]. This difficulty in naming a scent is thought to derive from a variety of reasons. One reason could be that in our evolutionary past naming an odor has not been essential for survival. It is usually enough to detect a scent and discriminate it [56.25]. A different reason might be that in everyday life odors most frequently occur within a context. It has thus been argued that the weak performance in odor naming tasks is due to a lack of context [56.26]. A third reason for the lacking ability to name scents may lie with the relatively weak semantic link between an odor and its name. It is conceivable that the areas of the brain responsible for processing words are located apart from the olfactory processing areas, at least further than for other sensory modalities [56.27]. In addition, odor processing and language processing engage identical cortical resources [56.28]. Therefore, simultaneous processing of language and olfaction leads to a competition for processing resources, hereby making it difficult to name odors [56.20]. Some of these limitations, however, may be overcome as people can be trained to enhance their ability to name and describe odors more accurately [56.29].

A scent's *pleasantness* is sometimes viewed as a property almost as important as its character [56.30] because people routinely and quickly establish if they like an odor or not. Past consumer research even suggests that odors are primarily perceived in terms of their pleasantness or unpleasantness, the individually and positively evaluated stimulation of the olfactory senses [56.31]. Beyond pleasantness, individuals perceive and respond to a scent's *familiarity* and *intensity*. While the evaluation of pleasantness and familiarity is often highly correlated (familiar scents are evaluated as more pleasant and vice versa) [56.32], the relationship between pleasantness and intensity is more complex [56.33]. Familiar odors are better liked than unfamiliar odors; also, pleasant odors are often found to be more familiar. In comparison, the relationship between odor intensity and pleasantness often can be characterized as an inverted-U function, depending on the specific scent. A perfume smells pleasant, as intensity increases, but only up to a point. Beyond that point the scent intensity becomes so strong as to be evaluated as unpleasant. Yet, with some scents, the relationship between intensity and pleasantness may be linear rather than bell shaped. Whereas a light fish odor may be acceptable, the evaluation may become continually more unpleasant as intensity increases [56.11].

The issue whether hedonic differentiation in human perception of odors is innate or learned is still under discussion. Support for the claim that hedonic discrimination is innate derives from research on taste perception which suggests that this mechanism is mostly hardwired [56.34]. However, there is also evidence for the associative learning theory [56.27]. According to this theory, one item (a scent) becomes connected to another (the judgment of pleasantness) as a function of an individual's past experience [56.35]. Supporters of the associative learning theory claim that newborns lack any odor preferences. Over a life time, odors occur bound to experiences and emotions, and hedonic responses are developed [56.30]. Therefore, the hedonic perception should depend on individual experiences.

56.2.2 Scent Processing

Given that other chapters are dedicated exclusively to the human processing of scent, we just briefly highlight a few issues with unique relevance for marketing. First, scent is special in that some scholars consider it the slowest sense when it comes to information processing [56.11]. Second, different from all other sensory inputs, olfactory information is relayed directly to the amygdala-hippocampal complex without any mediators in between. Input obtained through all other senses is routed first to the thalamus [56.11], which then relays the information to higher cortical areas. Further relevant to scent processing is the close proximity of the olfactory nerve and the amygdala (which are only about two synapses apart) and the hippocampus (three synapses) [56.11]. The amygdala is critical for processing of emotion and emotional memory [56.36, 37], and plays a key role in classical conditioning [56.38], and associative learning [56.39]. The hippocampus is a key player in short- and long-term memory and is involved in a variety of declarative memory functions [56.11]. Furthermore, the hippocampus is where most of the cross-modal integration takes place, that is, the combination of information obtained through two or more sensory modalities [56.40–42]. Finally, there is evidence pointing to olfaction and emotion as being intimately connected during neuro-evolution [56.43]. Both brain areas involved in the processing of scent, amygdala and hippocampus, evolved from a tissue that was formerly olfactory cortex, thus indicating that structures now dedicated primarily to emotion and associative learning were previously exclusively dedicated to scent processing.

Taken together, the unique ways of human olfactory information processing suggest that no other sense has such an instantaneous and explicit connection to the brain areas related to emotion, memory, and learning. This special processing combined with the large number of options available in influencing effective properties of scent is perhaps the major reason for scents holding appeal to marketing.

56.3 Consumer Response to Scent

Using scent for marketing products and services is an emerging idea. Not all effects are fully understood yet and some aspects are still heatedly debated [56.44]. The following sections detail specific consumer responses to scent, including changes in affective states, evaluative judgment, intention, behavior, and memory, and offer some integrative models.

56.3.1 Change in Affective States

Human affective states include both emotions and moods [56.45]. Marketing researchers commonly differentiate both concepts along the dimensions intensity, durability, and reference [56.46]. Moods are less intense, longer lasting, and largely unintentional in that they occur in the absence of a reference object. Emotions, in contrast, are more intense, short-term, and object-related, and incorporate some element of cognitive processing; in other words, emotions are *moderately mediated* by cognition [56.47]. In line with the dispositional theory of moods [56.45] and the affect-as-information paradigm [56.48] affective states are recognized for their role in impacting consumer behavior.

Among the most frequently discussed responses to scent are shifts in consumer mood and general affect [56.31, 49–52], often measured in terms of the *pleasure* and *arousal* dimensions of the PAD (pleasure – arousal – dominance) scale developed by *Mehrabian* and *Russell* [56.53]. Reported outcomes are not consistent, though. Reviewing twenty-two studies on ambient scent in retailing and consumer services, *Bone* and *Ellen* [56.44] found large variance in effects evidenced by more than 200 different consumer reactions; only three studies, however, demonstrated a significant effect of an ambient scent's presence on consumer mood. Other studies similarly failed to provide corroborating evidence [56.9, 54, 55], providing little to no support for what popular press repeatedly refers to as the myth of seducing shoppers through ambient scent [56.4, 12]. Strong negative evidence is further provided through *Chebat* and *Michon*'s study as shoppers' positive mood was completely unrelated to ambient scent and pleasure did not mediate the effects of ambient scent on behavior. It was therefore concluded that effects initially attributed to ambient scent may, in fact, be due to an interaction between scent and other factors rather than exposure to scent only [56.56–58]. Consistent with this interpretation, *Orth* and *Bourrain* [56.7] showed that the pleasantness of an ambient scent enhances the influence of consumer affective states (mood) on behavior, thereby stimulating exploratory tendencies, such as the exploration of unfamiliar offers and consumer acquisition of radically new offers.

Contrasting the lack of evidence in shopping contexts, the direct influence of scent on mood has been one of the major principles in aromatherapy; some fragrances even trigger physiological reactions (change in heart rate) in close correspondence with shifting moods [56.59]. Experimental (but not field) studies have generated corroborating evidence. For example, when completing a mood questionnaire in a room scented either with lavender and lemon (pleasant scents) or dimethyl sulfide (unpleasant scent), panelists in the pleasantly scented condition reported significantly more positive moods than did panelists in the unpleasantly scented condition [56.51]. In line with this finding, women seated in a dentist's waiting area exhibited more positive moods when the room was scented with an orange aroma than when it was not scented [56.60]. Contrasting the majority of retailing studies, *Doucé* and *Janssens* [56.61] found a positive effect on consumer mood (greater pleasure and higher arousal) when an upscale clothing store was scented with a minty lemon aroma. The authors emphasize, however, that their results likely trace back to their examination of high-involvement products (prestigious clothing) rather than the low-involvement products studied in previous research (school supplies, decor items, toiletry, and household cleaning products).

In summary, changes in affective states do not always emerge clearly from studies trying to link scent to mood. Although some studies have been conducted in realistic settings, such as a casino [56.62], a fashion store [56.61], or a shopping mall [56.56], most research has been conducted in artificial laboratory situations [56.9, 63] with differences in findings suggesting that effects may depend on additional individual or situational factors. We will revisit this issue later when discussing conditions and moderators of consumer response to scent.

56.3.2 Evaluative Judgments

Conveying meaning to consumers is one of the central objectives in marketing. To convey meaning (such as a product's quality, a brand's personality, or a store's price level) retailers, servicescape and brand managers around the globe employ a variety of visual, haptic, auditory, and olfactory cues. Quality is important because it represents the cognitive evaluation of an offer's intrinsic core benefit [56.64]. At times when this functional benefit is difficult to judge consumers employ extrinsic cues, including scent to infer quality [56.65]. Brand personality is important because it systematically captures and categorizes facets of brands in terms of generalizable symbolic benefits [56.66], enabling consumers to express themselves [56.67], and assisting managers in differentiating offers [56.68]. Companies that succeed in conveying a certain meaning, therefore enjoy advantages in terms of heightened consumer attention [56.69], better recognition [56.70], more favorable behavioral intentions [56.65, 71–73], extra advantageous positioning [56.74–76], and superior financial performance [56.77]. Although marketers – in principle – recognize the potential of conveying meaning through scent, the literature investigating this topic is still limited, with the majority of studies focusing on scent as a secondary product attribute, and only a few studies examining ambient scent.

Focusing on *scent as a secondary product attribute*, research on evaluative responses has commonly examined effects on consumer judgment of product attributes and overall quality [56.44]. Pioneering research in this field, *Laird* [56.78] instructed housewives to rate the quality of panty hoses in a home use test. Three of the hoses were lightly scented (aromas of flowers and fruits), the fourth was not. Regardless of the specific scent, study participants rated the scented hoses significantly better in terms of quality (as more durable, pearly sheen, and stronger); an effect that extended to higher preference. In addition, differences between the scents emerged as half of the participants preferred the panty scented with a narcissus scent. Examining the question whether or not a scent has to fit the product to elicit positive evaluation, *Bone* and *Jantrania* [56.8] found that consumers, in fact, evaluated a product more favorable if they judged a scent to be appropriate. Nevertheless, a broad range of key product attributes were uniformly rated more positive when the product was scented rather than unscented, regardless of whether the scent was deemed appropriate or not.

Examining consumer response to *ambient scents*, researchers selected scents according to their affective charge (pleasant, unpleasant) for use in a laboratory setting. The scents were diffused in a room designed to resemble a one-stop shopping outlet for students with merchandise consisting primarily of household items and school supplies. Adding a scent significantly improved visitor ratings of merchandise quality compared to the no-scent condition [56.54]. This finding was later replicated in a field study where the presence of a pleasant scent improved the consumer perception of an actual shopping mall and increased the amount of money spent by shoppers [56.56]. Merging research on ambient scent with studies of scent as a secondary product attribute, and extending the focus to brands, *Morrin* and *Ratneshwar* [56.55] requested members of a consumer panel to rate familiar and unfamiliar brand names in the presence of select ambient scents. The findings indicate that consumers evaluated the brands more positively when a pleasant scent was present.

Taken together extant research substantiates scent's ability to influence consumer evaluative judgments related to products, brands, and shopping environments. Differences in researchers' focus on effective scent properties (presence, valence, aroma), contexts (products, brands, environments), and dependent variables (overall quality, specific attributes) render it difficult, though, to deduct broad conclusions or infer general guidelines. In addition, making study participants aware of scents, an approach employed in several of the studies (respondents explicitly submitted scores on scent properties), may generate exaggerated or skewed findings [56.79].

56.3.3 Memories

Recent research highlights evoking involuntary autobiographical memories as a possible mechanism for successfully marketing to consumers [56.80]. Involuntary memories, one of Ebbinghaus's three basic kinds of memory, are memories of personal experiences that come to mind spontaneously – that is with no preceding attempt at retrieval [56.81]. Different than voluntary recall (deliberately retrieving memories), the activation of involuntary memories involves little executive control [56.82] as implicit memories are brought to mind automatically by a variety of cues including scent [56.83, 84] (Chap. 42). When implicit memory becomes accessible, the resulting experience shares many of the properties of the original experience. It is vivid, accompanied by subjective feelings and physiological changes, and commandeers attention, thought, and behavior [56.82].

Due to their unique processing, scents are considered superior to other sensory input in evoking vivid and complex memories [56.41, 85]. Implicit memories triggered by olfactory cues are usually highly personal, consist of fewer cognitions, and are accom-

panied by strong affective charges [56.86]. In comparison to autobiographical memories evoked by visual cues, scent-evoked memories trace back farther into one's past [56.87], and are accompanied by more and stronger feelings [56.85]. Integrating research on atmospherics with autobiographical memories, *Orth* and *Bourrain* [56.7] investigated the influence of scent-evoked nostalgic memories on consumer exploratory behavior in a laboratory environment scented with natural and manmade odors. The findings indicate that ambient scent evokes nostalgic memories, which in turn positively influence consumer sensation seeking with downstream effects extending onto exploratory tendencies, namely risk taking, variety seeking, and curiosity-motivated behaviors.

Most of marketing and consumer research studies have concentrated on examining scent as a secondary product attribute and as a means to promote brands. Scenting *products* with an appropriate scent can enhance individuals' memory for information about the product as well as associated attributes [56.88]. For example, when consumers were presented with a pencil, either scented with a common scent (pine), an uncommon scent (tea tree), or unscented, they remembered the information included on the uncommonly scented pencil best after a period of two weeks had passed [56.89]. Because the information on the commonly scented pencil was also remembered better than the information on the unscented one, the researchers concluded that scenting products improves consumer remembering of product information, regardless of the specific aroma.

Accompanying an *advertisement* with a pleasant and appropriate scent (diffusing rose or sandalwood aroma during a commercial promotion of a spa in a movie theatre) enhanced consumer recall of attributes (unaided as well as aided) compared to a no-scent condition [56.90]. Similarly, when seated in a room with an ambient scent, consumers recalled unfamiliar brand names better when the scent was pleasant rather than in an unscented condition. Further analysis suggested that this finding relates to a scent's pleasantness increasing attention as consumers spent more time evaluating the offer.

The literature discussed advocates that both ambient and product-related scents influence consumers' memories and behavior. Specific effects, however, hinge on the individual recollection of and personalized meaning attached to scent-evoked memories.

56.3.4 Behavior and Intention

One of the key effects of olfactory stimuli is their directive function. Ambient scents alert the organism to the existence of agents in the air, to check their quality for the guidance of behavior on the basis of previous encounters, to avoid or approach certain states [56.91]. This capacity of scents to heighten awareness is thought to be the primary cause for people's basic approach and avoidance responses [56.54]. Given that perceiving odors requires little, if any cognitive effort [56.31], no conscious attempt is required for fundamental behavioral responses, such as taking a deeper breath when a pleasant scent is present or holding the breath in the presence of an unpleasant scent [56.92].

The literature holds ample evidence for ambient scent's influence on consumer behavior. Behavioral responses assessed by researchers center on approach-avoidance, with specific measures including the length of a person's stay in an environment, spending, and behavioral intention. For example, in the presence of a pleasant and congruent scent, the amount of time consumers spent in a store increased in comparison to an incongruent pleasant scent condition [56.93]. This finding similarly holds for restaurants, where a pleasant lavender ambient scent related to consumers staying for a longer time, and spending a larger amount of money [56.94]. In casinos, diffusing an ambient scent increased the amount of gambling [56.62]. More recent findings, however, suggest that ambient scent can exert divergent effects depending on what type of shopping behavior is examined [56.95]. While ambient chocolate scent facilitates general approach behavior and increases the amount of money patrons spend in a bookstore, it also leads to less goal-directed behavior. Specifically, positive effects of the chocolate scent occur only when the product is thematically congruent with the scent. Accordingly, the sale of congruent books (e.g., cooking or baking guides) increased, whereas the sale of incongruent books decreased. Further support for the possibly important role of congruence stems from *Fiore* et al.'s [56.96] study testing the effect of ambient scent on consumer response to a product display. The findings indicate that consumers exhibited a significantly higher purchase intention and were willing to pay a higher price when the scent was both pleasant *and* congruent with the product displayed compared with a no-scent condition and a pleasant-scent-only condition.

In sum, the presence of an (pleasant) ambient scent impacts consumer behavior in terms of the time spent in the environment and the amount of money spent. Yet, some scholars caution that empirical evidence does not always support these outcomes [56.97]. Particularly, the psychological mechanisms of the reported effects on approach-avoidance behavior are not well understood and need further investigation [56.55].

56.4 Cross-Modal Effects

Our previous discussion of olfactory effects indicates that scent can impact consumer responses in a variety of ways. Readers should note, however, that the majority of studies have either examined scent in laboratory experiments (with other sensory input considered a distorting factor to be minimized) or have examined consumer response to scent in field studies that did not account for variation in other sensory input. These approaches do not fully reflect the reality of consumers experiencing the world through all of their five senses; in the marketplace individual judgments about a store, its products, and even its personnel, are driven by a concert of smells encountered, things heard, objects touched, taste experienced, and the products seen [56.17]. It is therefore imperative to consider cross-modal effects involving scent.

Humans are equipped with five basic senses (their olfactory, auditory, tactile, gustatory, and visual systems) with each modality providing sensory information about the environment. Although, overall, vision is considered the most informative and olfaction a less informative modality [56.5], the relative importance of each modality depends on the individual situation. For example, olfaction is more functional in providing information about a product being clean and safe [56.6], and a lack of olfactory input negatively impacts consumers' product experience [56.98]. In many cases (such as with the color and scent of food), specific product properties need to co-occur to signal safe use. This association in input received through two or more sensory modalities, termed *cross-modal correspondence* [56.99], is still an emerging area in research on consumer behavior. While the initial studies in this area have focused more narrowly on the interaction of two sensory modalities, such as color and scent [56.100–102] or music and scent [56.103, 104], a few studies have started examining multisensory effects in marketing contexts, such as interactive effects of scent and touch on product evaluation [56.89] or effect of scent and vision on memory for a product [56.89]. The next sections discuss noteworthy cross-modal effect studies in more detail.

56.4.1 Olfaction and Vision

Many sensory studies converge on the finding that the sense of vision dominates the other senses, as is the case in the interaction between visual and olfactory stimuli. Although there is large variance in effective properties for both, scent and vision research has mainly focused on the interaction of scent aroma and object color. Two streams of research relate to (1) color cues influencing scent perception and (2) visual and olfactory cues jointly influencing the individual evaluation of and response to products.

Although the influence of one sensory modality (vision) on another modality's (scent) input is not a straightforward marketing issue, much research has been dedicated to how colors affect scent perception, yielding some important insights for marketing [56.105]. For example, when fragrant liquids are colored appropriately rather than inappropriately (cherry-scented liquid that is colored red versus green), humans are more likely to identify the scent correctly [56.106]. This finding holds not only with abstract stimuli, but also extends to consumer products, as wine experts use descriptors that are typical for red wine for describing a white wine when it is colored red [56.107]. According to *Demattè* et al. [56.100, 105] the dominance of the sense of vision over olfaction is so strong that people are biased by color even when explicitly instructed to ignore visual cues. For example, when asked to identify scents and ignore visual cues (color) presented jointly with the olfactory cue, participants' responses were biased in that their identification of scents depended on the color. This biasing effect of color not only applies to scent identification [56.108], but also extends to the individual judgment of scent intensity [56.102] as visual lightness and odor intensity appear to correlate.

Investigating possible causes for the biasing capacity of vision, researchers posit that the interaction of color and scent takes place at the perceptual level [56.108]. Functional magnetic resonance imaging corroborates this view, indicating that the brain regions activated for processing pairings of scent and color vary as a function of the perceived congruence of these pairings [56.109]. Labeled a *perceptual illusion* [56.110–112], the concept reflects the fact that – following sensory input – a higher level of information processing (at the semantic level) relates to divergent olfactory perception. Further adding to the evidence of vision–scent congruence effects at the semantic level, individuals evaluated an identical scent as more pleasant when it was labeled positively (*cheddar cheese*) rather than negatively (*body odor*). In the latter case, the scent was also rated as more intense [56.113].

Putting the semantic congruence between vision and olfaction to practice, fragrance producers carefully match the colors of the vessel to the liquid, and design elaborate color schemes for packages and advertisements to better inform shoppers about their fragrance [56.99]. Package color has been identified as an especially effective tool for steering shopper ex-

pectations regarding levels of scent intensity (high: dark red; low: pastel green), sweetness (high: dark red; low: pastel green), and freshness (high: pastel green; low: dark red) [56.114]. Joining these findings with insights that the consumer perception of a deodorant's scent depends on the color of the package, it is therefore thought that people perceive the package and the content not separately but as a single integrated whole [56.99].

56.4.2 Olfaction and Other Sensory Input

Much less research has focused on the interaction of scent with other sensory input besides vision. A few studies have examined the cross-modal interaction of scent with auditory input (obtained through the sense of hearing) and haptic input (obtained through the sense of touch). As with the sense of vision, most studies focus on very basic relationships between sensory properties; only few examine outcomes more directly related to marketing.

Paralleling the previously discussed congruence effects between color and scent, a similar phenomenon has been established for the interaction between scent and auditory pitch. Qualitatively different odors correspond with different levels of pitch and sound volume. Fruity scents, for example, relate to higher pitch [56.103, 104], and the volume of a sound is positively associated with the perceived concentration of a scent [56.115]. More directly relevant to marketing environments, congruence in music and scent (their stimulating quality) leads visitors to rate an environment significantly more positive, with effects further extending to approach and impulse buying behavior, and an increase in satisfaction [56.116]. *Spangenberg* et al. [56.117] attribute this finding to the moderation effect of background music. In their study, consumer evaluations of the store were more favorable when the scent was congruent with the music (Christmas scent – Christmas music). Combining an identical scent with incongruent music (Christmas scent – non-Christmas music), however, leads to less favorable evaluation. Overall, carefully combining music and ambient scent may lead to an enhanced shopping experience and more profitable retail. Again, stimulus semantic congruence appears to be the key.

Regarding the cross-modal interaction of olfactory and haptic stimuli, only a few studies exist. Initial findings hint at the existence of a cross-modal link between scent and haptic similar to the previously discussed interaction effects of scent involving color and music [56.118]. Presenting a soft versus rough piece of cloth jointly with either a lemon or animalistic scent yielded softness ratings that varied significantly depending on the scent (were higher for the lemon scent). While this outcome was obtained using explicit measures (psychometric scales), a subsequent experiment confirmed that this effect also held when associations were assessed implicitly (using an implicit association test; [56.119]). Adding different scents to a shampoo yielded a significant effect of scent on the perception of haptic dimensions as participants – when washing their hair with the shampoo – evaluated both the fluid and their hair as softer. Similarly, using different scents influenced a product's haptic characteristic such as thickness [56.120]. Further strong evidence for scent's capacity to affect haptics stems from the research by *Krishna* et al. [56.10]. Scenting a cold versus a hot gel pack with a *hot* (pumpkin cinnamon) versus *cold* (sea-island cotton) scent elicited more positive evaluations of the gel pack with consumers when the scent matched the temperature. Perhaps even more important, participants evaluated the packs as more effective and faster in treating pain (sea-island-scented cold pack) and in warming one's hands (pumpkin-cinnamon-scented hot pack). Summarizing empirical studies on bimodal interactions involving scent yields that a major driver of consumer response is the semantic congruence between olfactory input and sensory information obtained through other modalities.

56.5 Moderators

Researchers and practitioners alike often seek to better understand what the conditions are for enhancing or attenuating (possibly even cancelling) effects of scent on consumer response. Addressing the corresponding question of *when*, moderation studies provide answers by generating insights into conditional values of moderator variables. While the preceding sections have implicitly referred to moderator variables (semantic congruence), the following paragraphs provide a more structured overview of salient variables in terms of conditions for scent to exert an effect.

56.5.1 Congruence

A recurring issue in studying how scent influences consumer behavior is the possibility that effects may depend on the congruence of the scent with other stimuli [56.8, 116, 117]. Of the many theoretical accounts

put forward to explain consumer response to scent, the vast majority acknowledges that responses may be moderated by the congruence between a scent and the product, the category, the shopping theme, or input received through another sensory modality. Congruence here is defined as the degree of fit or match among salient properties of two or more stimuli [56.10].

Product congruence effects were reported in *Bone* and *Jantrania*'s [56.8] study where a sunscreen lotion scented with a coconut aroma received more positive evaluations of product beliefs and attitude than the same lotion with a lemon scent. Similarly, consumers found a household-cleaning product combined with a lemon scent more appealing than one with a coconut scent. In both studies, approach behavior toward the product was enhanced by a higher product-scent congruence.

Regarding *congruence with other sensory input* music has received much of the attention in research with results indicating that congruence between music and scent leads to more positive evaluation of the shopping environment, an enhanced shopping experience, a higher level of approach behavior and more impulsive shopping in a mall. For example, studies on retail atmospherics indicate that when ambient scent and background music match in terms of arousing quality (high/high or low/low; [56.116]) or their consistency with a holiday (Christmas music-Christmas scent; [56.117]), consumers experience increased pleasure, evaluate the store more positively, and ultimately exhibit approach behavior.

A special case of congruence exists when consumers explicitly assess the fit in terms of semantic associations among stimulus properties [56.10]. Because of mutual associations with the experience, individuals assign sensory stimuli a semantic meaning; congruent associations then lead to a more positive evaluation of products and enhance the perception of this semantic meaning. Semantic congruence research is distinct in that it changes the focus from the perceptional to the cognitive processing level. A prominent example is *Krishna* et al.'s [56.10] testing of feminine versus masculine scents in combination with rough versus soft paper. The semantic congruence (assessed in terms of roughness–softness) moderated effects of the bimodal sensory input. In all, cue congruence appears to be a key concept in understanding consumer response to scent.

56.5.2 Individual Differences

There are a number of individual differences relating to both the perception and impact of scent. Individual differences include not only demographic variables (age, gender) but also chronic tendencies (personality traits) and situationally activated and less stable individual states (mood).

Regarding scent perception, a substantial body of evidence advocates that people differ in their ability to smell, that is, in their capacity to detect and identify olfactory input [56.121]. This individual difference traces back, in part, to differences in people's genetic information. In addition, prominent environmental factors, such as smoking habits and age affect the human ability to smell. Smoking has a negative impact as it reduces the number and sensitivity of receptors available for receiving scent molecules [56.122]. Similarly, aging has a negative impact as – over one's life span – receptors become dysfunctional or die [56.123]. Gender, in contrast, has no major effect; women and men appear equally capable of detecting and identifying olfactory input [56.124].

Regarding the impact of scent, age may have an effect as an ambient scent impacts individual spending during a shopping trip with young people but not with older people [56.125]. Adopting a more holistic perspective, *Davies* et al. [56.126] stipulate that the way in which an ambient scent is perceived depends on the objectively present ambient scent and the acuity of the consumers, which is influenced by individual characteristics, such as age and gender. This view is consistent with [56.60] finding of gender differences in mood shifts with men (no change) and women (mood shift) seated in a dentist's waiting area scented with an orange fragrance.

56.6 Ethical Aspects

Ethics generally distinguishes between the *right* or *good* and the *wrong* or *bad* [56.127]. The following paragraphs are intended to alert readers to a few of the challenges and pitfalls involved in (a) using scent for marketing purposes (marketing ethics) and (b) conducting research on consumer response to scent (ethics in consumer research).

Questions of *marketing ethics* arise when companies come to market with products aimed at consumers. Given the imperative of operating at a profit, commercial marketing activities can raise ethical questions [56.128]. Resolving these issues then depends on the perspective taken, especially whether one adopts a more deontological or teleological view-

point [56.129]. Quite commonly, resolving issues of marketing ethics also involves governmental regulation. Critical voices have expressed very specific concerns about marketers employing scent for misguidance, deception, and manipulation of the consumer. For example, it has been argued that using scent for enhancing the consumer perception of product quality [56.8] meets the standards of misguidance and deception because the product appears to be of better quality than it would ordinarily be (without scent). Another common application involves dispensing a *new car* fragrance in a used car to stimulate the perception of newness and possibly raise consumers' willingness to pay. Regarding marketing's use of ambient scent, it could be argued that the effects established could trick consumers into actions (spontaneous buying, exaggerated spending) they would not take if the scent was absent [56.130].

Issues of *ethics in consumer research*, by contrast, arise when researchers pursue self-interested goals possibly at odds with the needs of those either undergoing or sponsoring their study. These issues give rise to the requirement to protect *subjects* (consumers) against potentially harmful research practices and to preserve the integrity of findings intended to contribute to our knowledge. In many societies, there is a consensus that respondents are entitled to anonymity, peace of mind, candor, and freedom of choice; conversely, they agree that research practices deemed hurtful, deceptive, or treacherous should be outlawed [56.131]. Most prominent with scent are possible risks to health or well-being. Some fragrances are outright toxic [56.132] or carcinogenic [56.130], others have been identified as a major cause of allergies [56.133]. Given increasing numbers of people sensitive to scent, even mild reactions, such as headaches and asthma attacks [56.134] need to be considered when conducting research on human response to scent.

Responding to calls for state or federal governmental regulation, a number of national and international systems have been put into place including guidelines intended to regulate the identification and labeling of allergens in fragrances used in or on products. In addition to regulation, industry associations, such as the International Fragrance Association, have issued a detailed code of good practice for their member companies. Most regulations focus on making consumers aware of possibly harmful ingredients. However, testing over 700 products marketed to consumers in Germany for 26 supposedly (according to Article 10.1 of the 7th Amendment, Guideline 2003/15/EC) allergenic fragrances, *Klaschka* [56.135] found about half of all cosmetics, washing, and cleansing products contained at least one mandatory label ingredient, and 14% contained strong allergens. To date, consumers are still buying these products; the effect of companies voluntarily labeling their products to decrease the number of allergenic substances seems small.

56.7 Future Research

While the previous sections illustrate that much progress has been made toward a better understanding of how and when scent impacts consumer behavior, significant gaps in our knowledge still exist. From our perspective, there are at least three promising avenues for generating potentially valuable future insights: improving methods, advancing analytics, and adopting a more holistic perspective.

In spite of the variance in scent properties, marketing contexts, and consumer response variables investigated, the studies reviewed mostly rely on a single *method*, namely, the explicit assessment of constructs through psychometric rating scales. While widely applied, those scales are not without limitations in terms of design, analysis, and interpretation [56.136]. To overcome some of the limitations inherent to explicit measures (scale-based surveys or experiments), a few studies have started to rely on implicit association tests [56.119] and functional resonance imaging [56.109] to capture automatic thought and nonconscious responses. With the help of advanced or integrated techniques, applied to study effects of scent used for marketing, a more revealing view into the *black box* of the individual consumer should be feasible.

Benefitting from recent advances in the social sciences, marketing research has made great strides in terms of more sophisticated and powerful *analytics* in the past decade. For example, ever faster high-performance processors allow *big data* processing, computing large numbers of variables for large numbers of cases (consumers) in reasonably short periods of time [56.137]. Concluding that an analysis that focuses on answering only either *how* or *when* is inferior to one that examines both [56.138] researchers further developed methods, such as conditional process modeling to better quantify and simultaneously test hypotheses about how (through mediator variables) and when (conditional values of moderator variables) an independent variable influences a dependent variable [56.139]. It is plausible that the strength of the hypothesized indirect (mediation) effect is conditional on the value of specific moderators, or what has been termed conditional indi-

rect effects [56.140], alternatively known as moderated mediation. Collectively, many of the studies on scent in marketing suggest an indirect effects model, whereby the relationship between scent and consumer response (approach avoidance or intention to purchase) is transmitted by a mediator (a shift in affect or evaluative judgment), and is contingent upon a moderator (individual shopping goals, age, or gender). Given shortcomings identified for initial approaches [56.141], it is suggested that conditional process modeling yields far more complete results than do formal significance tests or bootstrapping alone [56.138, 141]. The application of these novel methods and analytical tools may therefore require practitioners and researchers to rethink how scent functions with consumers for more effectively market products and services.

Adopting a more holistic perspective naturally follows from establishing cross-modal effects of sensory input and further integrates sensory with marketing research. Past marketing and consumer research has concentrated on classical means of communicating products and brands (through advertising) including visual aspects. Marketing scholars considered vision the major source of consumer beliefs [56.142], but largely ignored the potential contribution offered by sensory science. More recently, however, researchers have started to embrace the concept of experiential marketing positing that a more holistic perspective may be appropriate for fully understanding consumer interaction with products [56.8], brands [56.9], or service environments. While the resulting multifaceted view of visual, tactile, olfactory, auditory, and taste input is deemed to possess greater explanatory power, new methods and analytics necessary for assessing the relationship between numerous variables are just recently becoming available (Chap. 47).

56.8 Conclusion

This chapter covered a lot of ground. Yet, it has barely begun to capture the number and diversity of phenomena directly or indirectly attributable to scent's use in marketing. Readers will inevitably identify many important areas of research, in consumer behavior, and related areas, that have been omitted here for the sake of brevity. As new research frameworks and controversies develop, updates, revisions, and extensions to the perspectives discussed here will become necessary in the coming years. We hope that the current chapter stimulates excitement and discussion about employing scent for marketing, and readers will contribute to the discipline through their own teaching, research, and practice. Scent marketing truly is an interdisciplinary field, and we are pleased to have the opportunity to provide these viewpoints.

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57. Sensual Perception in Architecture

Katinka Temme

From the five senses (according to Aristotle), the sense that first comes to mind when thinking about space is the visual sense. Indeed, much of spatial experience is derived from vision. In this article, it is postulated that an equal consideration of all senses however can lead to a refined architectural design method and designed space.

In particular, spiritual spaces offer often an experience beyond the visual. The space reception is thus multisensory; influenced by such factors as incense, darkness, coldness, and awe. All those stimuli constitute an intense, dense architectural atmosphere. If such an overlay of senses is possible, is it also possible to void a space of its stimuli? A study at the University of Applied Sciences in Augsburg came to the conclusion that a *neutral space* is impossible to design. Senses are essential to the human being and as soon as a space is emptied of this quality, its abstraction is perceived as nonhuman (*mad-house*) and thus as a negative space. Thus, the creation a positively perceived space was aimed for: one in which a majority of users feel welcomed at and comfortable in. This architectural answer can be utilized for test laboratories to avoid the potential negative perception by the sixth sense: that of anxiety when entering a testing environment.

From a chemist's or sensory scientist's point of view, the most important factor in an olfactory test room would probably be its functionality – for example odorous inertness, convenience in sample preparation and presentation as well as flexibility. These premises are strongly reflected in norms and settings of common sensory testing rooms that are precisely described in, for example, DIN EN ISO 8589:2014-10 [57.1]. Such test rooms are designed to allow for group discussions between panelists or to provide privacy in terms of isolated cabin-like booths, providing sinks for drinking water supply and to offer the possibility to spit out samples in case the samples need to be tasted. The rooms or booths may further comprise touchpads or computer workstations where panelists are supposed to record the data of their ranking. Light might be adjusted, for example with red light illumination to ensure that the color of samples does not falsify the rating results. A typical floor plan and a representative sensory panelist group discussion are shown in Fig. 57.1 and 57.2.

All in all, however, it becomes evident that such a testing room is not really related to real-life situations of everyday odor experience, and, moreover, does not fulfill the requirements of a scenic environment that is designed for providing a space for experiencing, enjoying or even celebrating smells.

Moreover, as soon as we add a psychologist's perspective on smell perception in a building or spatial surrounding, the prerequisites for such a room become much more complex. We know that multisensory integration and emotions can influence the perception of an odor subconsciously (Chaps. 40, 47). For a completely unbiased olfactory evaluation, it would hence be ideal if neither emotions nor other senses than the olfactory sense were triggered specifically, consequently perceiving the test environment as completely neutral. This raises the question what defines *neutral* in con-

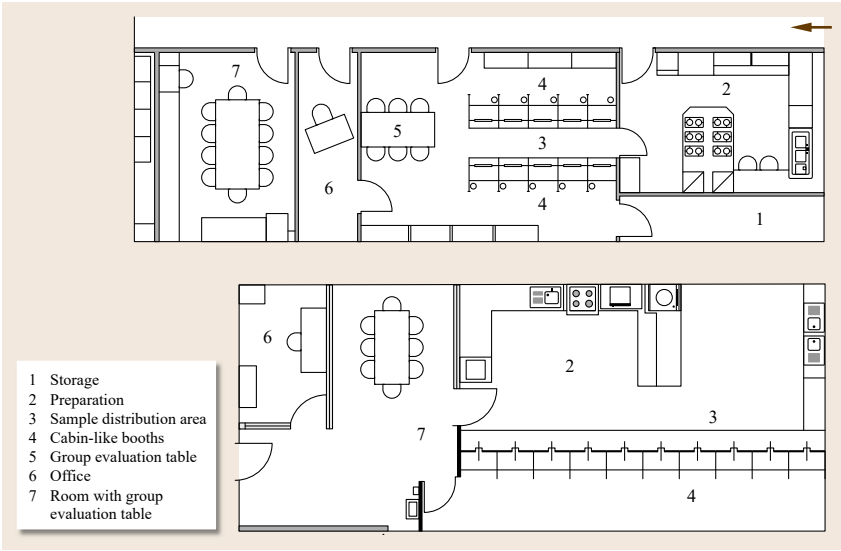

Fig. 57.1 Floor plan of a sensory testing room according to DIN EN ISO 8589:2014-10

Fig. 57.2 Expert panelist group discussion of presented smells (courtesy of Fraunhofer IVV) ◄

trast to a *sense-filled* space and whether it is possible to design an olfactory test setting that is *neutral*. This article deals with the question of how to design space for a sense such as odor and in what manner spatial experience is related to sensual perception and experience. It postulates that any successful design has to consider both the context of the building as well as the whole human body with its senses as valuable design perimeters.

57.1 Space and Limits of Space

Thinking about architectural design, one ultimately has to inquire into the architectural definitions of *space* and what constitutes or forms space. Space is defined on one hand by its limits indicating the architectural dimensions of the allocated space such as height, width, and depth. The porosity of those spatial borders evident in openings such as windows, doors, and skylights is essential as it allows light to penetrate space and accentuate the edges further. However, an enormous impact on the experience of space has to do with its sensual qualities evoked by its atmosphere and materiality. A wooden log cabin of small size might seem more intimate than a white tiled room of equivalent dimensions. These perceptions relate on one hand to the visual perception but it can be assumed that all other senses play a crucial role in identifying a spatial atmosphere as well.

Dom Hans van der Laan defines *architectonic space* as something experienced physically, sensually, and mindfully. Among the several definitions of *space* in relation to architecture there are fundamentally three tendencies: one that defines the object character of real, built space; one that deals with the experience of space as a complex situation; as well as one that, in a combination of the two previous positions, deals with the conceptual design or idea of space [57.2].

57.2 *Sensual* Space

In Japanese architecture, the empty room is the center of the Shinto shrine called *jinja* in which people perform religious duty. The graphic designer *Kenya Hara* points out in his book *White* [57.3], that this *jinja* space is homonymous to white (*shiroi*) and thus called *shiro* or *yashiro*. The design principle is that of an embracement of void. Placed along the cardinal points, four wooden pillars are erected on the ground, connected at their tips by a straw rope. That is the archetypal shrine. The space is simply regulated by a rope and the four pillar-points and contains nothing. In essence, because the space contains nothing, it has the enormous potential that *something* may enter; in a way like a sheet of white paper, waiting to be filled. The definition of a sacred space, such as a shrine as described by Hara, also appears in the land-pacifying ceremony known as *jichinsai*, in which offerings of produce and sake are placed on an altar within the sacred space. Note the four bamboo pillars and the straw rope, attached with ritual white sheets of paper. Although sake has no color (which reflects the *nothingness* of space), it has a light perfumed scent. In that sense, the void is rich and potent and this design principle is essential to Japanese aesthetics – symbolizing simplicity and subtlety (Fig. 57.3).

A similar approach towards spiritual space is evident in Western/Christian culture: spiritual space design is, in terms of decoration, very minimal. Contemplation, meditation, oneness with God is sought and objects superfluous or irritating are usually avoided. Religious buildings erected prior to the Middle Ages, in especially during the Romanesque Period (1000- about 1200) are still fine examples of sheer-walled environs, void of content other than faith, for example the Abbaye du Thoronet (Fig. 57.4). The abbey, erected between 1160 and 1190 is one of the *three provencal sisters*, famous Cistercian monasteries. The church is a Romanesque basilica with barrel vault above the central nave, yet without windows in the clerestory. The abundance of architectural sculptures leads to further formal clarity, order and minimal ideal space.

It is interesting that although visual or acoustic influences are minimized, odor on the other hand is widely used. Flower decorations, perfumed oils, incense sticks (in Buddhism, Shintoism and Hinduism) and burnt incense (in Catholic congregations) are symbols of respect towards the deity and heaven (see also Chap. 4). Incense is supposed to be the *scent of god*: as the incense smokes towards the sky, so does the prayer of the faithful servant of God [57.4] as described in the following psalm:

> *Psalm 141: "Come to Me Quickly! [1]A Psalm of David. O LORD, I call upon You; hasten to me! Give ear to my voice when I call to You! [2]May my prayer be counted as incense before You; The lifting up of my hands as the evening offering. [3]Set a guard, O LORD, over my mouth; Keep watch over the door of my lips. . . . ".*

In Egypt and Mesopotamia, incense may be purificatory, apotropaic, propitiatory, or mediatory [57.5] (Fig. 57.5).

Gustatory elements such as wine or harder liquor are rather common, be it the red wine for the Lord's Supper in the Catholic faith or the pox liquor, consumed in Mexican highland churches for an intense intoxicated state of mind, ready to embark on a journey to the *beyond* [57.6].

That being said, in order to find the ideal of an empty or neutral space in order to insert a function,

Fig. 57.3 Ritual space allocation at a *jichinsai* (land-pacifying ceremony when erecting a new building); photo by Sugimata Yasushi

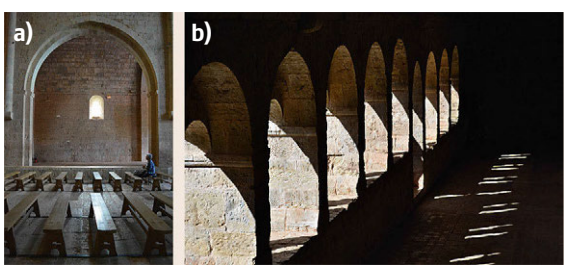

Fig. 57.4 Abbaye du Thoronet

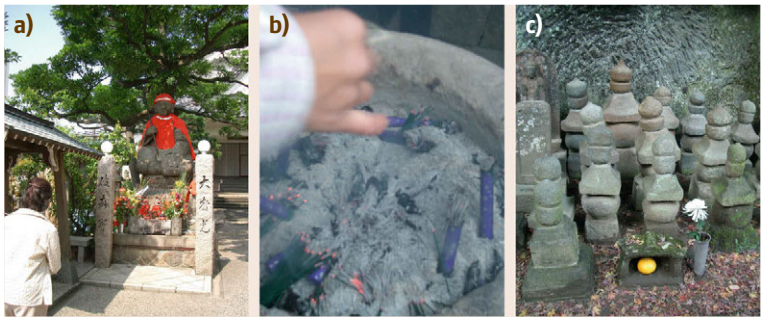

Fig. 57.5 Flowers (**a**), incense (**b**), and fruit (**c**) at spiritual places in Japan

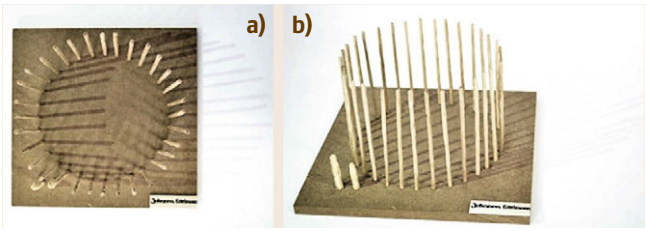

Fig. 57.6a,b Johanna Edelmann's neutral space is formed by wooden columns only; similar to the Japanese *jinja*

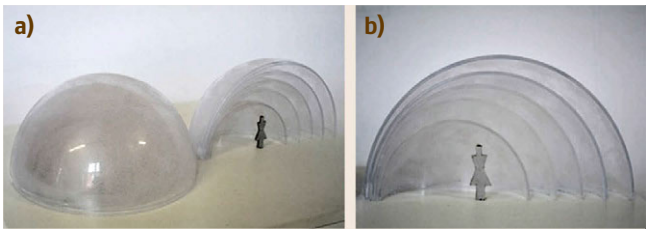

Fig. 57.7a,b Brigitte Kastner's neutral space is formed by dome shapes made of a glass-ricepaper sandwich creating thus a semi-transparent shell

such as an olfactoric laboratory, I conducted a series of design experiments in the summer semester 2014 at the University of Applied Sciences Augsburg. It was clear that the architectural space had to have more solid boundaries than those of a *jinja*. The idea of a void or emptiness had to accept the functional requirement: a space with little connotation in which test persons could concentrate on one sense.

57.2.1 The Search for *Neutral/Sense-Less* Space

The first experiment started with an analysis of *neutral space*. A freestanding pavilion not exceeding 30 m^2 in floor area had to be conceived including a mindful selection of material and dimensions of building members. The leading design question was whether or not it is possible to create space without qualities, a room void of any character, the zero-box with no emotional or sensual stimuli (Fig. 57.6–57.10).

It was very soon clear that a neutral built space does not and cannot exist. Even if we use non-colors such as white or black, and even if we use characterless materials such as a coating with no joints or surfaces, the space still triggers an emotional response. The less character it is given, the creepier the atmosphere. One student even showed an image of a psychiatric ward. The absence of sound, touch and smell makes us insane. The resulting design question was hence: *How minimal can space be without being irritatingly void?* Rather than further minimizing the qualities and atmosphere of space, the students investigated *positive* and *negative* associations regarding space. For example, most people would associate wood or warmer colors with a positive space; tiles and plastic as well as grayish colors with something negative. Concrete can be experienced as *brutal*, but as soon as the casting form with its wooden surface details the concrete with a more human scale, concrete can also appear as beautiful and positive.

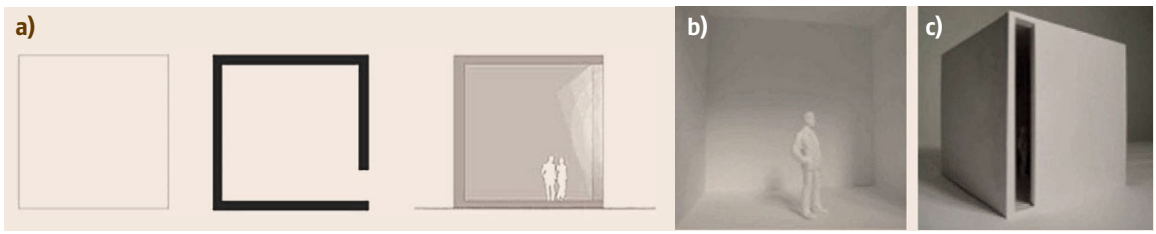

Fig. 57.8a–c Christoph Stegner considered the square to be the most *neutral* geometric form

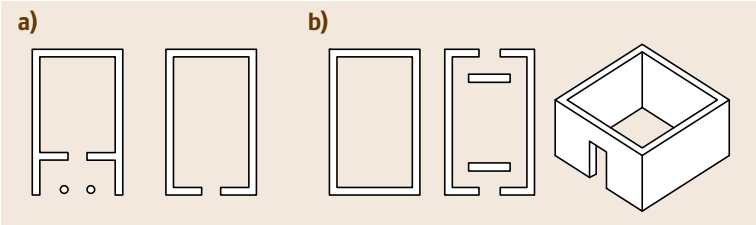

Fig. 57.9a,b Wolfgang Kramer analyzed **(a)** the ancient Greek *megaron* and *cella* spaces as neutral containers and **(b)** developed them further into the *white cube*, commonly considered best for art display

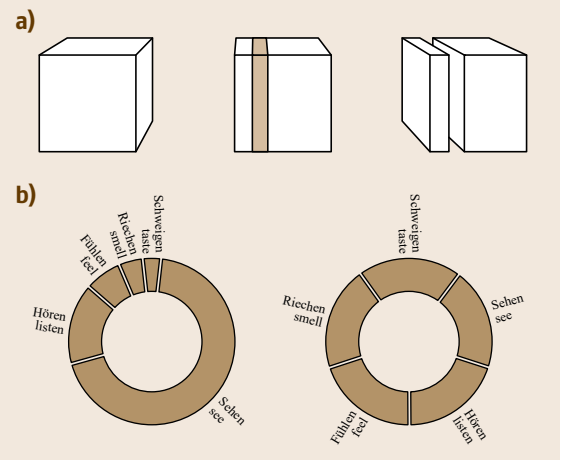

Fig. 57.10 **(a)** Marius Prechtl dissected the *perfect cube* to make the neutral space accessible. **(b)** His aim is to architecturally trigger all senses equally and cross-stimulate yet create harmony out of the sensory balance

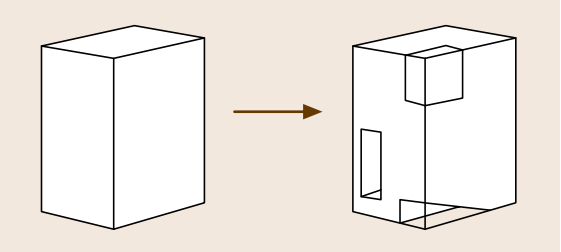

Fig. 57.11 Design task: sculpting a sensual space out of a given volume

In summary, it can be said that it is not possible to create a space void of emotion and atmosphere. Space immediately triggers our mind and memory to relate our body and soul to the architectural space. The more we try to empty space of character, the more frightened and negative its perception is. Rather than a *neutral* space, the ideal surrounding for a laboratory seems to be a space that makes most people feel comfortable; a positively filled room rather than an empty box, the living room or salon versus the lab cage.

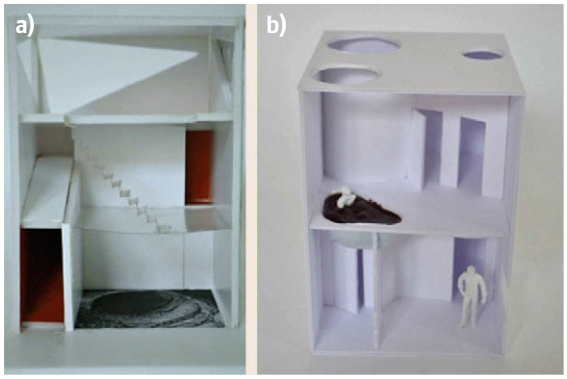

Fig. 57.12 **(a)** Sixth sense demonstrating several spaces of fear: a steep slope, a fragile glass stair across a seemingly large abyss, a narrow dark room. **(b)** The smell of the cocoa bath on the upper floor seductively leads through the building

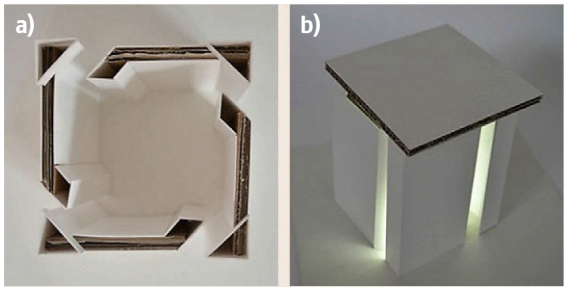

Fig. 57.13a,b The visual sense rarely resulted in successful designs

57.2.2 The Search for *Sense-Filled* Space

For the second experiment, first semester students had to each pick one sense and develop an architectural envelope for it. The ground floor area of 5×5 m as well as 7 m height was a given parameter to create a coherent volume in order to focus on the specific interventions. Together with the five classical senses (according to Aristotle), acoustics, optics, haptics, gustatorics and olfactorics, the sixth sense was added as a choice (Fig. 57.11–57.17).

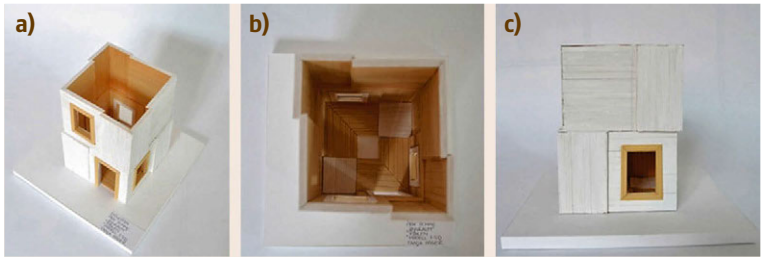

Fig. 57.14a–c Haptics and optics are combined and create a very intriguing sensual space

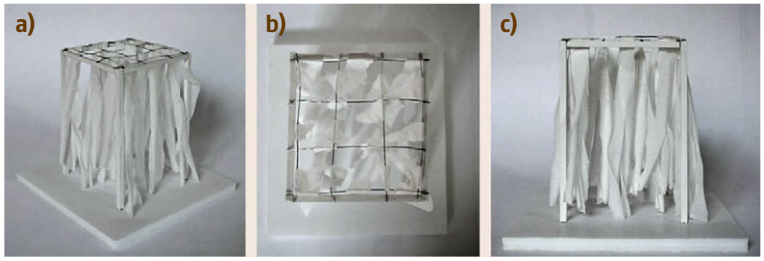

Fig. 57.15a–c A fresh breeze of air causes this building to enchant the visitor, its soft strips of fabric softly waving in space: a very poetic approach to a space that deserves to be touched and explored very physically

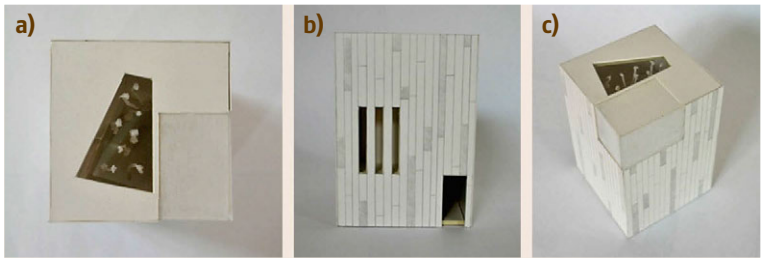

Fig. 57.16a–c Olfactoric sense: Small strips of light and the inserted atrium with skylight are the only means of light; the only guidance through space is the smell of trees and earth

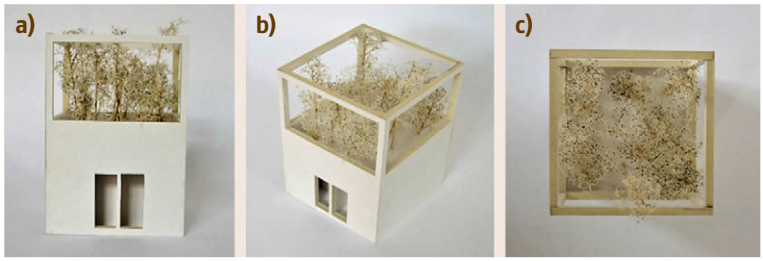

Fig. 57.17a–c This design visually hinted at the presence of trees. Once inside other, rather underrated senses are triggered: the smell of trees, the sound of earth beneath one's feet, the touch of the bare concrete wall, the taste of wet earth and the feeling of being somewhere very *rooted*

Each sense had to characterize the space and enable a focus on the specific sensual atmosphere: How does space sound? How can silence be built? How to concentrate on sound only? What do I see? How do I perceive? Is there too much visual stimuli so that I do not see the essence of the space? What is the smell of a space? When does odor turn to smell? Is it possible to sense materials differently if I approach them first by smell, by touch, by view? How does space smell? Can I taste a room? Can I trigger memories of a space by smell or taste? Does odor leave a trace on my tongue to *multi-sense* space? How do I move around space and can I be guided in my movements by smell in a space? What are the limits of space, of me in that space and of my perception of that space?

In regard to the sense-filled space, the students initially preferred to deal with the visual aspect. It seems that the supremacy of the eyes as a means of perception eased this decision. We are surrounded by visual stimuli. Our eyes are in constant demand, not just due to the increase of electronic gadgets such as smartphones, and tablets, but also the increased rapidity of cuts and sequences in film, media and advertising. In the end, many students failed to create a successful space for the visual senses. Most architectural spaces were overloaded or simply too blunt. It was not clear that the building had something specific about it (Fig. 57.13).

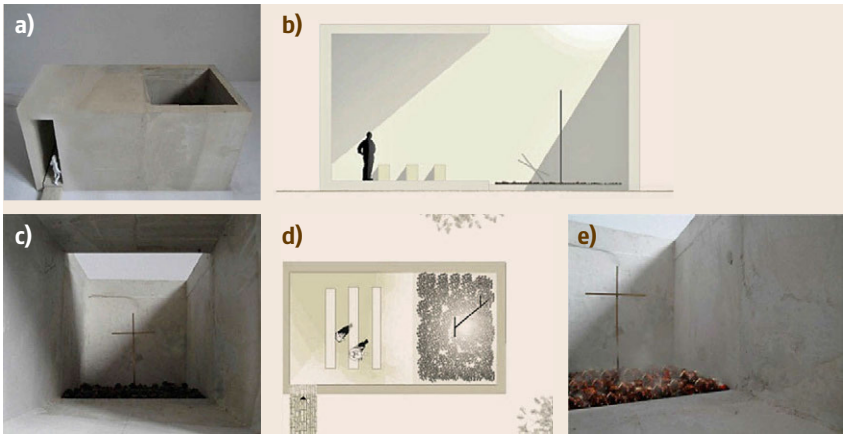

Fig. 57.18a–e Christoph Stegner composes a solemn space surrounded by concrete walls that show the wooden surface of the formworks. The altar is illuminated by the skylight and sensually connects the visitor to the outside surroundings. At the floor, pine cones are burned and not only cause an acoustic stimuli (shrinking under the influence of fire) but also an olfactoric one (the smell of burnt pine) as well as the visual sensation of slowly evaporating fumes

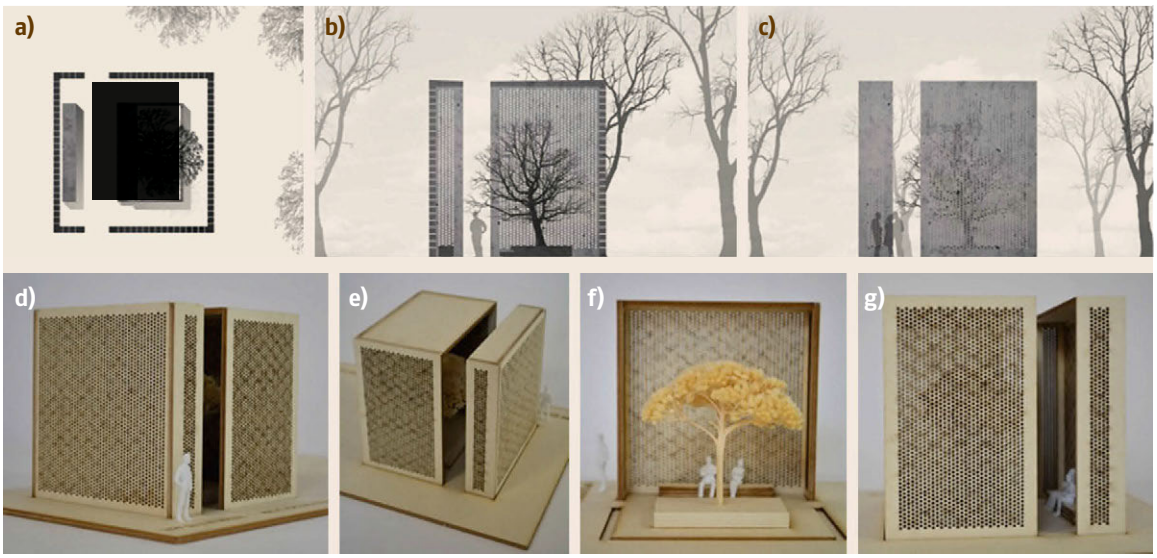

Fig. 57.19a–g Marius Prechtl balances all senses by a porous outer wall that allows light, sound and smell to penetrate through. Inside the sliced open cube, one can rest on a wooden bench (haptic stimuli) and reflect on the cherry tree that not only blossoms (visual and olfactoric stimuli) but also invites one to harvest (gustatoric stimuli) while listening to the wind and birds in the surrounding forest (acoustic stimuli). The overall atmosphere results in reconnecting all senses and ultimately body, mind and soul

The eyes are even in such dominance that perception with other senses almost has to be forced. Most students even doubted that space can be perceived by nose or mouth, and only on second thought did the acoustic and haptic perception appear as feasible. Thus, for all other senses but the visual, the buildings were rather successful. The difficulty of the design task challenged the students to think outside the box and to approach the design problem creatively and in a visionary, uncommon way (Figs. 57.14 and 57.15).

It was interesting that the students immediately wanted to deactivate the visual sense in order to activate touch or sound. In almost all cases, acoustic and haptic spaces were spaces without light in order to concentrate on the ears and hands (Fig. 57.16).

It can be noted that as soon as space had to deal with taste, odor and the sixth sense, two distinct design approaches occurred: those that *erased* all other senses to concentrate on a single sense and those that enabled all other senses to create a multisensory, complex sensual perception (Fig. 57.17). Reduction requires discipline so that the one conceptual idea can clearly be recognized and experienced in space. A multisensory and thus potentially multidimensional approach to space allows a more relaxed design and perception and is thus more suitable for everyday functions.

57.2.3 The Search for *Sense-Filled* Space; Multiple Senses

In a similar exercise, Master (fourth and fifth year) and Bachelor (third year) architecture students were given the same task yet without dimensional limitations. Rather than focusing on one sense alone, they were asked to design a *space for the senses*. In this combinatory experiment that also started with research into *neutral* space, the students were able to combine poetic architectural elements with the sensual experience (Figs. 57.18 and 57.19). It can only be assumed that it is indeed impossible to shut off any sense and that by nature, the whole body with all its senses has to be taken into consideration for any valuable and reflective, sensitive and sensual architecture.

57.3 Sensual Perception as a Design Method in Architecture

Although it seems very natural to utilize our own body and our senses to design and experience space, the lack of homogeneously distributed sensual awareness is indeed surprising. Little has been researched or written about the connection between sensual and spatial perception. In terms of odor, we know and can test how building materials evaporate toxins but it is unknown what positive odor does to the human mind and body. We know how to measure what potentially makes us sick, but we have not even started to research what sensual stimuli in the built environment make us happy. We know that color in the built environment might have a positive effect, however with the vague notion that this is perceived quite subjectively. While one might prefer a blue-colored room, another is infuriated by its coldness.

In regard to multisensory perception, we know even less. It can be assumed that one positive trigger might lessen the impact of another. However, there has been no further research into this cross-stimulating, at least not in the construction industry. We can only assume that the negative stimuli of a protruding smell might be weakened by a positive stimuli of a smooth touch for example. The result of such research could offer manufacturers and designers an enormous design potential. Cross-combining sensual stimuli might not only generate new products and material choices. The awareness of how *sensitive* each user is might additionally help to customize any design much further to clients' demands than to just please aesthetic (= visual) requirements.

Considering the need to investigate into that matter, it also has to be pointed out that both designers and users still lack this knowledge. Thus, not only the test person in a lab that has to focus on one specific sense is challenged, but also the architects of such a lab. When architects design space, they start with general dimensions, then continue to think about materiality and light, but most decisions taken stem from visual (or functional or budget) considerations. We render a designed space in 3-D, showing how it *looks* but never how it smells, feels or sounds. The other senses are rather relevant when it deals with eliminating those: avoiding hazardous building materials that evaporate an irritating smell, installing soundproof windows or non-slippery floor surfaces. Although clients place the same emphasis on the visual appearance (when talking about design objects, one usually refers to *the look* of things), in the end they do experience the designed spaces with the whole body and should be educated about those parameters in the design process.

Reducing ourselves to only one sense, we as well limit the spatial experience. While we allow our eyes to bathe in an intense arrangement of stimuli, all other senses are blocked or unused. Rarely, there are buildings (and architects) that emphasize senses other than the visual. Our memory might remind us that the smell of the bakery at the corner of our childhood home was compelling and the sound of rain and thunder is certainly something everybody can emotionally refer to. The sound of old wooden flooring, the smell of freshly mowed grass – there are many odor-triggered memories in relation to space and literature is full of those references. Rarely are senses, other than the visual, utilized as a design method or considered as valuable design parameters by architects; however there are few exceptions.

Peter Zumthor for example, a Swiss architect who designed the Thermal Baths in Vals, literally composed the baths as sensually rich spaces. Concerts take place in the thermal bath and due to the dimensions of each bath hall, the sound can be perceived very differently. Light shafts, openings in the massive gneiss stone walls (sometimes just slits, sometimes real openings to enter through), the temperature and mineral content of the bathwater all act as notes in the composition. Even time is taken into consideration as a design element. The mineral content of the water will change the appearance of the stone over time, the salt crystals will cover the joints and change the smooth appearance of the wall as well as the increasing patina on the brass mountings and fixtures. The vapor of the hot water bath perme-

ates through the walls and gives an optical hint to itself: a very clever design approach to signage in buildings. The stone floors are executed in a way so that not only the *pad-pad* of the wet feet can be heard but the imprint of the soles leaves also a visible dark mark on the floor. Those sensual perceptions all work, because as soon as one enters the space, the overall poetic becomes evident. The materials are strong in their appearance, solid like the surrounding rocks and mountains of the Vals valley but also simple in their expression. The spatial configuration is clear and the space laid out is easy to identify in. As the function of a thermal bath already suggests that of a relaxing space, a space to *let go*, sensual perception soon takes over from the mind-driven approach.

It can be assumed that a mindful concept for any space can be measured with the following design specifications: A material choice that makes sense and contextualizes the building within its surroundings (in architecture commonly called *genius loci*), a clear allocation of functions and division of rooms graspable by human scale, and a spatial quality that allows the mind to step back and allow us to perceive space with our whole body and senses.

57.4 The Ideal Laboratory

As a final experiment, Bachelor students were given the task to design a *sensual lab*. They could use their neutral and sense-filled experiments as a research base but had to develop the projects from rather abstract spatial experiments to a real architectural design with spaces for sanitary installations and technical systems, doors and windows, and the potential to be easily adaptable, moveable and extendable.

In order to approach the design task, students started focusing on the single cell first: the testing space. For the guest user (the tested person), a series of questions evolved: How do I enter the room? How do I experience the threshold between the *normal* exterior world and the *lab* space? Do I feel supervised (consciously or unconsciously) and *tested*? How and where do I interact with the lab personnel? How do I leave the testing space? Is there a rest area, allowing me to take a breath before exiting back to the *real* world? Can I see the *outside* while being inside? Do I have a chance to relax or is the spatial arrangement comparable to that of a conveyer belt?

For the main user (the testing person), the main questions were: How do I encounter the guest? Where do we meet, greet, chat and test? How can I operate my tools and machines? Where do I gather my results? How can I change the test surroundings? How much flexibility do I have with the test settings: Can I test more than one person, can I change the appearance, size, atmosphere or technical setting of a room by myself? And most importantly: Can I make sure that the guest is able to focus on the given task and is not irritated (positively or negatively) by the spatial surroundings?

While we did not go into detail about the technical limitations to any test setting, the students had to be aware that there are several possibilities to emit odor into the test cell. It was also clear, that there had to be a way to measure the responses from the tested person and that this already negatively influenced the emotional state of mind. It was also clear that too much flexibility in an architectural sense (removable wall panels and such) could easily lead to an *experimental atmosphere* further increasing the *lab* experience in a negative way.

In result, many designs came up with a pavilion like space (Fig. 57.20): a glass-surrounded building for which the roof is only supported by slender columns. Test labs, machine and sanitary rooms are inserted as *freestanding boxes* into the space so that anybody can wander around freely and always with a visual connection to the landscape surrounding the building (Fig. 57.20).

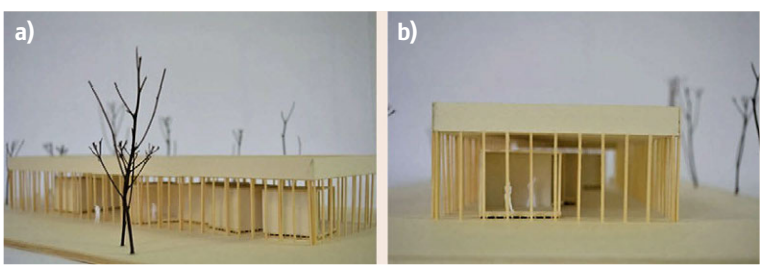

Fig. 57.20a,b A grid of columns lifts the roof above the ground and allows for a series of freestanding *lab boxes* to be freely arranged underneath and within the thermal and architectural enclosure

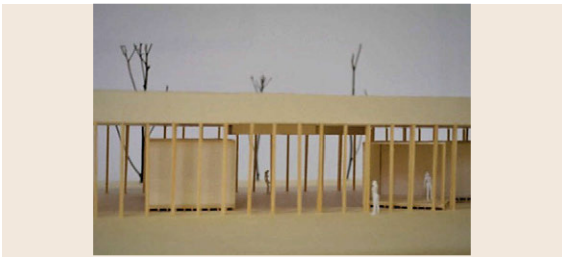

Fig. 57.21 The façade is structured by a row of columns. The inner core is flexible; technical installations are at floor level and in wall panels so that each lab module connects and disconnects as needed

Lounge-like areas for reading are randomly dispersed as well as tea kitchens and information counters. The overall atmosphere is that of an open, welcoming space, set in nature. The random placement of solid labs versus the overall open floor plan intensifies the transparent appearance, something not only felt physically but also psychologically: rather then entering the unknown, everything is evidently displayed. Lab personnel walking around interacting with the tested persons is normal and enhances the easy-going atmosphere of the space.

Due to this simple allocation, much focus can be given to the *freestanding boxes* (Fig. 57.22). The technical installations are either placed in the floor or the ceiling (less ideal) so that one can hook up easily and directly from the floor or indirectly through the walls. The placement of walls in a grid (in accordance to the placement of the structural columns) means that each wall can easily be extended within the structural grid to extend or limit the room size. Two functions or more can be joined without problems by simply taking out or inserting a wall panel (Fig. 57.23).

The above described combination of a fixed overall structural and architectural setting on one hand and that of a flexible lab system on the other hand is the most successful result. It combines the ease of a welcoming, living-room like space with the pragmatic and highly demanding technical requirements of a lab.

An architectural setting in which the architecture steps back in intensity and allows for an open, welcoming character in an almost democratic manner of a free plan is potentially the best answer to creating a lab space of the future: a lab that not only allows a positive emotional approach by the tested user but also by the testing user and is thus a lab that facilitates communications. In a way and looking back to Kenya Hara's observations to the void but potent shrine space, the ideal lab is a space that is defined by subtle architectural elements but has enormous potential for the functions to come.

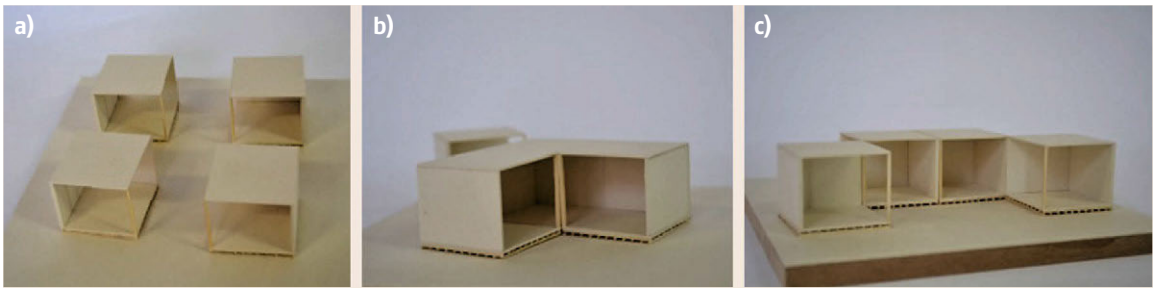

Fig. 57.22a–c Layout and combinations of lab modules

Fig. 57.23a–c Lab module details, showing the flexible wall panel if two or more modules have to be combined. The floor is composed of a *Euro Palett* and can easily be transported by a forklift

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58. Microdosing of Scents

Martin Richter

Microdosing systems based on micropumps, which are applied nearby the nose, will enable the change of scent impressions of human nose at every breath several times a minute. Due to the small dosing volume the scent impression can be detected only once, and no other person in the room can smell the scent. With that, for the first time scent scenarios become possible, analogous to *picture scenarios* (movies) and *sound scenarios* (music). Such vision of scent scenarios can be combined with audio and video for applications in the movie, games, and music industry. The technology could also enable new applications for other branches like training of tasters (wine, food) or training tools for sense of smell for kids or adults.

To realize this vision, both small and cost-efficient micropumps as well as a tight reservoir technology with small dead volume to provide scent molecules to be dosed are required. This chapter is discussing the *dosing chain* of scent from the reservoir to the nose, the dynamics of single-breath scent dosing as well as the state of the art of microdosing systems and micropumps.

58.1 Microdosing of Scent

The nose can be considered as a biodetection system for specific molecules, residing in a defined space in front of the nose. To smell a scent impression, the molecules have to be sucked into the nose by a breath intake. Depending on the type of the scent, just a few molecules can be enough for such a scent impression.

A conventional air freshener usually has a high dosing rate in order to fill the whole room with scent molecules. To replace this odor by another one, it would not be sufficient to dose other molecules into the room; this new odor impression would superimpose to the previous one, resulting in an unwanted mixed odor. For the pure smell of this new odor, the complete air in the whole room has to be removed and exchanged. Such a complete change of scent within short time is very difficult to achieve using conventional dosing technologies.

Novel technologies have been developed during the past years to dose small amounts of liquids or gases in a defined way. These technologies can not only handle small volumes of gas, but they are also very small in size and energy efficient. These new microdosing technologies can be mounted near the nose in portable, battery-driven systems.

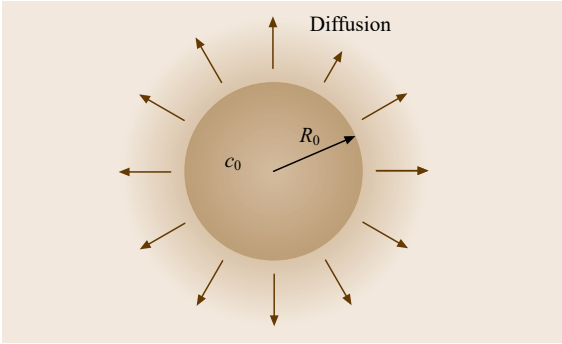

Fig. 58.1 Diffusion of a scent cloud in the air

Each single micro-dosed scent impression ideally disappears by diffusion after onebreath intake. This allows scents to be varied over a short time, enabling scent scenarios with different scents.

Air and sound can spread in space very quickly and disappear without any residuals. This can be technically exploited for providing *picture scenarios* (movies) or *sound scenarios* (music). Both audio and video are multibillion dollar markets, providing our eyes and ears with quickly changing pictures and sounds.

Together with these new microdosing devices, a completely new *scent scenario* technology for odors can be realized. The frequency of changing scents is defined only by the frequency of breath intake of human beings. For an adult this frequency is 12–15 breath intakes per minute [58.1]. With a microdosing technology, mounted near the nose and including several scent reservoirs, it is possible to realize a scent scenario with 15 different scent impressions in 1 min. Such scent scenarios can be combined with audio and video for applications in the movie and music industry. The technology could also enable new applications for other branches like training of tasters (wine, food) or training tools for sense of smell for kids or adults.

58.1.1 Transport Mechanisms for Scent Molecules

The transport mechanisms for scent molecules are diffusion and convection.

Diffusion

If there is a gradient in concentration, molecules travel from higher to lower concentration by diffusion. This mechanism is isotropic.

We consider a spheric scent cloud with radius R_0 with concentration of scent molecules c_0, which is surrounded by air without any scent molecules (Fig. 58.1).

The concentration $c(r,t)$, which depends on radius r and time t, is spreading by diffusion according to Fick's law [58.2, 3].

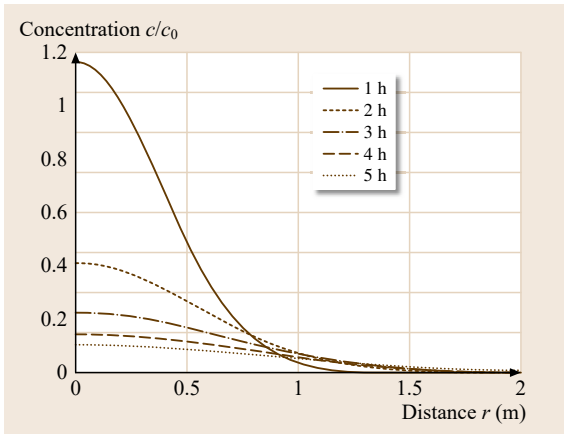

Fig. 58.2 Concentration profiles for diffusion of air as a function of radius r for different times from 1 to5 h

$$\mathbf{j} = -D\nabla c\,, \qquad \text{(1. Fick's law)}$$

$$\frac{\partial c}{\partial t} = D\nabla^2 c\,. \qquad \text{(2. Fick's law)}$$

Combining both equations and solving them gives the solution for the time- and radius-dependent concentration $c(r,t)$

$$c(r,t) = \frac{1}{8}N_0(\pi Dt)^{-\frac{3}{2}}\mathrm{e}^{-\frac{r^2}{4Dt}}\,. \qquad (58.1)$$

The constant N_0 describes the total amount of scent, which can be verified by integrating over the total space

$$\int_0^\infty \frac{c}{N_0}4\pi r^2\mathrm{d}r = \int_0^\infty \frac{1}{8}(\pi Dt)^{-\frac{3}{2}}\mathrm{e}^{-\frac{r^2}{4Dt}}4\pi r^2\mathrm{d}r$$

$$= \sqrt{\frac{1}{Dt}}\sqrt{Dt} = 1\,.$$

D is the coefficient of diffusion of the scent molecules (referring to ambient air). The unit of D is $[\mathrm{m}^2/\mathrm{s}]$. For diffusion of air in air (self-diffusion) D amounts to $D = 2\times10^{-5}\,\mathrm{m}^2/\mathrm{s}$. Molecules with higher atomic weight (e.g., scent molecules) normally have a smaller coefficient of diffusion D. The concentration is Gauss-shaped according to Fig. 58.2, in a space of 2 m around the source for a time up to 5 h.

At a given distance r_1, the concentration grows up to a maximum at the time t_1 and will fall down afterwards until all molecules are spread over the (infinite) room. This time t_1 of the local concentration maximum at distance r_1 can be calculated using the root of the

derivative of (58.1). to be

$$t_1 = \frac{r_1^2}{6D} .$$

In the air, the maximum concentration at a distance of 0.1 mm from the scent source is reached within 0.83 s, at a distance of 10 mm within 83 s, and at a distance of 1 m from the source that time amounts to 8333 s or 2.3 h, respectively.

These results show that diffusion is (for distances above around 1 mm) a very slow process. Only in the direct surrounding of the scent source diffusion is fast. Due to this fact, diffusion is not suitable for scent transportation over longer distances.

The amplitude $c(r_1)$ of the concentration maximum at r_1 amounts to

$$c(r_1) = \frac{1}{8} N_0 \left(\pi \frac{r_1^2}{6}\right)^{-\frac{3}{2}} \mathrm{e}^{-\frac{3}{2}} = \frac{1}{8} \left(\frac{\mathrm{e}\pi}{6}\right)^{-\frac{3}{2}} \frac{N_0}{r_1^3} .$$

The maximum of concentration is decreasing with the third power of the distance to the source.

Convection

If there is a pressure gradient, molecules flow from higher to lower pressure. Very small pressure gradients are sufficient to move air. Wind is one example for convection. In nature, air pressure drop of less than 1 kPa generates wind. Additionally, a temperature gradient in the air (by a heat source) generates a thermal convection flow, the heated air has a lower density and moves upward due to buoyancy. Moving bodies like human beings, opening a door, or a rotating fan generate convection, too. This is why transportation of scent molecules over larger distances in a short time is originated by convection, instead of diffusion.

Breathing is an example for this effect, an underpressure is generated in the lung, by sucking in the air, together with scent molecules. To complete the breath cycle, the used air is pushed out of the lung by an overpressure.

The volume of a typical breath comprises around a half liter of air. By sucking in the air the volume is taken from an almost spherical-shape region (here defined as volume V_1, Fig. 58.3). During exhalation the air is accelerated by the over-pressure in the lung and blows out in a directed flow due to the nozzle effect of the nose. One can verify this by putting the hand a few centimeter in front of the own nose for one breath cycle: During breath intake, there is no or just a little flow feeling, while during exhalation one can feel the air stream flowing out through the nozzle of the nose.

Due to this asymmetric flow, the nose sucks in *fresh air* during the next breath intake, instead of inhaling the

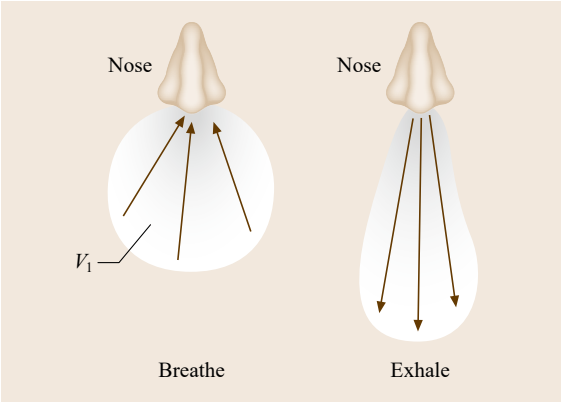

Fig. 58.3 Cycle of inhaling and exhaling as an example for convective transport. The breath is taken from the volume V_1

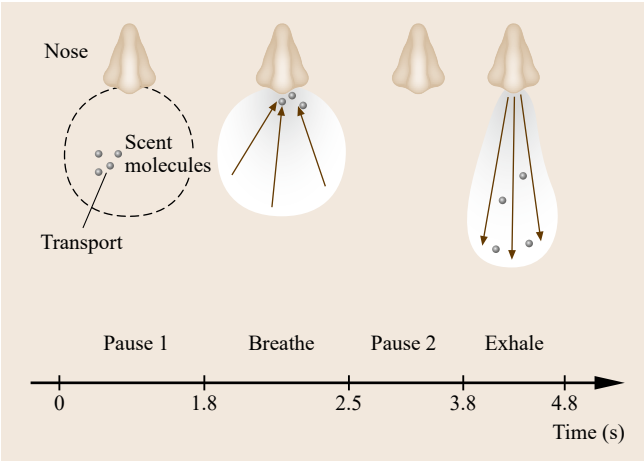

Fig. 58.4 Scent impression during one breathing cycle

air of the previous breath cycle. It is evident that this is very important for every human being in order to get sufficient oxygen from every breath cycle.

This cannot be exploited only for oxygen, but also for scent molecules.

The whole breathing cycle consists of

$$\text{inhale} \Rightarrow \text{pause}_1 \Rightarrow \text{exhale} \Rightarrow \text{pause}_2 \Rightarrow \text{inhale etc.}$$

To get a scent impression, scent molecules have to be transported to the volume V_1 (Fig. 58.4).

A microdosing unit, which can deliver a very small amount of different scent molecules into the volume V_1 in front of the nose, enables scent scenarios. At every breath stroke a different scent impression can be delivered.

After exhaling, the most scent molecules *type coffee* are transported out of the breath volume V_1. During

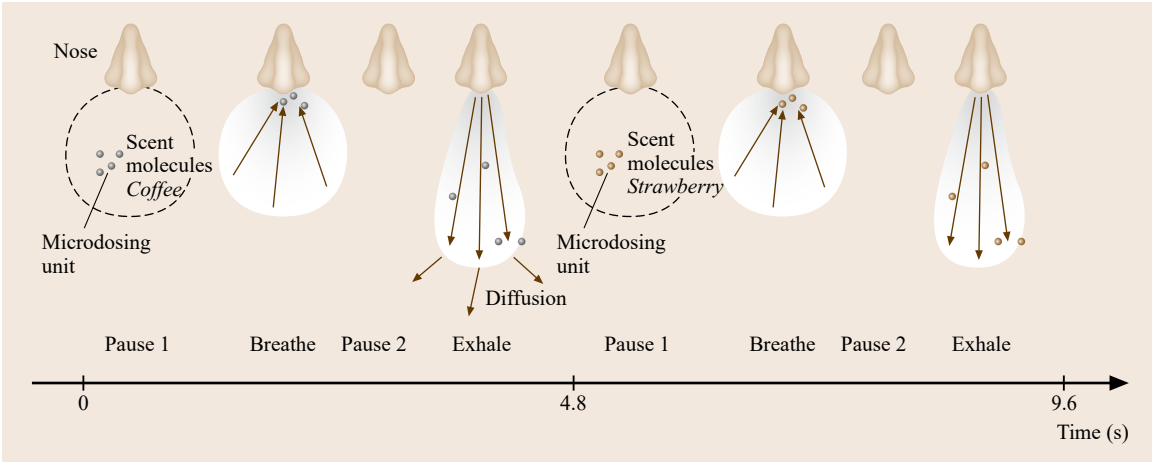

Fig. 58.5 Scent scenario, a microdosing unit delivers different scent molecules at different breath cycles to the nose. At the beginning of the first breath cycle (for example) coffee scent is dosed to the volume V_1, at the beginning of the second breath cycle strawberry scent is dosed to V_1

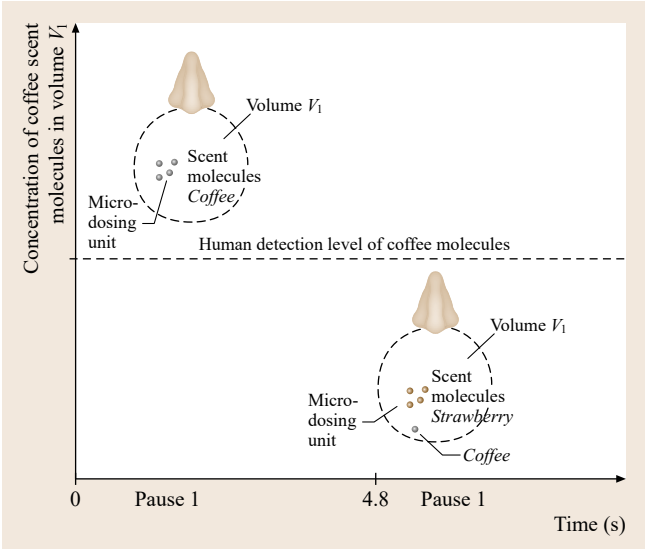

Fig. 58.6 Concentration of coffee scent molecules in volume V_1 at the beginning of both breathing cycles: Coffee can be olfactorily recognized only within one breath cycle

pause 1 of the next cycle, the molecules disappear by convection and diffusion. Just a few molecules of the coffee odor remain in the volume V_1. For that, the concentration of coffee scent molecules within the volume V_1 at the start of the second breath cycle is much lower and beyond the human recognition threshold compared to the start of the first breath cycle. The coffee scent impression is present just for one breath cycle. At the next breath cycle another scent (e.g., *strawberry*) can be dosed to V_1 by the microdosing unit (Fig. 58.5). As a result, the person would smell coffee in the first breath intake, and strawberry in the second, without smelling the previous coffee odor.

The dosing volume of the scent is adjusted to create such a concentration, which the nose will detect the scent after the delivery, and after exhaling and diffusion the concentration descends beyond the detection limit of the nose. This is illustrated in Fig. 58.6.

In principle, at every breath stroke a different scent impression could be delivered by the microdosing unit. Together with audio and video scenarios this enables a new dimension for consumer applications, such as scent memory games and training tools for the human nose.

Due to the dosing volume, low enough for only the user of the microdosing unit to get a scent impression at one breath, the other persons in the same room will not be able to detect this scent: Due to diffusion the concentration of the scent molecules is far beyond the detection level of other persons except the user. This enables micro-scent dosing units to be used also in public locations without bothering other people.

58.2 Generating Scent Molecules

To olfactorily detect a scent molecule, it has to be liberated into the air phase. Scent molecules, which are concentrated in a liquid or solid phase, can be released into the air by different mechanisms:

- Outgassing of a solid or liquid
- Evaporation of a liquid
- Nebulizing of a liquid.

58.2.1 Scent Reservoir

Pleasant scents like from flowers or perfumes, as well as unpleasant scents like those emanating from excrements, can be detected by the nose due to the fact that the scent molecules move first from a liquid or solid phase into the air. If the scent source is surrounded by a fixed volume (e.g., the gas volume in the colon, or the atmosphere in a closed fuel tank), within a certain time an equilibrium concentration builds up, meaning that the number of molecules, which exit the solid or liquid phase within a certain period of time, is the same as the number of molecules, which travel back by condensation to the liquid or solid phase. If the gas volume is continuously exchanged and the scent molecules constantly leave to the ambient air, the scent reservoir is depleted. The smell of a new car vanishes within a few years, as well as the scent of a new wooden chair, or a new carpet.

To realize a microdosing unit for scent, a scent source, a defined gas volume around the scent source to store the scent in a defined concentration, and a delivery unit to transport a small volume from this store into the volume V_1 in front of the nose are required. To ensure a defined number of scent molecules to be dosed to volume V_1 in order to give a scent impression just above the human recognition threshold for this particular scent, the concentration of scent molecules of the gas in the scent reservoir has to be known. During the time between two scent-dosing events (a multiple of a breathing cycle), the scent reservoir has to return to its original concentration. With that, during that time the same number of molecules which have been dosed before has to be emitted by the solid or liquid scent reservoir.

Given is a reservoir (volume V_0, temperature T_0) with a scent sample (solid or liquid) which has been completely purged at the time $t = t_0$. No scent molecule is in the reservoir at t_0. Assumed that the reservoir is airtight again after purging, what is the time-dependent concentration in the reservoir? (Figs. 58.7 and 58.8)

58.2.2 Resaturating of the Volume in the Reservoir with Scent Molecules

The calculation of the transient behaviour of the saturation of a closed and purged reservoir volume with scent molecules is very complex. Evaporation and condensation parameters depend on the type of the molecule, on temperature and pressure. Also the concentration distribution in the gaseous volume has to be taken into account.

Even for water, the substance which is very well known, this cannot be calculated easily. If a bottle with dry air is filled half with water and closed at the time $t = 0$, the humidity in air as a function of time cannot be easily calculated. The humidity concentration is not only a function of time, but also has a space distribution. The concentration of humidity is higher directly over the water level than higher above it. If diffusion would be the only mechanism to transport the humidity in the reservoir, the time to achieve equilibrium would be very long, as shown in Fig. 58.2.

However, it can be stated that even in a closed reservoir convection takes place: The evaporation of the water needs energy, which influences the temperature slightly, changing the density, leading to convection flow, leading to a uniform humidity within the air over the water.

Based on this, for scent reservoirs it can be assumed that due to convection flow a uniform concentration is everywhere in the space above the reservoir at a specific point of time.

Using this assumption, a very simplified model can be derived for calculating the time-dependent concentration of scent molecules after purging: Assuming a constant temperature and a constant free liquid or solid surface of the scent source, the number of molecules which move from liquid (or solid) to air is a constant α

$$\left.\frac{\mathrm{d}N}{\mathrm{d}t}\right|_{\text{liquid}\,\rightarrow\,\text{air}} = \alpha\,.$$

The inverse process of condensation is proportional to the number of scent molecules N in the air

$$\left.\frac{\mathrm{d}N}{\mathrm{d}t}\right|_{\text{air}\,\rightarrow\,\text{liquid}} = -\beta N\,,$$

and β is the condensation probability of one gaseous molecule to enter the liquid phase during the next time

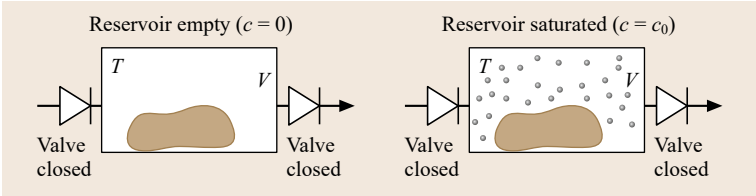

Fig. 58.7 Re-saturating of the reservoir with scent molecules after purging

step. Both the evaporation coefficient α and the condensation coefficient β are directly proportional to the free surface A between liquid or solid scent source and the air in the reservoir.

The net flow is given by the differential equation

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left.\frac{\mathrm{d}N}{\mathrm{d}t}\right|_{\text{liquid}\to\text{air}} + \left.\frac{\mathrm{d}N}{\mathrm{d}t}\right|_{\text{air}\to\text{liquid}} = \alpha - \beta N \,.$$

The solution of this differential equation is

$$N(t) = \frac{\alpha}{\beta}(1 - \mathrm{e}^{-\beta t}) \,.$$

Dividing by volume V_0 of the reservoir gives the time-dependent concentration $c(t)$ of scent molecules in the headspace

$$c(t) = \frac{\alpha}{V_0\beta}(1 - \mathrm{e}^{-\beta t}) \,.$$

The concentration is following an exponential law. Within a typical time constant τ

$$\tau = \frac{1}{\beta}$$

the headspace with a volume V_0 will be saturated with scent molecules to a concentration c_0. The time constant is inversely proportional to the condensation coefficient β

$$c_0 = \frac{\alpha}{V_0\beta} \,.$$

Figure 58.8 shows the time-dependent concentration.

The parameters α and β depend on the molecule properties, on pressure and temperature, as well as on the surface O of the liquid or solid scent source within the reservoir (Fig. 58.8).

$$c(t) = c_0(1 - \mathrm{e}^{\frac{t}{\tau}}) \,.$$

Assuming an ideal gas

$$pV = NkT \,.$$

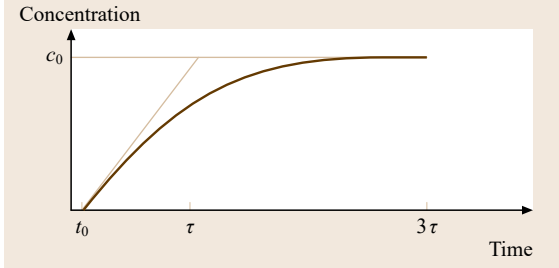

Fig. 58.8 Refilling the headspace: Time-dependent concentration of the scent

The equilibrium concentration c_0 can be described by the saturated partial pressure p_s

$$c_0 = \frac{N}{V} = \frac{p_s}{kT} \,.$$

The saturated concentration c_0 is proportional to the saturated vapor pressure p_s, which can be calculated from the equation of Clausius–Clapeyron. Assuming that the volume of the evaporated gas is much larger compared to the volume of the liquid, the saturated vapor pressure p_s can be integrated from

$$p_s(T) = p_0\mathrm{e}^{-\frac{Q_n}{RT}} \,.$$

With that, the time-dependent concentration would be

$$c(t) = \frac{p_0}{kT}\mathrm{e}^{-\frac{Q_n}{RT}}(1 - \mathrm{e}^{\frac{t}{\tau}}) \,.$$

The molar evaporation heat (or enthalpy of vaporization) Q_n has to be known for the particular odor material to quantify this equation. Q_n also depends slightly on temperature. From all materials, the material best investigated is (odorless) water.

After a time of 3 τ more than 99% of the saturated concentration has been achieved.

58.2.3 Recognition Threshold and Detection Threshold

The *odor detection threshold (ODT)* can be defined as the concentration of scent molecules which is necessary to smell a scent impression (*there is something smelling*), whereas the *odor recognition threshold*

(ORT) can be defined as the concentration of scent molecules which is necessary to identify the scent impression. The ODT concentration is about a factor of 100 lower than the ORT [58.4].

These odor thresholds of different fragrances for human beings can vary in a broad range: The human nose is very sensitive to odors such as hydrogen sulfide (rotten egg) or skatole (C_9H_9N, excrement). For these scents the ODT is only 10^7 molecule/ml air, whereas geraniol ($C_{10}H_{18}O$, essence of roses) the odor detection level is a factor of 10 million higher (10^{14} molecules/ml air) [58.4, p. 155].

This different sensitivity of the human nose to different fragrances has consequences to the microdosing systems: Assuming ideal gas behaviour of gaseous fragrances, in 1 mol (22.4 l) there are 6×10^{23} molecules of a gaseous fragrance. With that, the odor detection level of 10^7 molecules (e.g., to smell rotten eggs or excrements) correspond to a gaseous volume of

$$V_{\text{odor}} = \frac{22.4\,\text{l}\times 10^7\times 1000}{6\times10^{23}} = 3.7\times10^{-13}\,\text{l} = 0.37\,\text{pl}\,.$$

The odor volume $V_{\text{odor}} = 0.37$ pl (picoliter) corresponds to a cube with side length of 7.2 µm. To detect essence of roses (10^{14} molecules/ml air) the odor detection level would correspond to a gas volume of $V_{\text{odor}} = 3.7$ µl (cube with side length 1.5 mm). This means that even for odors with high detection levels the volumes of scent molecules are very small. These detection levels are values for trained people. The detection level of untrained people can be higher.

58.3 Microdosing Systems: Concepts

The microdosing chain according to Fig. 58.9 consists of a micropump or microfan for transporting the odor molecules from a headspace inside a reservoir to the volume in front of the nose. Two check valves at inlet and outlet of the reservoir avoid incorrect dosing when the pump is switched off.

If the micropump is arranged before the reservoir (left side of Fig. 58.10), it will not be contaminated with the odor. This might be the preferred solution if the reservoir has to be exchanged for another scent type. However, the dosing accuracy of the left arrangement is lower compared to the situation, where the micropump is between the reservoir and the nose: The micropump has to achieve a certain over-pressure for opening the check valves, which increases the dosed volume. The arrangement on the right hand side can deliver very small gaseous volumes of pure odor molecules and might be the preferred solution, if very small amounts of the sample are to be dosed.

Normally, a fan can achieve only a very small backpressure of a few pascal (Pa). Using a fan, instead of a micropump, is only possible, if on the one hand the check valves are replaced by active valves, and on the other hand there are huge cross-sections. Furthermore, no pressure drop along the flow path has to occure.

In other cases, a micropump that can achieve a certain back pressure is needed.

The micropump would be the key component for realizing small microdosing technologies according to Fig. 58.10. It must be able to handle small amounts of gases, and in some applications the micropump itself has to be very small and energy efficient.

58.3.1 Micropumps

During the past 25 years, many different types of micropumps have been developed. Actuation principles like electromagnetic, piezoelectric, osmotic, magneto-hydrodynamic, thermopneumatic, electrostatic or electro hydrodynamic and more have been investigated. For gaseous scent dosing, the displacement of the gas, followed by transportation through valves, is required. Some of the actuation principles mentioned above cannot be applied for pumping air (osmotic, electro-hydrodynamic, magneto-hydrodynamic), as these principles need the properties of a liquid for actuation. The stroke of electrostatic actuation is too small to compress gas to a sufficient compression ratio. Ther-

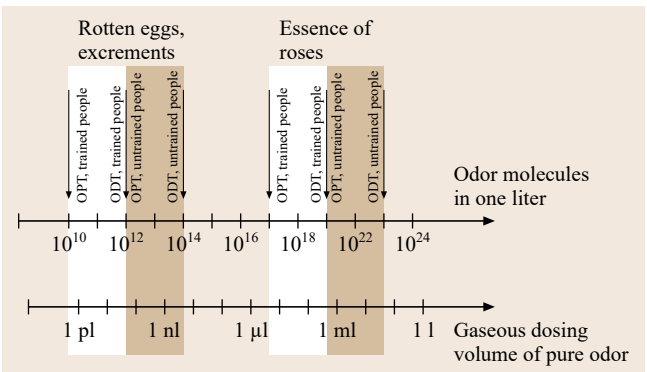

Fig. 58.9 Low ORT *(light green)* and ODT *(dark grey)* for human beings for two different odors, for untrained people. The *bottom axis* shows the corresponding gas volume of the odor for an ideal gas

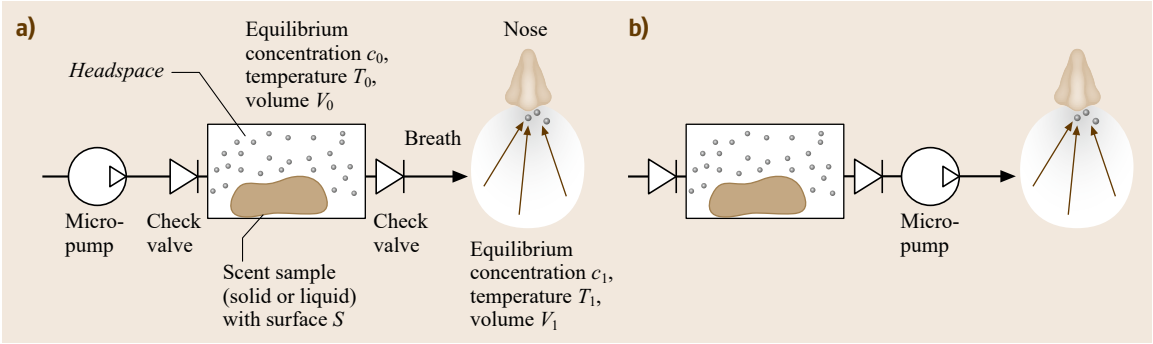

Fig. 58.10a,b Microdosing chain for scent molecules: **(a)** pump is arranged before scent reservoir, **(b)** pump is between the scent reservoir and the nose

mopneumatic actuation exerts higher strokes but huge energy consumption and low pump frequencies are associated drawbacks.

For these reasons, especially piezoelectrically driven micropumps seem suitable for the purpose:

- The most common type is the *microdiaphragm pump with passive check valves* (Fig. 58.11): A piezoceramics mounted on a diaphragm forms the actuation unit and borders the upper part of the pump chamber. Two passive check valves are arranged beyond the pump chamber, directing the flow as inlet and outlet valve. Upon application of a negative voltage the diaphragm expands the pump chamber, leading to a negative pressure, which sucks the gas through the inlet valve into the pump chamber (supply mode, Fig. 58.11b). When a positive voltage is applied at the piezo, the diaphragm moves down, compressing the pump chamber. The corresponding over pressure opens the outlet valve and pushes the stroke volume out of the pump chamber into the outlet (pump mode, Fig. 58.11c), completing the pump cycle. After that, a new pump cycle can start.
- Another common type is a *quasi peristaltic micropump*: Here three piezoceramics are glued onto a diaphragm forming a pump chamber and two active valves: Actuating the piezo in a phase-shifted way, the fluid (liquid or gas) can be transported from the inlet valve via the pump chamber to the outlet valve. Due to the symmetric arrangement, this pump type can operate in a bidirectional way (Fig. 58.14).

These pump principles have been realized with different technologies:

- Silicon micropumps
- Plastic micropumps
- Metal micropumps.

Silicon Micropumps

Debiotech S.A. (Switzerland) has a long experience in developing silicon microdiaphragm pumps for medical applications, especially for the therapy of diabetes. For medical applications, the *nanopump* with a chip size of $10 \times 6\,\mathrm{mm}^2$ has been developed [58.5, 6]. It is optimized for low flow; a dosing rate of 2.5 ml/h can be delivered with an accuracy better than 5%.

Different types of piezo-driven silicon microdiaphragm pumps have been developed at Fraunhofer EMFT within the past 24 years (Fig. 58.11d). Most types have a chip size of $7 \times 7 \times 1\,\mathrm{mm}^3$. One type is optimized for high flow rate (max. air flow rate 40 ml/min, liquid flow rate 5 ml/min, air back pressure 5 kPa, liquid back pressure 50 kPa). Another type has been developed for low flow rate and high pressure (max. air flow rate 0.5 ml/min, liquid flow rate 0.2 ml/min, air back pressure 90 kPa, liquid back pressure 600 kPa) [58.7].

The micropumps are manufactured by silicon micromachining (Fig. 58.12): Three monocrystalline silicon wafers (100 oriented) are structured by double-sided lithography and etched by silicon wet etching (using potassium hydroxide solution KOH). Two wafers form the valve unit, and the third one the actuation diaphragm. The connection between the structured wafer layers is realized by silicon fusion bond (Fig. 58.13). This bonding technology needs very smooth surfaces (roughness lower than 0.3 nm) and very high temperatures (up to 1100 °C) to perform a direct silicon–silicon bond between the wafer layers. Silicon fusion bond provides two benefits: There is no bonding layer, which might be attacked by the fluid to be pumped, and without a bonding layer the vertical pump design parameters (especially pump chamber height) are well defined.

An important design rule for a micropump to achieve a high compression ratio (defined as ratio

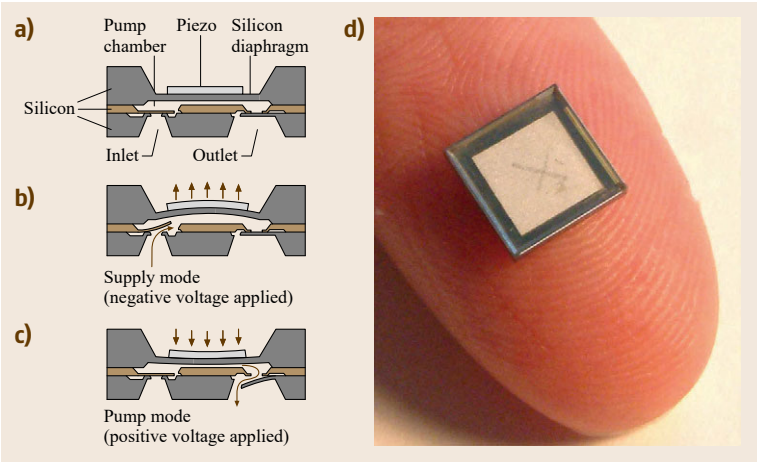

Fig. 58.11a–d Schematic view to a micropump **(a)** with piezoactuator and passive check valves. The pump cycle consists of supply mode **(b)** and pump mode **(c)**. **(d)** A micropump chip (size $7 \times 7 \times 1\,\mathrm{mm}^3$) placed on a fingertip (courtesy of Fraunhofer EMFT)

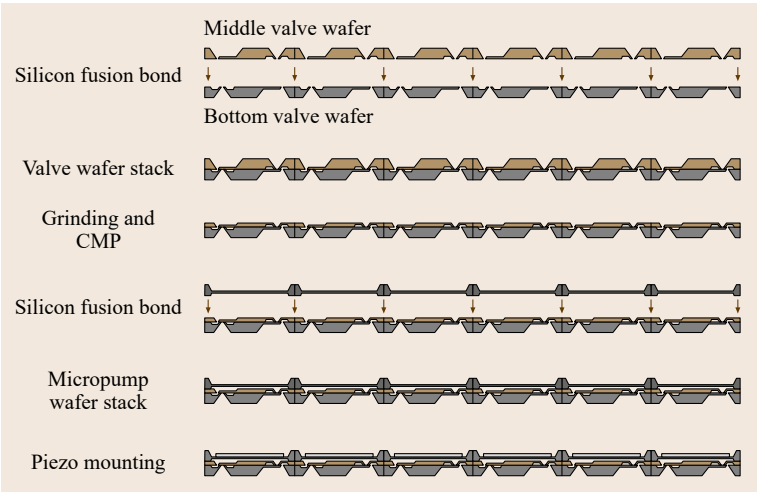

Fig. 58.12 Process flow of micropump manufacturing by silicon micromachining

between stroke volume and dead volume), which is needed to pump gas, is to reduce the dead volume. For silicon pumps as shown in Fig. 58.12 the middle valve wafer is thinned by a grinding process, followed by a CMP (chemical mechanical polishing) process step to enable the second silicon fusion wafer bond to complete the silicon wafer stack.

Plastic Micropumps

One general problem using plastic parts in microdosing systems for scent is the adsorption of scent molecules to plastic materials. For that, the use of plastic materials is only recommended if the micropump will not be used to deliver different scent molecules. Furthermore there is the problem of corrosion induced by some scent molecules for many plastic materials.

Since 2008, Bartels Mikrotechnik has established the serial production of a plastic micropump [58.8]. To improve the bubble tolerance, two micropumps are assembled in series in one housing. This micropump can achieve flow rates up to 7 ml/min (water) and 18 ml/min (gas), and maximum back pressures of 60 kPa (water) and 10 kPa (gas). The energy consumption of the pump including the driver is less than 200 mW. The size (without the driver unit) is $30 \times 15 \times 3.8\,\mathrm{mm}^3$, the weight 2 g.

Micro Jet (Taiwan) offers a series of different piezo-driven microdiaphragm pumps made of plastics [58.9]. The flow rate of the micropump PS31U (dimensions $34.5 \times 34.5 \times 12.3\,\mathrm{mm}^3$) is up to 100 ml/min (liquids) and back pressures up to 35 kPa. The gas flow micropump GS51C (dimensions $53.5 \times 53.5 \times 14.5\,\mathrm{mm}^3$) achieves air flow rate of 200 ml/min and back pressure of 5 kPa.

At Fraunhofer EMFT, a three-chamber plastic micropump has been developed in cooperation with RKT GmbH [58.10]. It consists of an injection-molded plastic body, a metal diaphragm glued onto the metal body,

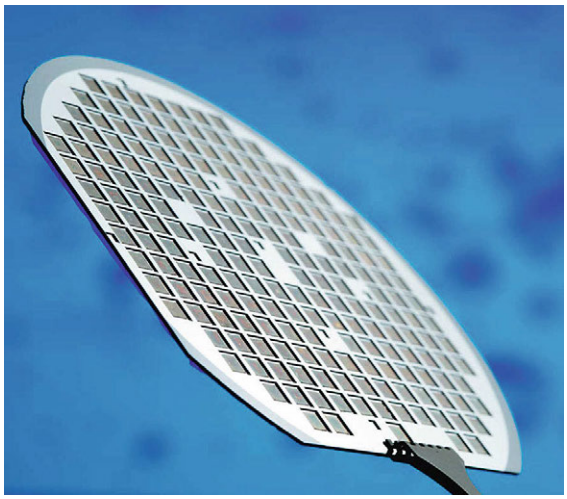

Fig. 58.13 Micropump wafer stack with mounted piezos and 190 micropumps (each $7 \times 7\,\mathrm{mm}^2$) (courtesy of Fraunhofer EMFT)

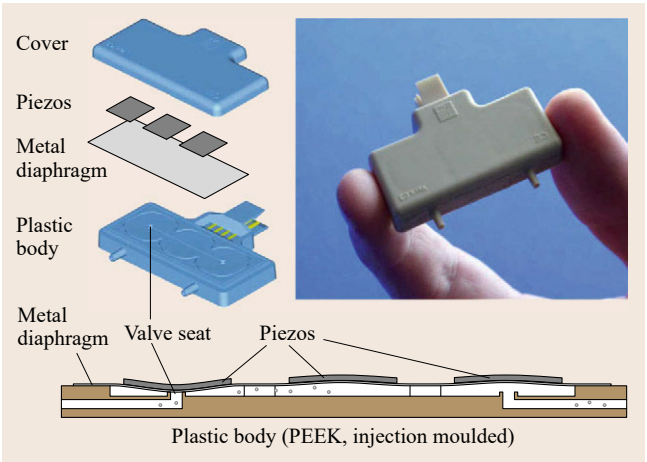

Fig. 58.14 Plastic micropump with three piezoactuation units (quasi peristaltic operation) (courtesy of Fraunhofer EMFT)

and three piezoceramics glued onto the metal foil. The middle piezo is for the actuation of the stroke volume, whereas the outer two piezos can open and close a valve seat each. The pump can operate bidirectionally (Fig. 58.14).

This pump can achieve flow rate of 3 ml/min (Fig. 58.15, *left*) with water and 30 ml/min with air (Fig. 58.15, *right*).

Multimaterial Micropump

Together with Fraunhofer EMFT, RKT GmbH and PI Ceramics Paritec GmbH have developed a multimaterial micropump (Fig. 58.16). This pump has a steel actuation diaphragm driven by a piezo and a plastic body with silicon check valves integrated by hot embossing. This pump has been developed for high performance [58.11], and has diameter of 30 mm, thickness of 4 mm, and can achieve flow rates up to 150 ml/min with water and 350 ml/min with air, respectively.

Figure 58.17 shows the performance of the multimaterial micropump with a double-layer piezo. Due to its outstanding performance, this micropump can be considered as a benchmark for this class of micropumps. On the other hand, due to the combination of several materials (silicon, metal, plastic), this micropump has a limited potential for low cost manufacturing.

Metal Micropumps

Kikuchi Seisakusho Co., Ltd. has developed a piezo-driven microdiaphragm pump made of metal foils [58.12]. The size of the metal pump is $7 \times 7 \times 1.6\,\mathrm{mm}^3$, max. flow rate, 3.5 ml/min, max. back pressure (liquids) of 90 kPa. This micropump can be considered as the currently smallest metal micropump worldwide.

For high-flow applications, Fraunhofer EMFT has developed a micropump consisting of steel foils (Fig. 58.18). A cross-section of the parts of this micropump is shown in Fig. 58.19, in order to explain the laser welding technology used for joining the metal foils together.

The diameter of the micropump µP303 is $d =$ 25.6 mm, the thickness of the actuation diaphragm 100 µm, the thickness of the piezo 200 µm [58.13]. The pump achieves a back pressure of 20 kPa (Fig. 58.20).

58.3.2 Evaporation

The transition of odor molecules from the liquid to gaseous phase can be enforced by heating up the liquid above the boiling point. Scent molecules evaporate and can be transported, for example, by a fan to the human nose. One example is the *Sniffman* scent dispenser [58.14]: Driven by a piezoactuator a small droplet is jetted to a heater plate and evaporated (Fig. 58.21). After that, the scent molecules are transported by a fan through an outlet to the human nose.

The droplet-generation mechanism is similar to a piezo-driven ink jet printer: The pump chamber has to be primed completely with liquid scent without gas bubbles. The actuated piezo generates a pressure wave, which travels to a nozzle, generating a droplet with a very small volume of about 50 pl. This dosing principle is similar to other dosing dispensers, for example, from microdrop [58.15] and Gesim [58.16].

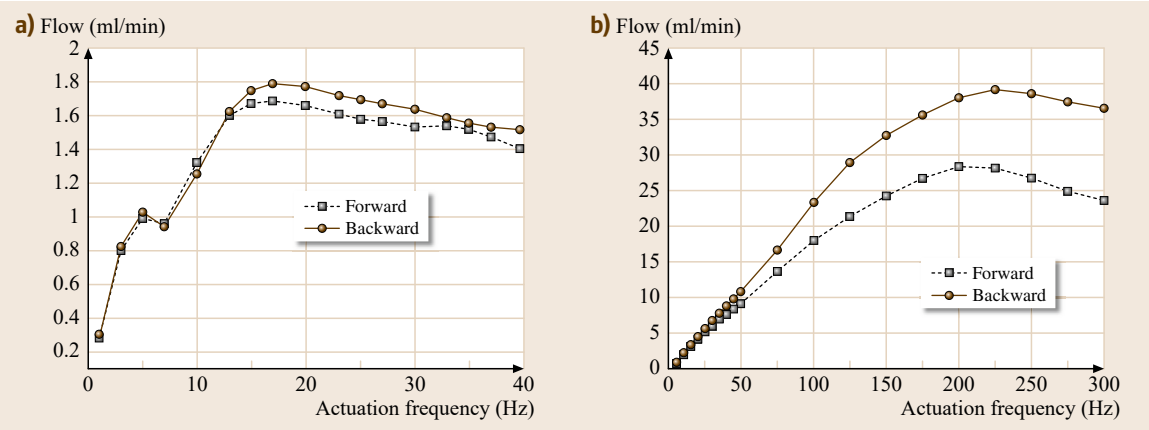

Fig. 58.15a,b Frequency-dependent flow characteristic of the quasi peristaltic plastic pump for water **(a)** and air **(b)**

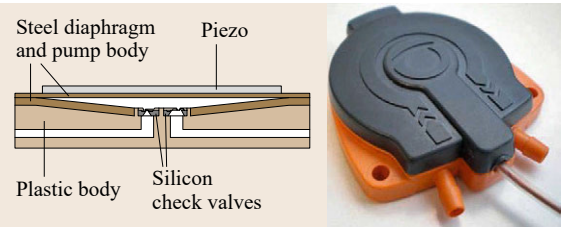

Fig. 58.16 Multimaterial micropump micro-run from Paritec (courtesy of Fraunhofer EMFT)

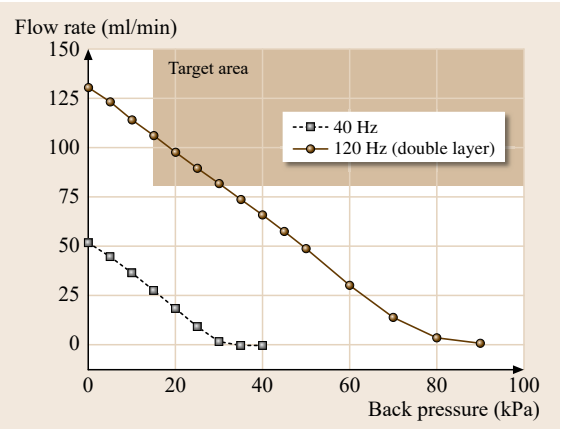

Fig. 58.17 Back-pressure-dependent flow rate of the micro-run micropump with water

Very high droplet frequencies of more than 1000 Hz can be achieved.

Drawbacks of the Sniffman principle are unwanted odors due to the open reservoir at the filling port, and bubbles inside the pump chamber resulting in a malfunction of the jetter (no bubble tolerance). The lack of bubble tolerance is a general drawback of this *ink jet* method of droplet generation.

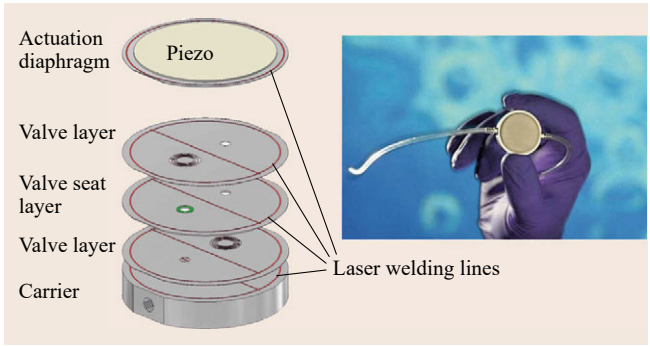

Fig. 58.18 Metal micropump consisting of steel foils structured by laser cutting and joined together by laser welding

58.3.3 Integrated Scent Microdosing System

Another approach for realizing an integrated scent dosing system has been developed at Fraunhofer EMFT. A nanoliter micropump delivers a small amount of liquid scent from a reservoir to a heater chamber. The heater is activated and evaporates the scent. An air micropump with high flow rate pushes the evaporated scent through a micro nozzle (Fig. 58.22). Prototypes of the system have been realized (Fig. 58.23).

Drawbacks of the evaporation method are on the one hand the energy consumption of the heater, and on the other hand the possible unwanted recondensation of gaseous scent at cold walls inside or at the scent dosing device. Furthermore, the heating energy has to be specifically optimized for every type of liquid scent.

58.3.4 Free Jet Dispenser Based on Volume Displacement

There is another jet technology with a different jet generation principle: The actuator can be designed to

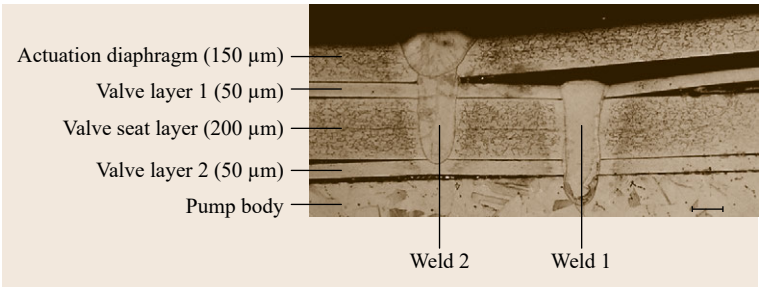

Fig. 58.19 Cross-section of the metal micropump with the weld grooves from laser welding. The thickness of the four steel layers is 450 µm, together with the piezo thickness of 300 µm the active thickness of the micropump (without body) is less than 1 mm

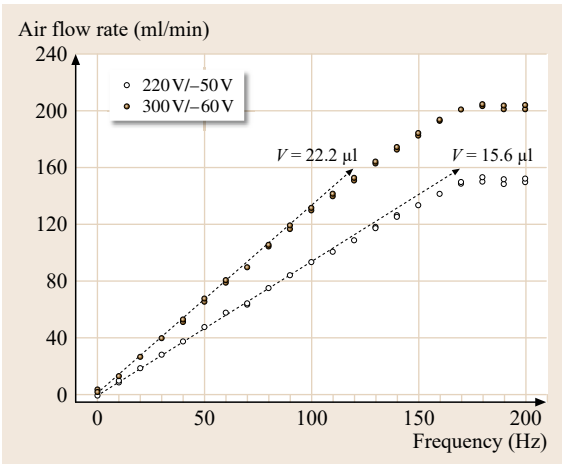

Fig. 58.20 Air flow rate of the Fraunhofer EMFT metal micropump, depending on the operation frequency, at two different actuation voltages (no back pressure)

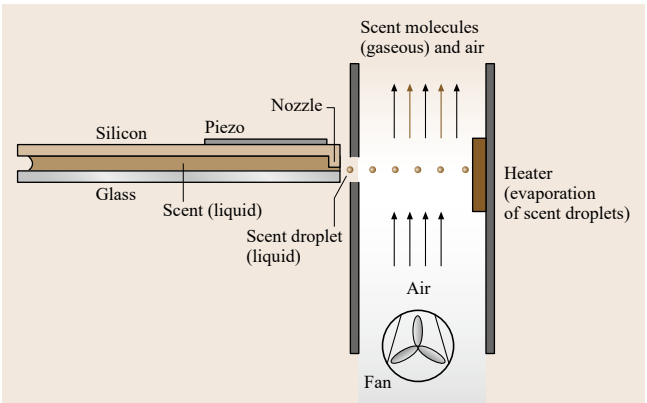

Fig. 58.21 Principle of the *Sniffman* scent dispenser

generate not only a pressure wave, but also to displace a stroke volume in the pump chamber, resulting in a jet ejection outside the nozzle. The dosing volume of that jet ejection is about 50 nl and thus three orders of magnitude larger than in the ink jet technology. An example of this dosing principle is the pipe-jet from Biofluidix, Germany [58.17].

This jetter has a better bubble tolerance compared to the ink jet principle; however the refilling frequency of the pump chamber is limited by the surface forces: During the refilling of the chamber, the surface forces of the liquid at the nozzle have to be at flow stop to avoid sucking in air through the nozzle. That is why, depending on the surface force and the nozzle diameter, a jetting frequency of max. 100 Hz can be achieved.

One possibility to increase the jetting frequency is the combination of a micropump with a nozzle [58.18]. The check valves of the micropump enable a very quick refilling (Fig. 58.24). With that, several 100 Hz of jetting frequency can be achieved. Furthermore, this jetter with integrated micropump is self-priming and bubble tolerant.

58.3.5 Nebulizers

Beside the headspace concept (evaporation or outgassing), the scent molecules can also be nebulized. With this technology, small droplets are generated. Using microactuators, especially piezo ultrasonic nebulizers driven at several hundred kilohertz are used. The liquid (e.g., perfume) is in contact with a metal diaphragm driven by a piezo, and an aerosol is generated which can be transported to the nose. Depending on the piezo frequency, the droplet size has a certain distribution and can vary between submicrometer and some tenths of micrometer.

Recent developments for medical inhalers use a metal diaphragm, which is perforated by laser drilling with a defined hole diameter forming a metal sieve. Around the metal sieve a ring piezoceramics is glued. Beyond the sieve, the drug is in contact with the holes of the sieve. After actuation of the piezo with an actuation voltage with an ultrasonic frequency, the sieve vibrates generating a fog of drug droplets above the sieve. The diameter of the droplets have a sharp size distribution according to the diameter of the holes. The size of the droplets define their ability to be inhaled into the human lung: while droplets with a diameter of 3 µm and below can be deeply inhaled, diameters of 15 µm stay outside. This is why the inhaler eFlow from Pari has a metal

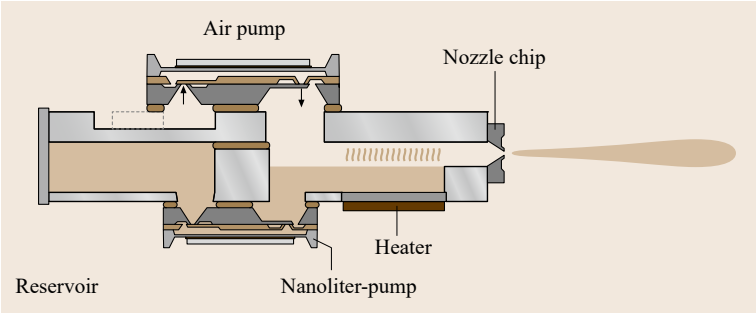

Fig. 58.22 Microdosing system for scent, developed by Fraunhofer EMFT

diaphragm with 3 µm nozzles. In contrast to medical applications, scent droplets shall not be inhaled into the lung in order to prevent potential health damage by the scent.

Dispensing scent by nebulizers has two drawbacks:

- It must be ensured that the liquid droplets of the scent do not harm the user. Thus the diameter of the droplets generated by the nebulizer technology must be large enough so that they cannot be inhaled into the lung.
- Due to the large aerosol diameters, the consumption of scent is much higher compared to evaporation or outgassing. This means that a larger amount of scent is needed for the same scent impression. Next, the big scent droplets contaminate the environment around the user, resulting to an unwanted permanent odor.

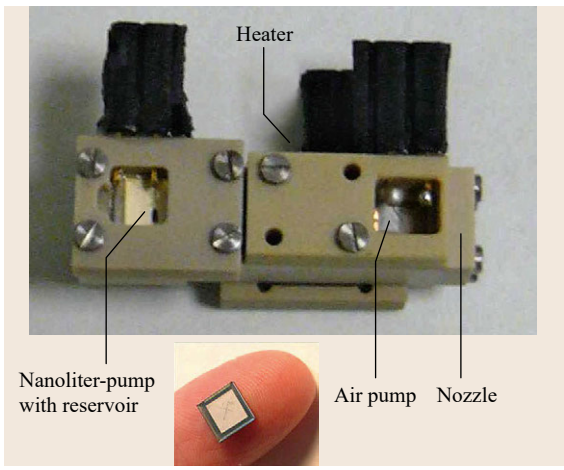

Fig. 58.23 Demonstrator of microdosing system (after [58.19], courtesy of Fraunhofer EMFT)

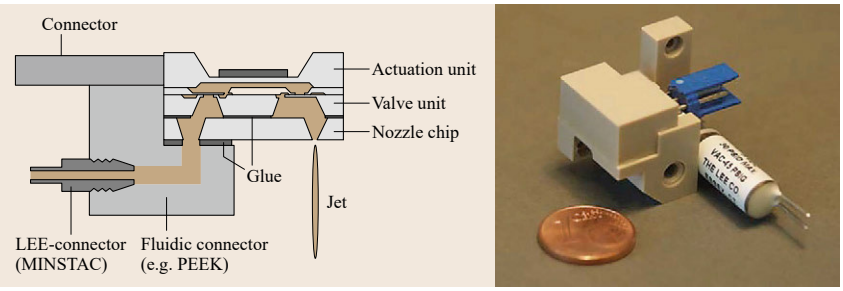

Fig. 58.24 Principle and prototype of a jet dispenser with integrated valves (after [58.18], courtesy of Fraunhofer EMFT)

58.4 New Applications for Microdosing of Scent

Dosing of small volumes of scent molecules enables new features: due to the small dosing volumes:

- The change of the scent impression within short time (even between two breath cycles) becomes possible.
- The concentration of scent molecules is so small that only the user of the dosing unit and not the person right beside can smell the scent.

The application scenarios behind these technologies are mostly not yet realized. In this chapter new potential applications are discussed.

58.4.1 New and Accurate Olfactometers

The accurate dosing of gaseous scent samples can be used to deliver scent in a defined way, in order to improve the accuracy of conventional olfactometers. Transferring the scent to a carrier gas (normally air) by bubbling the air through a scent liquid (state of the art of olfactometers, Fig. 58.25a) is not very accurate nor defined. A more accurate way would be to sample the scent from the headspace of a scent liquid by a micropump (Fig. 58.25b) in a defined way, and dose this to the carrier gas stream. Knowing the concentration of scent molecules of the headspace, the concentration of scent in the carrier stream can be adjusted precisely.

The micropump type μP001FW from Fraunhofer EMFT has a stroke volume of 250 nl, a flow rate of 15 μl/min at 1 Hz operation frequency, and a max. flow rate of 40 ml/min. This allows the concentration to be adjusted in a wide range of more than 3 orders of magnitudes. To ensure a high dosing accuracy, it is important to realize a small dead volume in the micropump and its housing and fluidic tubings between headspace and carrier stream.

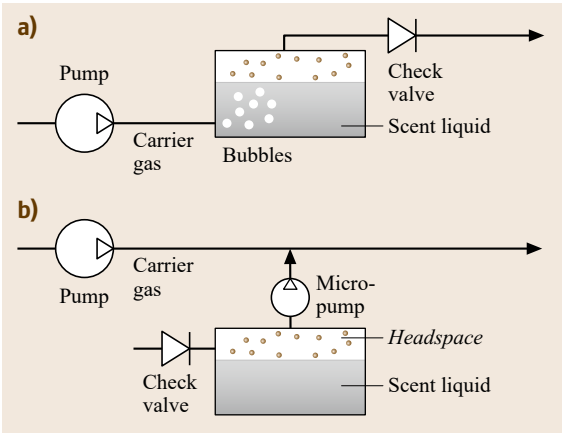

Fig. 58.25a,b Fluidic schematics of a conventional olfactometer **(a)** and a new olfactometer **(b)** using a micropump to deliver scent to a carrier stream

58.4.2 Training of Sniffer Dogs with Microdosing Units

Dogs can be trained to find people after earthquakes or avalanches; they can detect illegal drugs or explosives at airports or stations or can help to find and remove mines in mine-contaminated areas. Currently, it takes approximately 4 months to train a sniffer dog in a dog school. Microdosing units mounted at the dog's muzzle can help to train sniffer dogs more quickly and precisely.

Medical Application: Early Detection of Lung Cancer (LC) by Micropump-Trained Sniffer Dogs

The vision behind this idea is to establish a noninvasive analysis of exhaled breath of patients as a new tool for early in vitro diagnosis of lung cancer.

Early diagnosis is essential to improve the prognosis and healing rate of the patient. However, up to now, there is no established method for lung cancer screening. For lung cancer, the 1 year survival rate in UK is 29.4%, falling to 7.8% after 5 years. Lung cancer is rarely curable at late stages. However, if lung cancer is found in an early stage (currently often only by chance), according to the American Cancer Society (ACS) the survival rate is much higher at 47% (instead below 10% for all stages).

Current methods for diagnosis of lung cancer are x-ray detection and bronchoscopy. However, the random diagnosis of early lung cancer (stage 1) by x-ray detection is difficult. Round focals from 5 to 10 mm can easily be overlooked. The new diagnostics approach using sniffer dogs could be a major step forward to detect lung cancer in an early stage.

For lung cancer detection from breath samples using diagnostic sensor devices, such as electrochemical sensors or pattern recognition sensors, no clear target has been identified yet. Animals' noses are an alternative to detect biomarkers for lung cancer. Here, multidisciplinary scientific and technological knowledge from diverse fields is needed to improve and establish *living sensors* for clinical applications. Prospective clinical trials have convincingly shown that animals' noses (especially sniffer dogs) have the potential to detect lung cancer from patient's breath [58.6, 20]. To establish the diagnostic capabilities of sniffer dogs,

new multidisciplinary methods have to be developed to quantify and scale the training of dogs regarding organic compound targeting in the patient's breath.

It can be hypothesized from that work that there are organic compounds transported with the human breath with the potential to act as biomarkers for LC. A quantitative reference of the relevant biomarker amount could be established with the help of technical sensors analysing the breath samples. That would allow the quantification of the sensitivity of a dog's nose for this purpose.

In a previous prospective clinical trial conventionally trained sniffer dogs were used to discriminate breath samples of lung cancer patients and healthy individuals: In 220 patients lung cancer was detected with 71% sensitivity and 93% specificity. With that, the presence of lung cancer specific organic compounds in the breath of patients was hypothesized. Comparing these results with established multimodal diagnostic capabilities, it is apparent that dogs can detect lung cancer better than conventional diagnostic tools.

However, one big drawback using dogs for lung cancer diagnosis is the missing acceptance of an animal nose as a lung cancer sensor both for physicians and for patients. The diagnosis of a life-threatening cancer disease currently cannot be based on a not verifiable animal decision. Additionally, medical industry, especially big and expensive diagnostic analysis tool producers, state that there are approved and validated methods, which can be traced back to an objective calibration standard. For sniffer dogs there is a lack of traceability.

Up to date training methods of dogs are the same as 100 years ago: A toy is contaminated with the target scent and hidden. The dog searches for that hidden toy. He will get a reward if he finds the hidden toy. It is not possible to measure during the training how much target molecules arrive at the dog's nose. Accordingly, up to now, it is very difficult to introduce a *scaling method* for the dog. In order to increase the acceptance of diagnosis by dogs, there is an urgent need to train dogs in a way that enables every dog's nose to be calibrated by the dog's trainer to the breath sample and the organic compound which is representative for lung cancer.

Microdosing systems could be a breakthrough in solving this problem: A micropump with a connected reservoir of patient's breath sample can be mounted onto the muzzle of the dog. The dosing system is so small that it does not disturb the dog. The dog's trainer can activate the microdosing system and deliver tiny amounts of breath sample directly to the snout of the dog (Fig. 58.26).

A tiny remote-controlled microdosing system to be mounted on a dog's muzzle will be used to train the

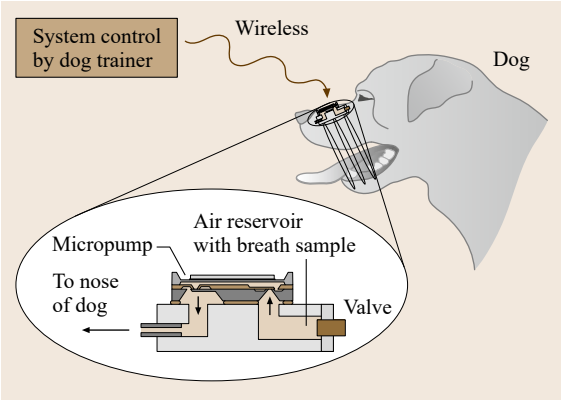

Fig. 58.26 Concept of dog training with microdosing systems

dogs at dog school Lisar and to sample patient's breath. Fraunhofer EMFT and Lisar have jointly developed a demonstrator of a microdosing system including a micropump, electronics, a reservoir, and a check valve (Fig. 58.27) and successfully tested the system at the dog school Lisar (proof of concept).

With this microdosing tool, dogs can be trained much faster (4 weeks instead of 4 months). On the other hand, the dogs can be trained to a lower detection level, and dogs can be calibrated to a defined concentration.

Especially for lung cancer detection, if the patient's breath is stored in the reservoir, the dog trainer can repeat the dosing several times to ensure the response of the dog. The dog trainer can also mount the same microdosing system to another dog to ensure the feedback of the first dog.

These microdosing tools can thus help to establish sniffer dogs for medical diagnostics.

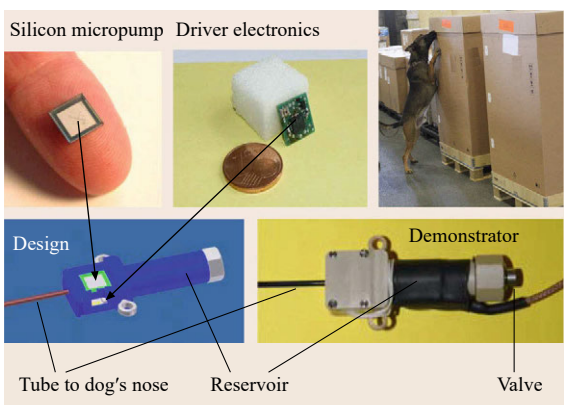

Fig. 58.27 Demonstrator of microdosing system for dog training (courtesy of Fraunhofer EMFT and dog school Lisar)

58.4.3 Scent in Cars

Peugeot and Citroen offered controllable scent sticks already 10 years ago; currently the models Peugeot 308 or Citroen DS3 Cabrio have scent-dispensing technologies. The acceptance on the market has been limited in the past, but the situation is currently changing, due to the car market increase in Asia [58.21]. Recently, the S-Class Mercedes started to offer scent-dosing technology [58.22]. Other car manufacturers now begin to work on own solutions for scent dosing in the car.

The scent delivery technology is similar to the headspace concept of Fig. 58.25: The scent cartridge generates a headspace scent atmosphere in a closed volume. To release the scent, a flap valve, driven by a stepper motor, is opened and closed (Fig. 58.28). When the flap is open, the air stream of the car's air conditioning takes the scent headspace and transports it inside the car [58.23].

58.4.4 Scent for Games

Scent scenarios can be combined with video and audio for games. For this, a headset with integrated multiple scent-dosing systems (e.g., 32 different scents) driven by battery-powered programmable electronics is needed. The game software can control the dosing unit in the headset wirelessly (e.g., via bluetooth), enabling scent scenarios for the user depending on the current game event.

To play different games, disposable scent cartridges can be exchanged and mounted nearby the nose (e.g., at to mouthpiece of a headset). The scent cartridge of a video game related to formula 1 race (scent of tunnel, forest, breaks, fuel, . . .) has different scents than a game to train memory and to learn to distinguish flowers, or an adventure game to explore an old castle.

58.4.5 Scent for *Point of Sales*

Another future application field for microdosing units is scent dosing at *point of sales* in super markets or shopping malls. One of the pioneers in this field is Airplay [58.24]. The buying decision of many goods is supported by scent impressions (drugstore articles, fruits, juice, etc.) a customer can choose the product at a user interface panel, and beside the product information at the monitor the scent-dosing unit delivers a scent impression of the selected product to the customer. Once the customer makes the buying decision, he gets a light signal helping him to find the product quickly.

Another future possibility of scent microdosing is the replacement of room scenting in shopping malls (which fills the whole room) with customer-specific scenting. Different customers might prefer specific scent impressions. Depending on the customer, a specific scent can be delivered, and this scent can be replaced by another fragrance for the next customer. Here, a technology similar to the car scent dosing technology is needed.

58.4.6 Scent in Mobile Phone Applications

During the last decade, several attempts to integrate scent to mobile phones have been made. Motorola submitted an invention for scent delivery in portable electronic devices in January 2003 (US 20040203412 A1), whereas in 2004 Samsung patented a mobile phone with scent delivery function [58.25]. In 2005, Hyundai launched the mp 280 perfume cell phone, with a user-refillable scent reservoir [58.26]. In 2007, a mobile phone from Sony Ericsson (SO703i) entered the market with scent delivery function. In 2008, isi and Convisual developed a scent function for mobile phones [58.27]. Here, a scent can be released by a scent cartridge with different liquid reservoirs. Recently, Scentee (Tokyo) developed a scent dosing module, which can be adapted at the headphone port of the mobile phone [58.28]. Every scent capsule has a capacitance for 100 bursts, the user can select between various scents.

There are many challenges to be solved for integration of scent delivery functions in mobile phone applications: On the one hand it is difficult to miniaturize both the delivery technology and the scent reservoirs, especially, if more than one scent is to be delivered. On the other hand, the energy consumption of the delivery technology has to be low to meet the mobile phone requirements. Next the scent reservoirs have to be tightly

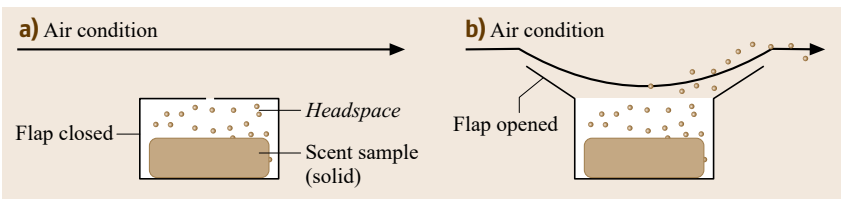

Fig. 58.28a,b Principle of scent dosing unit in automotive (after [58.23]): **(a)** closed; **(b)** open

closed, when the delivery function is off. And last but not least, the scent reservoir has to be changeable by the user if it is empty, and all parts have to be very cost-efficient.

One possibility is to provide *scent cards* with different liquid or solid scent materials attached on the card. There is a heater for each reservoir, which can be controlled by system electronics. To release the specific scent, the heater which is located nearby the scent material is activated. This method does not need mechanical parts like pumps or valves. Drawbacks are high energy consumption for the heaters, and false scents due to untight reservoirs, as well as condensation of scent vapor on the mobile phone.

To overcome these drawbacks, miniaturized and cost-efficient micropumps, microfans, and microvalve technologies are needed. Micropumps and microvalves made of silicon seem to have the highest potential to meet these miniaturization and cost requirements in the future.

58.5 Conclusions

Micro dosing of scent is a new and emerging field enabling many new applications. Delivery systems for very small volumes of one or several different scent pulses in close proximity to the human nose are needed to realize this vision. Considering all scent delivery technologies, micropump-based systems appear to be most suitable. Very small micropump arrays, which are mounted nearby the nose (e.g., at the mouthpiece of a headset), will deliver very small volumes of scent which can be sniffed just once by the person. This technology enables the perception of *scent scenarios* analogous to *picture scenarios* (movies) or *sound scenarios* (music) for human beings.

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Chapter A.4

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Jane K. Parker is a Physical-Organic Chemist and received her PhD from Cambridge University. After having worked in both the chemical and the flavor industry, she moved to the University of Reading to train as a Flavor Chemist. She is a Senior Research Fellow at the University of Reading and Founder and Director of The Flavour Centre, the University's interface between flavor research and the food industry.

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Valentina Parma is a postdoctoral fellow at the Monell Chemical Senses Center, Philadelphia. Her research activity and recent publications have been focused towards the study of olfactory processing and the effect of odors on typical and atypical human behaviors.

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Bettina M. Pause is Professor of Biological and Social Psychology at the University of Düsseldorf. She uses chemosensory stimuli as a methodological approach for understanding phylogenetically ancient emotions. Her applied research interests are related to deviant emotional processes, as manifested in diverse psychological disorders. She was awarded the Prize for Outstanding Science by the Heinrich–Heine–Universität in Düsseldorf in 2009.

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Chapter E.45

Ofer Perl studied Life Sciences at the Hebrew University of Jerusalem. During his graduate studies in the group headed by Prof Noam Sobel at the Weizmann Institute of Science, he discovered his interest in the influences of odors during sleep in both health and disease. He is currently pursuing his PhD in the same group, investigating the roles of sniffing in human cognitive functions.

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Magali Philippeau obtained a MSc in Biotechnology from ENSTBB. She worked with Novartis and in the Laboratory of Histology, Neuroanatomy, and Neuropathology at the Free University of Brussels. In 2004, she joined Chemcom. She leads the high throughput screening department that is active in deorphanizing olfactory receptors, identifying antagonists or enhancers, and in achieving structure–activity relationship studies for receptors of industrial interest.

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Chapter 1

Wilhelm Pickenhagen received his PhD from the University of Paris. After postdoctoral studies at MIT, he became a Research Chemist and then Department Head at the Research Division, and later Vice President General-Manager for Flavor Technology at Firmenich. He has also worked with PPP Geneva SA and Dragoco AG (now Symrise AG) and is Lecturer and Honorary Professor at the University of Göttingen and at the University of Versailles-St. Quentin.

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Anne Plotto has a degree in Agricultural Engineering from Montpellier Sup Agro and a PhD in Horticulture and Plant Physiology from Oregon State University. She has worked towards improving eating quality of fruit by using horticultural practices. Her current research at the US Horticultural Research Laboratoryin Fort Pierce focuses on flavor analysis and sensory evaluation of fruit and fruit products.

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Christina Regenbogen obtained her PhD at RWTH Aachen University, where she studied emotion processing and empathy in healthy individuals, and patients with schizophrenia and depression. She works at Karolinska Institutet, where she is responsible for designing, running, and analyzing studies that share common ground on finding out how huamns deal with multisensory input that reaches them via nose, eyes, and ears.

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Martin Richter studied Physics at the Technical University of Munich. He received his PhD in Electrical Engineering for work in the field of microfluidic systems. He managed several research projects in the field of microdosing systems, and since 2000 he has been responsible for the Department of Micromechanics, Actuators, and Fluidics at Fraunhofer EMFT in Munich. He focuses on micropumps and microdosing systems.

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S. Craig Roberts holds a Personal Chair at the University of Stirling. His early research was in mammalian chemical communication and behavioral ecology, but he now studies human behavior within an evolutionary framework, particularly mechanisms involved in social communication and mate choice.

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Philippe Rombaux is Head of the Dept. or Otorhinolaryngolgy at the University of Louvain in Brussels. His principal interest is medical and surgical rhinology with a special focus on the chemosensory perception. He developed with his collaborators a Smell and Taste Center where all the common techniques to measure the chemosensory function in the clinic are performed such as psychophysic, electrophysiology and imagery.

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Patrizia Rubiolo studied Pharmacy at the University of Turin and spent research periods at the Laboratory of Medicinal Chemistry and Microbiology of the University of Antwerp and the Institute of Organic Chemistry of TU Berlin. She joined the faculty of the University of Turin in 1993 and has held a full professorship since 2011. Her research is directed to exhaustive studies of biologically active fractions from plants.

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Michael Rychlik is the Head of Analytical Food Chemistry and the R&D Division of the Bioanalytics Department at the Technical University of Munich (TUM). He graduated in Food Chemistry from the University of Kaiserslautern and he was appointed Full Professor at TUM in 2010. His group has been working for 15 years in the field of developing analytical methods for bioactive food components, such as odorants.

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David Sander studied Mathematics and Psychology at the University René Descartes (Paris, France), and received a PhD in Cognitive Sciences from the University Louis Lumière (Lyon, France). In 2002, he joined the Department of Psychology at the University of Geneva (Switzerland). He is now Full Professor in this Department where he directs the Laboratory for the Study of Emotion Elicitation and Expression. In 2012, he was appointed Director of the Swiss Center for Affective Sciences, and of the National Center of Competence in Research (NCCR) in Affective Sciences. He is mainly interested in the mechanisms involved in emotion elicitation, and how these mechanisms modulate attention, memory, and decision-making.

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Dr Antje Schellenberg studied Food Chemistry at the Technical University of Munich. She completed her PhD studies in Flavor Chemistry at the Technical University of Munich in 2002. Afterwards she was responsible for stability analysis of drugs in a pharmaceutical laboratory until 2007. Since 2007 she has been employed at the Bavarian Health and Food Safety Authority and is responsible for stabile isotope analysis.

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Han-Seok Seo studied Food Science in Korea, before his training to become an Otorhinolaryngologist at TU Dresden's Smell and Taste Clinic. Since 2012 he has been Assistant Professor at the University of Arkansas in the Department of Food Science. His research interests are chemosensory perception, contextual influences on taste and smell perception, and the relationship between emotion and olfaction.

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Janina Seubert completed her postdoctoral training in Human Chemosensation at the Monell Chemical Senses Center in Philadelphia. At Karolinska Institutet, her research goal is to understand the unique informational value carried by olfactory perception in humans, and how it adaptively complements other sensory modalities during interactions with the environment.

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After receiving her MSc in Industrial Biotechnology from the University of Technology of Compiègne, Anne-Marie Seuvre obtained her PhD in Macromolecular Physico-Chemistry and Solution Chemistry. She received her habilitation in 2007. She is currently an Associate Professor at the University of Burgundy, where she teaches biological physico-chemistry in the food industry section.

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Noam completed a PhD in Neuroscience at Stanford University (1999) and a postdoctoral fellowship at Caltech (2000), before taking the position of Assistant (2001) and later Associate (2005) Professor of Neuroscience at UC Berkeley. In 2007, Noam moved back to Israel, where he took the position of Professor of Neurobiology at the Weizmann Institute. Noam's lab studies human olfaction.

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Veronika Somoza graduated from the University of Giessen and received her *venia legendi* for Food Sciences from the University of Kiel. She was Vice Chair at the German Research Centre for Food Chemistry before she accepted a tenured professorship at the University of Madison. Currently, she is Head of the Department of Nutritional and Physiological Chemistry at the University of Vienna.

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Marc Spehr received his PhD from Ruhr-University Bochum in 2002. After a postdoctoral position at the University of Maryland, School of Medicine in Baltimore, he was appointed Independent Research Group Leader at Ruhr-University Bochum. In 2009, Marc Spehr was appointed a Lichtenberg-Professor in Chemosensation at RWTH Aachen University. He works on signaling mechanisms in mouse chemodetection and the physiological basis of spermatogenesis.

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Christian Starkenmann received his PhD in Organic Chemistry from Geneva University, Switzerland, in 1990. He currently works as Distinguished Scientist at Firmenich S.A. in flavor and fragrances research and development. His main focus is the understanding of food flavors, the modulation of taste perception via mouth microflora, and also the study of malodor formation in the environment.

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Jörg Strotmann studied Biology at the University of Osnabrück and received his diploma in 1989. He obtained his PhD in 1992 from the University of Hohenheim in Stuttgart. From 1995–1996 he engaged in postdoctoral work at Rockefeller University in the Department of Developmental Biology and Neurogenetics. He has been Associate Professor in the Institute of Physiology in Hohenheim since 2005.

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Katinka Temme studied Architecture at the University of Karlsruhe and at Arizona State University. After several years in various international architectural firms and after teaching experience at the University of Darmstadt and the Bauhaus-University Weimar, Katinka has held the Chair for Analog Architecture and Design at the University of Applied Sciences in Augsburg, Germany, since 2013. She collaborates with Daniel Reisch in their architectural think tank studio3architekten.

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Alex Veithen obtained degrees in Biology at the University of Louvain and in Cell Biology at the de Duve Institute. After postdoctoral work at the Pasteur Institute of Lille, he joined ChemCom. He works on functional assay development for olfactory and taste receptors and also manages structure–activity relationship studies for agonists and antagonists of olfactory receptors.

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Chapter B.13

Emeritus Professor and Doctor of Science since 1986, Andrée Voilley has always taught and researched in the same field. She was part of the research group Food Science and Engineering at the University of Burgundy. Her area of expertise extends to physico-chemical properties of small molecules (especially mass transfer of aroma compounds) at the interfaces in complex food systems.

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Chapter D.35

Jessica Walker graduated in Food Chemistry and Toxicology and completed her dissertation at the Technical University of Kaiserslautern in 2008. She worked in the Department of Food Science at the University of Madison. Currently, she investigates the anti-inflammatory impact of food constituents on models of periodontal and systemic inflammation as a Research Assistant at the University of Vienna, Austria.

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Chapter E.41

Donald A. Wilson is Professor at the Nathan Kline Institute and NYU School of Medicine. He is interested in how the mammalian brain processes and remembers information. Using electrophysiological, behavioral, genetic, neuroanatomical, and pharmacological approaches his group explores the neurobiology of memory and the role of experience in sensory system function.

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Chapter A.2

Matthias Wüst received his PhD in Food Chemistry. After postdoctoral studies at Washington State University, he joined the Institute of Food Chemistry of the Goethe University Frankfurt. In 2003, he was appointed Professor of Food Chemistry at the University of Applied Sciences Western Switzerland. He has been Full Professor of Bioanalytics/Food Chemistry at the University of Bonn since 2009.

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Dr Wiebke Zander studied Chemistry at Leibniz University of Hannover and finished her diploma in 2008. For her PhD she studied natural product chemistry. She was Research Assistant in the group Microbial Drugs in Braunschweig before taking up a position at the Analytical Research Center of Symrise AG in Holzminden, where she was responsible for preparation and structure elucidation. She has been Laboratory Manager at Cargill GmbH, Hamburg, since 2015.

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Chapter C.18

Dr Erika Zardin has a background in Environmental Sciences (MSc, University of Venice, 2004) and Analytical Chemistry (PhD, University of Western Australia, 2011). She worked as a Research Scientist on the chemical analysis of volatile aroma compounds and is currently a Business Field Manager in sensory processes and technologies; both positions being at the Fraunhofer Institute for Process Engineering and Packaging IVV in Freising (Germany).

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