Chapter 2
Identification of Volatile Compounds from Flowers and Aromatic Plants: How and Why?

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Abstract When working on volatile compounds from plants, the objectives are multiples and can be summarized in four points: (1) Research of bioactive molecules, (2) Chemotaxonomic studies, (3) Applications in perfume industry, (4) Plant-insect interactions. Each of these four points will be discussed and illustrated by one or several examples of research projects conducted in the Chemistry Laboratory of Natural Substances and Food Sciences. The first two points exclusively concern volatile compounds generated by essential oils extracted from endemic or indigenous plants of Reunion, Mauritius and Madagascar islands. The two last points are dedicated to volatiles found in the airspace (headspace) surrounding flowers. This paper will also present a selection of sampling methods for volatile compounds that range from conventional, inexpensive, solvent-free, quick sampling methods to innovative methods, as well as an overview of detection and identification methods of volatiles including GC-FID and GC-MS.

2.1 Introduction

Aromatic plants are often confused with medicinal plants because they secrete chemicals which sometimes have pharmacological effects. But rigorously, aromatic plants are considered as plants which secrete volatiles by, at least, one vegetative or reproductive organ, often leaves but also roots, stems, bark, seeds, fruits and flowers. These volatiles may act as aroma and flavour molecules due to their interactions with human receptors. The primary functions of these compounds released into the atmosphere are to defend plants against herbivores and pathogens or to provide a reproductive

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advantage by attracting pollinators and seed dispersers [1]. Today, a total of 1,700 volatile compounds have been described from more than 90 plant families [2]. These volatiles constitute about 1 % of plant secondary metabolites known to date and are typically classified into four major categories: terpenoids, phenylpropanoids/benzenoids, fatty acid and amino acid derivatives [3]. Knowledge of the identity and relative amounts of the volatile substances released by plants is of great importance to several fields of basic and applied research in biology, chemistry and many other disciplines. Obtaining this knowledge requires overcoming many analytical challenges posed by these complex mixtures, because they present large variations in component amounts, chemical structures and functionalities.

After a short presentation of the functions of plant volatiles, the chemical compounds classes in plant volatiles, and a discussion on Sect. 2.3, this chapter will cover several practical approaches to plant volatiles analysis: isolation techniques, separation and detection techniques, and compound identification procedures. A few examples will be presented next, to highlight some of the research topics focused on plant volatiles and developed by the Chemistry Laboratory of Natural Substances and Food Sciences (LCSNSA).

2.2 Plant Volatiles

2.2.1 Functions of Plant Volatiles

It is recognized that these compounds stored in specialized secretory structures such as glandular trichomes or resin ducts [4, 5] are not only emitted by plants in response to abiotic stress such as light and temperature changes, flooding and drought, ultraviolet radiation and oxidants, but they are also used as a sophisticated "language" by plants to have a dialogue with other organisms: microbes, animals, and even other plants [1, 3, 4, 6–10].

Some compounds may attract beneficial insects such as pollinators, whereas others are involved in different modes of defense: direct defense, indirect defense and inter-plant priming.

Direct defense involves the production of compounds that inhibit microbial growth, also kill or repel herbivores. *Indirect defenses* involve the production of compounds that minimize infestations of herbivores by attracting natural enemies preying upon or parasitizing herbivores according to the proverb "The enemy of my enemy is my friend". Finally, chemical volatile signals released from injured plants not only affect herbivores and pathogens but may also signal alarm to neighbouring plants by triggering defense responses. This is called *inter-plant priming*.

Many of these compounds have been referred to as "secondary metabolites" to distinguish them from the "primary metabolites" required for the growth of plants. These secondary metabolites however, are likely to be essential for successful competition or reproduction.

2.2.2 Chemical Compounds Classes in Plant Volatiles

The volatile compounds emitted by plants are generally lipophilic and belong to several different classes but are united by their low molecular weight (from 30 to 300 amu) and vapour pressure sufficient to be released and dispersed into the air under normal pressure and temperature. In aromatic and scented plants, they originate from four categories of chemicals: terpenes derivatives, aromatic derivatives, fatty acid derivatives and amino-acid derivatives. Other groups seem to be more sporadic [3, 4, 10–13].

Terpenes Derivatives

Terpenes derivatives, also called isoprenoids, are defined as materials derived from the head-to-tail linkage of the isoprene moiety (2-methylbutane). The isopropyl part of 2-methylbutane is defined as the head, and the ethyl residue as the tail. Depending on the number of isoprene subunits one differentiates between hemi- (C_5), mono- (C_{10}), sesqui- (C_{15}), di- (C_{20}), sester- (C_{25}), tri- (C_{30}), tetraterpenes (C_{40}) and polyterpenes $(C_5)_n$ with $n > 8$. In mono-, sesqui-, di- and sesterterpenes, the isoprene units are linked to each other from head-to-tail; tri- and tetraterpenes contain one tail-to-tail connection in the centre (Fig. 2.1).

Sometimes skeletal rearrangements occur and fragmentation or degradation reactions can reduce the number of carbon atoms so that the empirical formula does not contain a simple multiple of five carbons, thus providing irregular terpenes. Nonetheless, the natural product chemist quickly recognizes the characteristic terpene framework of the structure.

Terpenes encountered among the volatile compounds from plants are exclusively mono-, sesqui- and diterpenes, as well as irregular ones.

Monoterpenes. These substances can be further divided into three groups depending on whether they are acyclic (e.g. myrcene), monocyclic (e.g. limonene) or bicyclic (e.g. α-pinene). Within each group, the monoterpenes may be simple unsaturated hydrocarbons (e.g. limonene) or may have functional groups and be alcohols (e.g. α-terpineol), aldehydes (e.g. citronellal), ketones (e.g. carvone), esters (e.g. linalyl acetate) (Fig. 2.2).

Sesquiterpenes. Like monoterpenes, the sesquiterpenes fall chemically into groups according to the basic carbon skeleton; the common ones are either acyclic (e.g. α-farnesene), monocyclic (e.g. γ-bisabolene) or bicyclic (e.g. α-guaiene) (Fig. 2.3). However, within each group there are many different compounds known. Today, several thousands sesquiterpenoids with well-defined structures, belonging to some 200 skeletal types, are listed.

Diterpenes. Very few diterpenes are reported in floral scents; this may be due to their general low volatility (Fig. 2.4).

Irregular terpenes. The irregular terpenes include compounds varying in the number of carbon atoms from 8 to 18. Among these are apocarotenoids, which are biodegradation products of carotenoid compounds (C_{40}) like β-carotene. Ionones

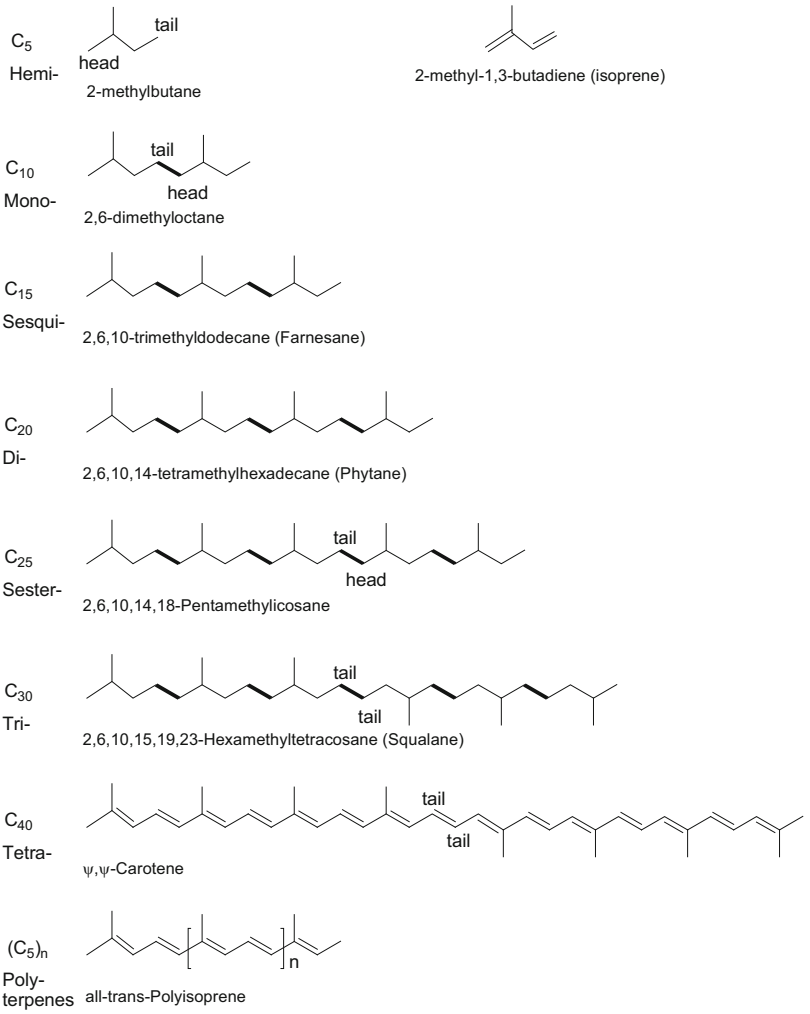

Fig. 2.1 Parent hydrocarbons of terpenes (isoprenoids)

such as dihydro-β-ionone, 6-methyl-5-hepten-2-one and geranyl acetone are the compounds the most often cited (Fig. 2.5).

Aromatic Derivatives

The second category of volatile organic compounds, aromatic derivatives also named as phenolic compounds or benzenoids, are mainly synthesized *via* the shikimate pathway. This pathway got its name from shikimic acid, which is the key step in the formation of the aromatic compounds. Examples of these include: acetophenone, *ortho*-vanillin, cinnamyl alcohol (Fig. 2.6).

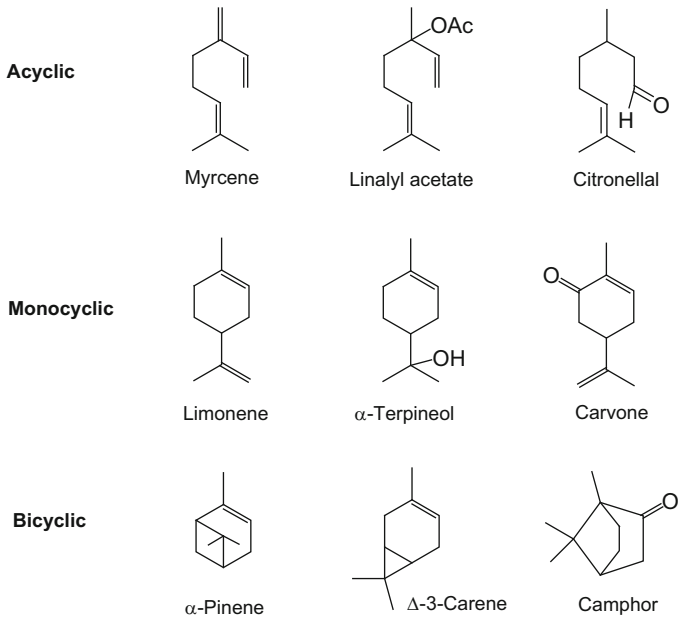

Fig. 2.2 Chemical structures of some monoterpenic compounds

Fatty Acid Derivatives

Fatty acid derivatives are often associated with green leaf odour emitted immediately following the breakdown and lipoxygenation of lipid membranes after mechanical damage. However, these green leaf volatiles are sometimes also produced by flowers. Among the fatty acid derivatives, both saturated and unsaturated hydrocarbons are fairly common, the majority having between 2 and 17 carbon atoms (Fig. 2.7). Aldehydes, alcohols and ketones are also common. Free acids are less common, whereas esters encompass the largest number of different chemical structures. Special mention should be made of the six carbon-compounds known as "green-leaf" volatiles like (*Z*)-3-hexenyl acetate found in vegetative as well as floral scents of numerous plants. This compound probably plays a role in plant defense [1, 7, 8].

Amino Acids Derivatives

Many plant volatiles including aldehydes, alcohols, esters, acids and nitrogen- and sulfur containing compounds are derived from amino acids such as alanine, valine, leucine, isoleucine and methionine, which play an important role in plant defense by recruiting the natural enemies of the attacking herbivore. Amino acids on de-amination form α-keto acid, which in turn forms formaldehyde, acids, alcohols

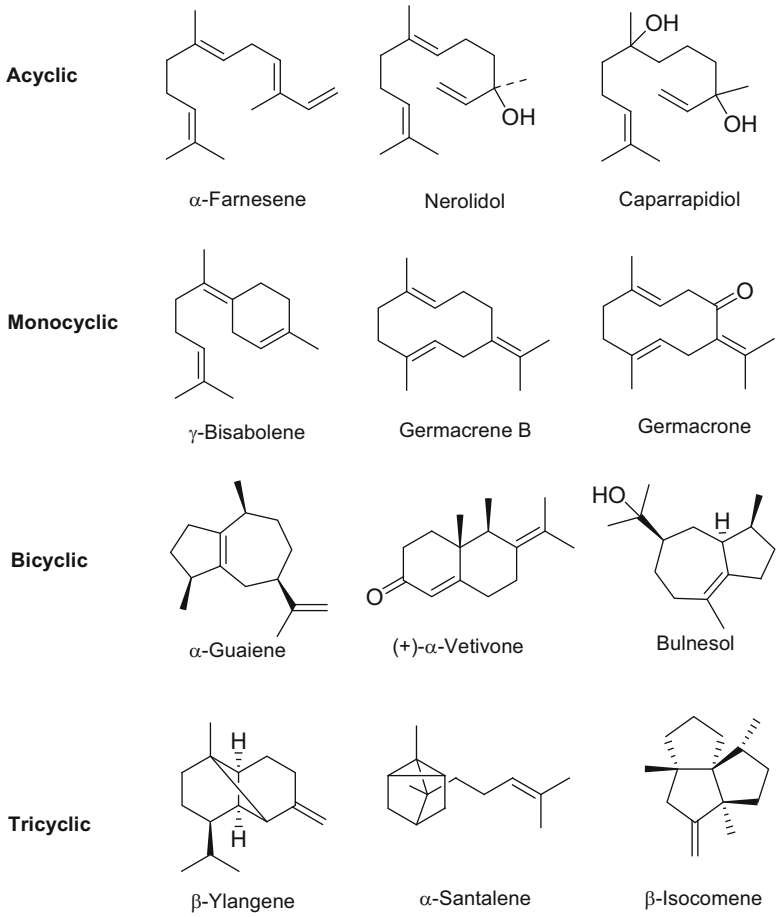

Fig. 2.3 Chemical structures of some sesquiterpenic compounds

and esters on decarboxylation, reduction, oxidation and esterification. Methionine and cysteine have been found to be the precursor of sulfur containing volatiles such as methanethiol, dimethyl disulfide and thioesters responsible for the odour of garlic, onions and boiled potatoes (Fig. 2.8).

2.2.3 Variation in Plant Volatiles

The presence, yield and composition of secondary metabolites in plants, in particular volatile compounds, can be affected in a number of ways, from their formation in the plant to their final isolation. Factors affecting volatile compounds production

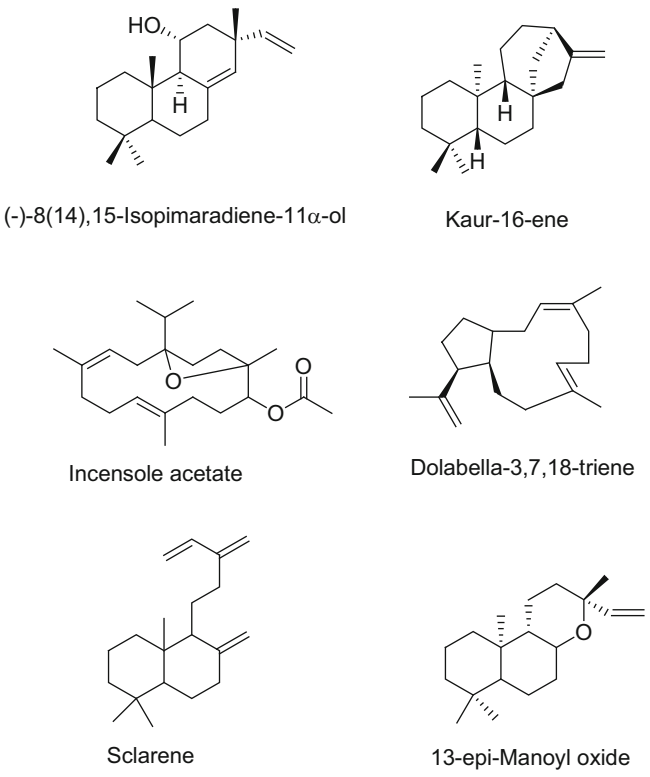

Fig. 2.4 Chemical structures of some diterpenic compounds

include: (1) *physiological variations* such as organ development, pollinator activity cycle, type of plant material (leaves, flowers, etc.), type of secretory structure, seasonal variation, mechanical and chemical injuries; (2) *environmental conditions* like climate, pollution, diseases and pests, edaphic factors; (3) *geographic variation*; (4) *genetic factors and evolution*; (5) *storage* [14].

2.3 Why Investigate Plant Volatiles?

Knowledge of the identity and relative amounts of the volatile substances emitted by plants is of great importance to several fields of basic and applied research mainly in chemistry and biology. So, they are studied for different purposes.

The first one is purely *economic* as plant volatiles can be used in a wide variety of consumer goods such as detergents, soaps, toilet products, cosmetics, pharmaceuticals, perfumes, confectionery food products, soft drinks, distilled alcoholic beverages (hard drinks) and insecticides.

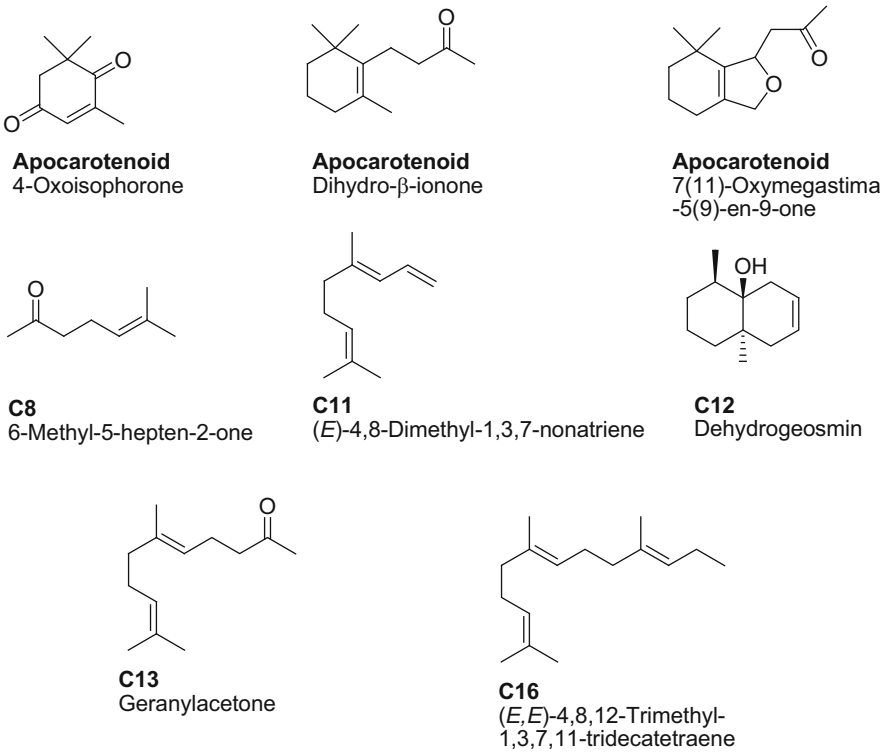

Fig. 2.5 Chemical structures of some irregular terpenes

The second one concerns more specifically *ecology* and focuses for example on plant-plant communication, plant-insect interaction, plant pollination and defense, thermo-tolerance and other environmental stress adaptation.

Studying volatile compounds from plants may also help to understand the *phylogeny or systematic* of some plants through chemotaxonomy. The chemotaxonomy (from chemistry and taxonomy), also called chemosystematics, is the attempt to classify and identify organisms (such as plants), according to demonstrable differences and similarities in their chemical compositions.

At last, volatile compounds are also studied for their *biosynthesis*. Although many of the volatile constituents of plants have been identified, many of the enzymes and genes involved in their biosynthesis are indeed still not known. Such investigation could be interesting for biotechnological process. Indeed, for some years now the demand for natural aroma chemicals is growing fast, in response to both consumers, who are asking for a return to nature, as well as perfumers and flavorists looking for novel creative ingredients. However, the quality and supply of traditional natural flavour and fragrance chemicals are often limited. So, in addition to extraction from natural sources, viable alternative and innovative ways to provide flavour and fragrance chemicals include today

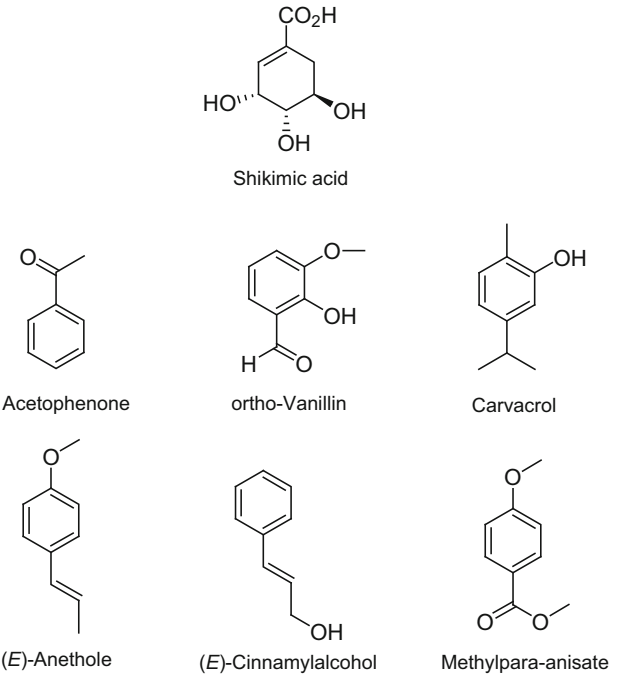

Fig. 2.6 Chemical structures of some aromatic compounds

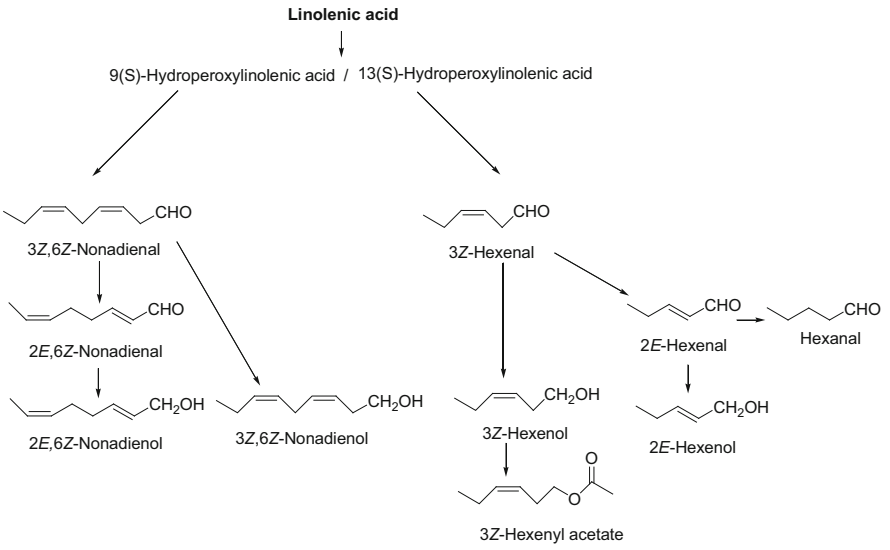

Fig. 2.7 Chemical structures of some fatty acid derivatives

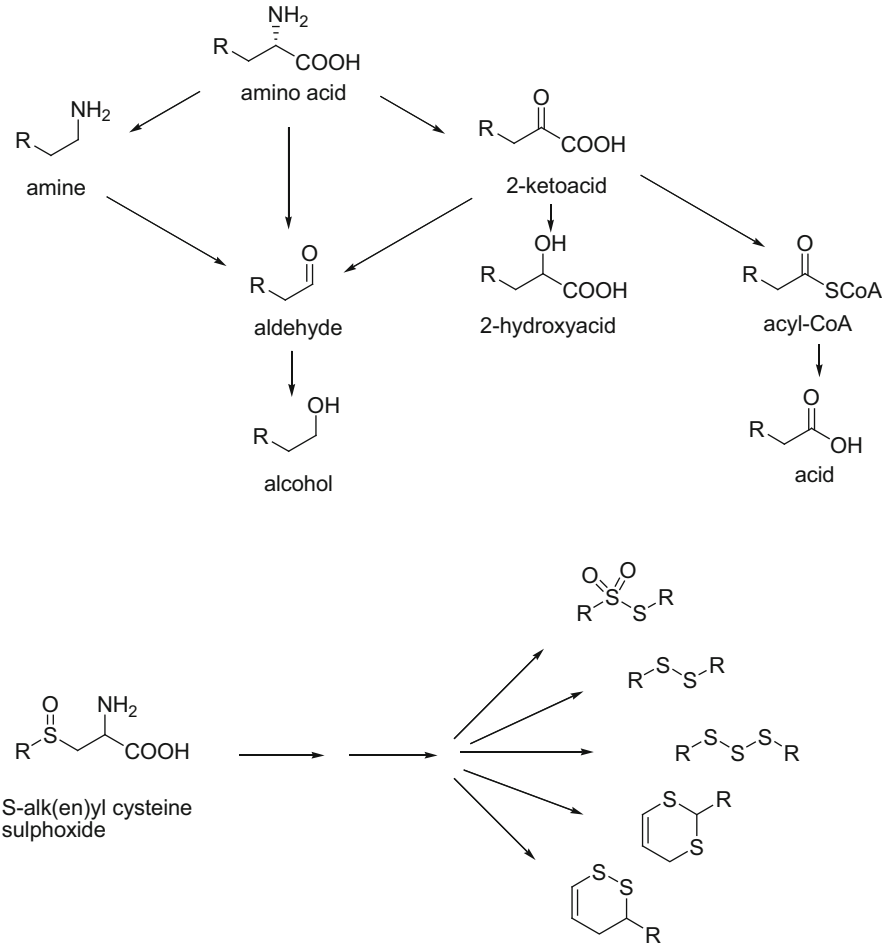

Fig. 2.8 Chemical structures of some amino acid derivatives

biotechnological routes, *i.e*. microbial fermentation, biotransformation using whole cells, biocatalysis using enzymes, plant tissue culture and transgenic plants. So, production or modification of flavour by genetic engineering is thus dependent on the knowledge and availability of genes that encode enzymes of key reactions that influence or divert the biosynthetic pathways of plant-derived volatiles.

This increasing scientific interest in plant volatile compounds has led to the development of a variety of systems for the collection and analysis of volatiles. The choice of which system to use in a particular experiment for collection and analysis obviously depends on the objective which is set. One must consider all the advantages and disadvantages of each technique.

2.4 How to Investigate Plant Volatiles?

2.4.1 Sampling Techniques

Extracting and trapping methods for volatile compounds stored in plants are very numerous. The more commonly used methods will be described hereafter.

Extracting Methods

Extracting methods [15–17] can be divided into two groups: (1) traditional and (2) innovative methods. In relation to the technique used, the qualitative and quantitative composition of essential oils extracted from a same part of a plant is quite different.

Traditional Methods

Distillation. This is the most popular, widely used and cost-effective method for producing essential oils throughout the world. Distillation simply implies vaporizing or liberating the volatile compounds from the plant cellular membranes in the presence of moisture, by applying high temperature and then cooling the vapour mixture to separate the oil from the water on the basis of the immiscibility and density of the essential oil with respect to water. There are different techniques of distillation: hydrodistillation, water and steam distillation, direct steam distillation, distillation with cohobation and hydrodiffusion.

Hydrodistillation. Hydrodistillation is the simplest and oldest process available for obtaining essential oils from plants. Hydrodistillation differs from steam distillation mainly in that the plant material is almost entirely covered with water in the still which is placed on a furnace. An important factor to consider in water distillation is that the water present in the tank must always be enough to last throughout the distillation process; otherwise the plant material may overheat and char. In this method, water is made to boil and the essential oil is carried over to the condenser with the steam which is formed. Water-distilled oil is slightly darker in colour and has much stronger still notes than oils produced by other methods. Hydrodistillation is extensively used by small-scale producers of essential oil.

Water and steam distillation. To eliminate some of the drawbacks of water distillation, some modifications were made to the distillation units. A perforated grid was introduced in the still to support the plant material and to avoid its direct contact with the hot furnace bottom. When the water level is kept below the grid, the essential oil is distilled by the rising steam from the boiling water.

Direct steam distillation. In direct steam distillation, the plant material is distilled with steam generated outside the tank in a steam generator or boiler. As water and steam distillation, the plant material is supported on a perforated grid above the steam

inlet. In water and steam distillation, the steam is at atmospheric pressure and hence its maximum temperature is 100 °C, whereas steam in a modern pressure boiler operating at, for example, 50 psi pressure will have a temperature correspondingly higher. Moreover, there is no limitation to the steam generation when an external boiler is used as a source of steam.

Distillation with cohobation. Cohobation is a technique that can be used for hydrodistillation or for water and steam distillation. It uses the process of returning the distillate water to the still after the oil has been separated from it so that it can be re-boiled. This method has been developed for oils which have partial solubility in water. Indeed, although most of the essential oils have finite solubility in water, some oils like those of rose, lavender and geranium have comparatively higher solubility. In such extractions, the loss with the outgoing water of distillation can become alarmingly high. This problem can be solved by returning the condensate water from the separator back to the still: this is known as cohobation. It is evident that this cannot be done with steam distillation as the water level in the still will keep building up to continuous steam injection.

Hydrodiffusion. This system was first described in 1983. Unlike traditional steam distillation, hydrodiffusion works on the diffusion principle of allowing steam to enter the top of the plant charge and diffuse oil from the oil glands. The system is connected to a steam source, and low pressure steam is passed into the plant material from a boiler. The condenser, which is directly under the basket within the still, is of the tube type. The oil and water are collected below the condenser in a typical oil separator (such as a Florentin flask). The yield of oil is generally high and the process is advantageous because of reduced steam consumption, shorter distillation time and absence of hydrolysis, as the raw material does not come in contact with boiling water.

Cold expression. This technique is used for the very delicate type of *Citrus* family oils which are easily destroyed even by moderate heat and damaged by the steam because of their terpene and aldehyde composition. The peels of ripe *Citrus* fruits are first squeezed by hand or by using special devices that press at room temperature. Secondly, the oil is rinsed off in cold running water. The essence is then collected after decantation.

Maceration in organic solvents. For delicate flowers such as Rose, Jasmine, or Violet, volatile compounds can also be extracted by maceration in organic solvents. This technique permits recovery of many volatile compounds that are lost during distillation due to high temperature. The solvent dissolves all extractable matter from the plant which includes waxes, pigments and highly volatile compounds. The extraction efficiency depends on organic solvents (e.g. pentane, or petroleum ether), the use of agitation and choice of temperature (between 25 °C and 30 °C) to increase the solubility. The solution containing solvent and dissolvable plant material is then filtered and the filtrate subjected to low pressure distillation to recover the solvent for further use. The remaining waxy mass is called *concrete*.

The concentrated concretes are processed further to remove the waxy materials which dilute the volatile compounds. To achieve this, the waxy concrete is warmed and stirred with alcohol (absolute ethanol). During the heating and stirring process

the concrete breaks up into minute globules. Since the aromatic molecules are more soluble in alcohol than is wax, an efficient separation of the two takes place. But along with volatile molecules a certain amount of wax also becomes dissolved and this can only be removed by agitating and freezing the solution at very low temperatures. In this way most of the wax precipitates out. As a final precaution the purified solution is cold filtered leaving only the wax-free material which after solvent evaporation is called *absolute*.

Enfleurage. This is a method of extraction involving the absorption of the volatile compounds into a fatty substance. It is usually used to extract volatile compounds from fairly soft plant materials, e.g. flowers, buds, petals and so on. In the "Enfleurage" method the flowers or other plant materials are spread in a glass dish which contains a thin layer of scentless and purified fat. In less modern times, purified lard was often used for this purpose. The plant material is then left for 24, 48 or 72 h, and then replaced by fresh plant material at 24, 48 or 72 h intervals. This process is repeated time and time again until the fat medium is "saturated" with volatile compounds. The resulting fat is called a *pomade*.

Like concretes, this pomade is dissolved in a solvent (often ethanol). The fatty substance itself does not dissolve, but sinks to the bottom of the vessel, while the volatile components are dissolved in the alcohol. The alcohol is then gently heated to give the absolute. Volatile compounds from Rose, Tuberose, Jasmine and Neroli are collected in this way.

Innovative Methods

Important progress has been made in the development of novel separation techniques with shortened extraction times, reduced solvent consumption, and enhanced prevention of oxidation and isomerization, especially for thermolabile and chemically highly active constituents.

Microwave extraction. Microwaves are increasingly being used as the heat source to assist the extraction of volatile compounds. The developed microwave extraction methods include the Solvent-Free Microwave extraction (SFME) and the Microwave Steam Diffusion (MSD). The Solvent-Free Microwave Extraction in particular is the most commonly used. This method involves placing the fresh plant material in the microwave reactor without addition of water or any other solvent. The internal heating of the water within the sample distends its cells and leads to rupture of the glands and oleiferous receptacles. This process thus frees essential oil, which is evaporated by the *in-situ* water of the plant material. A cooling system outside the microwave oven continuously condenses the vapours which are collected on specific glassware. The excess of water is refluxed back to the extraction vessel in order to restore the *in-situ* water to the sample [18–20].

Supercritical fluid extraction. This is a solvent-free extraction method, usually carried out using CO_2 due to its advantages as a solvent for volatile compounds. Under high pressure, CO_2 turns into a liquid and acts as a solvent that can be used to extract volatile compounds from plant material. CO_2 is forced into a stainless steel

tank containing plant material, then the pressure is released. As pressure decreases, CO_2 returns to a gaseous state and only the plant extract remains. One of the main drawbacks of solvent-free extraction is its limitation to non-polar and medium-polar substances, since it is mostly applied with CO_2.

Ultrasound-assisted extraction. This extraction method can be used for the extraction of volatile compounds localized in both surface glands, where a mild ultrasonic treatment is enough, and inside the cells, where stronger treatment is needed. Ultrasound-assisted extraction increases the performance of solvents and is performed at lower temperature, which is less likely to result in losses of thermally unstable compounds, but isomerisation and decomposition may occur for chemically unstable compounds. Ultrasound-assisted extraction provides smaller extraction yields than most classical methods such as hydrodistillation or steam distillation and some recent extraction methods.

Trapping Methods

Several fields of study include analyses of floral volatiles in relation to pollination biology, measurements of volatiles such as isoprene released from phytosynthetic tissues in response to changes in light and temperature, and volatile emissions induced by herbivore damage. In most cases, the emitted volatiles have to be sampled and concentrated prior to subsequent analysis. Such investigation is often referred to as *headspace* analysis [21–23]. Headspace sampling is a non-destructive method for collecting volatiles in the airspace (or headspace) surrounding above-ground plant parts. Compared with solvent extractions of volatiles from plant tissues, headspace analysis gives a more realistic picture of the volatile profile emitted by plants and detected by insects that respond to plant volatiles, making this method most suitable for many ecologically relevant applications.

There are different headspace sampling techniques: the *static headspace sampling* (no air circulation) with the use of Solid Phase Micro-Extraction (SPME); and the *dynamic headspace sampling* which includes Close-Loop Stripping (air is continuously recycled), Pull and Push-Pull Systems (air is constantly taken up from the outside, passed over the plant sample and through an absorbent trap). In all these volatile collection methods, the used chamber for headspace collection should be free of material that retains volatiles or cause bleeding of compounds that may contaminate the system. Good choices for materials include glass, Teflon and metal which are easy to clean and do not show bleeding, whereas materials such as rubber, plastic, glues, adhesives and wood should be avoided.

Concerning more specifically static headspace analysis, the plant or its parts are enclosed in a container and emitted volatiles are trapped onto an adsorbent. The air surrounding the plant remains “static” which means it is not circulated in the chamber. Volatiles are enriched on the adsorbing matrix without sampling impurities of a continuous air stream that may obscure the detection of

low-abundant volatile compounds. Thus, this method is advantageous for sampling volatile compounds from low emitting plants.

An important advance in static headspace analysis was the development of SPME which is a true "green revolution" in sample preparation techniques. Cheap, fast, simple and solvent-free, this method enables the collection of volatiles at detection limits in the ppbv (parts per billion by volume) range. Solid phase micro-extraction is based on ad/absorption and desorption of volatiles from an inert fiber coated with different types of ad/absorbents. The fiber is attached within the needle of a modified syringe and volatiles can be sampled by inserting the needle through a septum of a headspace collection container and pushing the plunger to expose the fiber. Following equilibration between the fiber and the volatile sample (a few minutes to half an hour), the fiber is retracted into the needle and can be transferred to a gas chromatograph injector for direct thermal desorption. Solid phase microextraction fibers can be reused approximately 100 times. By carefully selecting the polarity and thickness of the fiber coating, compounds of different polarity and volatility ranging from high-boiling or semi-volatile to volatile compounds can be sampled. Solid micro-extraction does not provide directly quantitative information since the adsorbed amount depends on the fiber-coating affinity for the compound in addition to its concentration in the headspace. Thus, the results obtained may misrepresent some volatiles and over-represent others. However, quantification of volatiles by SPME may be possible by the application of internal or external calibration.

2.5 Analysis Techniques

Plant volatile compounds extracted or trapped on ad/absorbing matrices are routinely analysed by the standard technique of gas chromatography [24, 25]. A chromatographic system includes four fundamental blocks: (1) An injector; (2) a column placed in an oven; (3) a detector and (4) a data system.

Injector. Samples are either injected as solvent extracts into the heated injector in a split or splitless mode or desorbed from the adsorbent such as the SPME fiber by placing it directly in a thermal desorption tube, heated to 250–300 °C. The injection system then transfers the mixture of volatile compounds into the chromatographic column. Ideally, this must be done in a quantitative manner, without discrimination due to molecular weight or component volatility and without chemical alteration of any constituent substance. So, an injector is a sort of entrance door into the column where component separation is achieved.

Column. Volatiles from plants are commonly separated on fused capillary columns with different stationary phases, such as the non-polar dimethyl polysiloxanes (e.g. OV-1, DB-1, DB-5, CPSil 5) and the more polar polyethylene glycol polymers, including Carbowax 20 M, DB-Wax and HP-20 M. Columns are usually 30–60 m long (and have a stationary phase film thickness of 0.2–0.3 μm and an internal diameter of 0.25 mm or 0.32 mm).

Detector. Following separation on a GC column, volatile compounds can be analysed by two different types of detectors. The first type, for example, a *flame ionization detector* (FID) provides only information on retention times, while detectors of the second type, such as *Mass Spectrometry* allow additional structure evaluation.

Flame ionization detectors. With such a detector, organic compounds are ionized in a hydrogen/air flame, producing a signal proportional to the mass flow of carbon. They are commonly used for quantitative analysis because of their wide linear dynamic range, their very stable response and their high sensitivity with detection limits of the order of picograms and nanograms per compound.

Mass Spectrometry detectors. These are the most popular type of detector for routine plant volatile GC analysis. In the mass spectrometers of most standard GC-MS bench-top instruments, compounds exiting the GC column are ionized by electron impact (EI) and the resulting positively charged molecules and molecule fragments are selected according to their mass-to-charge (m/z) ratio by entering a quadrupole mass filter. Total ion chromatograms are obtained, which provide information on the retention time of each compound and its mass spectrum consisting of a characteristic ion fragmentation pattern.

Data systems. For identification of compounds in GC-MS analysis, suggestions can be obtained from popular computerized mass spectral libraries such as Wiley [26], NIST [27] or MassFinder [28] and other databases not computerized developed by Adams [29] or Joulain and König [30].

Concerning the computerized MS library search, it is usually performed using PBM (probability – based matching) algorithm, a library-search routine that uses a reverse search to verify that peaks in the reference spectrum are present in the unknown spectrum. Extra peaks in the unknown are ignored, thus allowing the analysis of a spectrum resulting from a mixture of compounds. The PBM search results displays the list of the best 20 matches that resulted from the library search showing the name of each compound, the molecular weight and the correlation coefficient or quality. The correlation coefficient is used as the first identification criterion. However, mass spectra libraries cannot be used as unique and absolute criteria for the identification of chromatogram peaks. This process is not always straightforward but complicated by several issues.

First of all, even under highly reproducible mass spectrometry conditions, two mass spectra of the same compound are not absolutely identical, but only similar to each other. Measured on the same mass spectrometer under identical experimental conditions, the similarity is usually excellent. However, in most cases, the reference spectrum contained in the library has been recorded with another mass spectrometer than the unknown mass spectrum, and the spectra may differ to a certain degree. Using several types of spectrometer (quadrupole, ion trap. . .) result in many cases in notable differences. Impurities of co-eluting peaks or air may also cause significantly different spectra.

Furthermore, the essential feature of mass spectrometry for essential oils is that mass spectra are not particularly unique in many cases. Within the broad class of monoterpenes and sesquiterpenes found in essential oils, a large number of isomers

of the same molecular formula but with different structure exist. Many times, their mass spectra are very similar making the peak identification somewhat difficult and sometimes impossible. The issue of substances with almost identical mass spectra can only be resolved by using additional experimental data that are able to distinguish between them. The gas chromatography is indisputably the complementary technique used to differentiate such compounds. The first solution consists of identifying the components by co-chromatography with standards. If authentic material is not available, the second solution is to combine the experimental values for retention times and mass spectra that are two independent parameters: substances with same mass spectra usually exhibit different retention times, or in other words, co-eluting peaks could only pure chance give rise to identical mass spectra. However, the retention time of the separated compounds cannot be utilised as such owing to their dependence on many factors such as column length, column polarity, carrier gas velocity and oven temperature program. All these elements prevent comparison of the retention times obtained with those of other scientists. A superior approach is then to use a derived value from the retention time called *Retention Index* or *Kovats Index*. These have the advantage of being fairly insensitive to experimental conditions and can therefore be replicated for a given stationary phase. They represent a common language among chromatographers.

The Retention Index (RI) or Kovats Index (KI) is a relative value determined by comparing the retention time of the compounds with retention times of two standard compounds. Several homologous series of organic compounds can be used as standards, but *n*-alkanes have been used exclusively. The formula for RI calculation depends on temperature conditions.

For isothermal conditions:

$$\mathrm{RI(x)} = 100\frac{\log\left(t_{R(x)}\right) - \log\left(t_{R(z)}\right)}{\log\left(t_{R(z+1)}\right) - \log\left(t_{R(z)}\right)} + 100z$$

For linear temperature program conditions:

$$\mathrm{RI(x)} = 100\frac{t_{R(x)} - t_{R(z)}}{t_{R(z+1)} - t_{R(z)}} + 100z$$

where:

RI(x) is the retention index of compound x
z is the number of carbon atoms in alkane z
$t_{R(x)}$ is the retention time of compound x
$t_{R(z)}$ is the retention time of alkane z
$t_{R(z+1)}$ is the retention time of alkane z + 1

To use the retention index system, a measure of gas chromatograms of all *n*-alkanes covering the desired retention time range needs to be done; e.g. to investigate monoterpenic and sesquiterpenic compounds a range of C_9–C_{26} is

needed, so all terpene peaks will elute between known alkanes. In order to measure the complete alkane pattern, with only a single injection, mixtures of *n*-alkanes are usually prepared from pure chemicals at 5 % concentration in pentane. The measurement of the alkane pattern obviously has to be carried out under exactly the same experimental conditions as the future analyses. Different GC-MS systems will exhibit slightly different alkane patterns, so for each system the alkane pattern has to be determined separately. Few compilations of retention indices are currently available. Those which should be mentioned are:

Davies [31], a compilation of RI of some 400 monoterpenes and sesquiterpenes on either or both types of stationary phase (non-polar and polar).

Kondjoyan and Berdagué [32], a compilation of RI of more than 2,000 volatile compounds on non-polar (type DB-1 or DB-5) and polar (type Carbowax) stationary phases.

Adams [29] and Joulain and König [30] list the retention indices of compounds on non-polar stationary phases (DB-5 and CPSil-5) and their mass spectra.

Massfinder [28] also represents another excellent two dimensional search algorithm that takes both retention index on non-polar stationary phase (DB-5) and mass spectrum similarity into account. The library of this program covers approximately 2,000 compounds commonly found in essential oils, particularly monoterpenic and sesquiterpenic compounds, diterpenes and related aromatic and aliphatic constituents like esters or lactones. Most spectra acquired on quadrupole spectrometers will be very similar and fully compatible with Massfinder mass spectral collection. Mass spectra acquired on ion traps may exhibit close similarity, but depending on the measurement conditions may differ slightly.

To sum up, a correct identification of a volatile compound requires at least the determination of Retention Indices on two columns with different polarities and a good match quality of the mass spectrum of the compound of interest with that of authentic standard.

2.6 Investigation on Volatile Compounds by LCSNSA

2.6.1 Environment Protection and Biodiversity Conservation

It is recognized that tropical oceanic islands contribute disproportionably for their area to global biodiversity. However, these islands are also specifically vulnerable to many threats including habitat loss, habitat degradation and fragmentation, and invasion of exotic species. Tropical islands have therefore been considered biodiversity hotspots containing many plant and animal species that are not found elsewhere but that are under severe threat. A series of islands scattered in the western Indian Ocean along the southeast coast of Africa has been recognized as a global biodiversity hotspot. Dominated by the nation of Madagascar, the fourth largest island on earth, this

hotspot also includes the independent nations: Seychelles (including Aldabra), the Comoros, Mauritius (including Rodrigues), and the French overseas departments: Reunion, Mayotte (one of the Comoros) and the Iles Eparses. Three of them (Reunion, Mauritius and Rodrigues) are grouped together and make up the Mascarene Islands. Reunion Island (2,512 km^2) is the biggest of the three and also contains the largest proportion of intact habitat types (ca 30 %) because of its rugged topography which has precluded agriculture and large-scale urbanization. Today, it appears obvious that all initiatives that can ensure biodiversity protection on this volcanic island will enable to preserve representative tracts of Mascarene ecosystems. Systematic conservation planning is widely considered the most effective approach for designing protected area and other ecological network. However, effective systematic conservation planning requires expert knowledge on the distribution of plant and animal species, patterns of species richness and composition, and the way they are interrelated. Thus, in this context, biologists, botanists and chemists from the University of Reunion Island decided to collaborate and contribute to a better knowledge of the fauna and flora of Reunion Island.

Among the several ongoing scientific projects, the phytochemical study about species of the genera *Psiadia* and *Melicope* was envisaged by the LCSNSA for chemotaxonomic purpose [33–36].

The genus *Psiadia* (Asteraceae) comprising aromatic plants is widely distributed in tropical and subtropical regions. The taxonomy of this genus is rather complex. Thus, in the Mascarenes, the taxonomic scheme established by Scott is built on the segregation of the genus into five groups mostly based on morphological characters. Nine species endemic to Reunion Island and Mauritius were investigated.

The genus *Melicope* (Rutaceae) encompasses more than 200 species occurring from the Malagasy and Indo-Himalayan regions, east to Hawaiian and Marquesas Islands and south to New Zealand. In the revision by Hartley, all *Euodia* species from the Malagasy region, including the Mascarene Islands were transferred to the *Melicope* species. Six of these species are endemic to Reunion Island. The six species including sub-species and varieties were investigated.

For all these species, the chemical composition of the essential oil obtained from their leaves by hydrodistillation has been examined. The chromatographic analyses by GC-FID and GC-MS allowed the quantification and identification of their volatile constituents. However, some of them could not be identified by computer matching with MS libraries (laboratory-made and commercial) and Retention Index. So, their complete structure was elucidated by means of mono- and bidimensional NMR spectroscopic techniques after isolation and purification by repeated column chromatography. Thus, several new molecules were identified. This is the case of an acetylated monoterpene (1) and a sesquiterpene alcohol (2), both detected at a high percentage in the essential oils of *Psiadia anchusifolia*, *Psiadia argentea*, *Psiadia boivinii* and *Psiadia salaziana*. The occurrence of these unusual terpenoids in these four species supposes that the latter have the same biosynthetic pathway and it may also suggest that the two new molecules could be used as a chemotaxonomic tool for the characterization of some *Psiadia* species. By NMR spectroscopy, we have also identified a new oxygenated sesquiterpene (3), major constituent of *Melicope obscura* essential oil (Fig. 2.9).

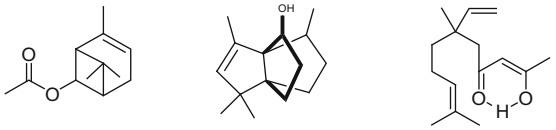

Fig. 2.9 New compounds isolated from *Psiadia* and *Melicope* species

Environment protection and biodiversity conservation requires also a better knowledge of inter-specific interactions between plants and their pollinators. No other plant family shows as wide a range of pollinator-linked floral forms as Orchidaceae, which exhibit pollination systems among most diverse, specialized and complex of all angiosperms. Orchid pollination mechanisms have primarily involved the insect orders Hymenoptera (bees, wasps and ants; these pollinate roughly 60 % of orchid species), Diptera (flies and mosquitoes), Lepdoptera (moths, hawkmoths and butterflies) and Coleoptera (beetles). Approximately 3 % of orchid species are estimated to be pollinated by birds and 5–20 % of species are thought to be self-pollinating. However, in spite of the large size of this model family and a long history orchid pollination biology, the identity and specificity of most orchid pollinators remain inadequately studied, especially in the tropics where the family has undergone extensive diversification. Thus, in order to better understand the reproductive biology of the tropical orchids on Reunion Island, biologists and chemists from The University of Reunion investigated the pollination syndromes i.e. floral morphology, breeding system, pollinator diversity, floral scent profile and fruity success of orchid species of the genera *Angraecum* [37, 38], *Jumellea* [39] and *Bulbophyllum* [40]. Floral scents were studied using headspace solid phase micro-extraction combined with GC-MS analyses. On the basis of this work, it has been demonstrated that one species of *Angraecum* (*A. cadetii*) is pollinated by a highly unexpected pollinator, an undescribed species of raspy cricket (*Glomeremus orchidophilus*, Gryllacrididae). Although Orthoptera are well known for herbivory, this represents the first clearly supported case of orthopteran-pollination.

2.6.2 Commercial Goals

The second objective of the investigation of the LCSNSA on aromatic plants is more commercial. It concerns not only essential oils but also the composition of headspace from fragrant flowers.

Essential Oils

Essential oils are valuable natural products used as raw materials in many fields, including perfumes, cosmetics, aromatherapy, spices and nutrition, etc. Aromatherapy is the therapeutic use of fragrances or volatile compounds to cure, mitigate or prevent diseases and infections by means of inhalation. This has attracted our attention and encouraged us to screen plants to study the biological

activities of their oils from chemical and pharmacological investigations to therapeutic aspects. Hopefully, this will lead to new information on plant applications and new perspective on the potential use of these natural products. Of course, it is clear that only a detailed knowledge of the constituents of the essential oil will allow for a better and specially directed application. Among the aromatic plants investigated as potential biological and pharmacological resources, there are:

Cedrelopsis grevei native to Madagascar is a small tree, known under various common names such as Katafray, Matahora, Bemafaitra and particularly exploited not only for its wood but for its medicinal properties as well. The essential oils extracted from its bark and its leaves and commercially produced by traditional distilleries in Madagascar, are in particular commonly used in folk medicine as fortifying, tonic, relaxing and postnatal medication. The bark essential oil is also used to cure rheumatism and muscular pains and is known to exert antifungal and antibiotic activities. The essential oils from the bark and leaves commercially available have been examined separately [41]. A total of 55 compounds have been identified constituting around 77 % and 92 % of the volatile compounds for respectively bark and leaf essential oils. Both oils were found to have a similar composition rich in terpenes. However the relative percentages of some compounds notably differed. The bark essential oil contained β-pinene, *cis*-sesquisabinene hydrate and caryophyllene oxide as the main components whereas the leaf essential oil was largely dominated by *trans*-β-farnesene, β-pinene, *cis*-sesquisabinene hydrate and *ar*-curcumene.

Ayapana triplinervis commonly known as Ayapana in Hindi, is native to South America and can be found in the Amazon region of Brazil, Ecuador, Peru, the three Guianas, Puerto Rico, Hawaii but is also well represented in other countries such as India, Vietnam and the Mascarene Islands (Reunion, Mauritius, Rodrigues). Since it is widely used in folk medicine, the plant has been extensively investigated for its biological and pharmacological properties. However, till now the information about its chemical composition has remained poor. Concerning more specifically volatile compounds from *A. triplinervis*, few investigations exclusively devoted to essential oil samples of Brazilian, Indian and Vietnamese origins have been carried out. Thus, we decided to investigate the chemical composition of the volatile oil of *A. triplinervis* from Reunion Island, where this plant is locally really appreciated for its healing virtues in particular its digestive properties. Our study [42] includes the analysis of the leaf essential oil of three specimens of *A. triplinervis* collected at two distant locations (North of the island for samples 1 and 2, South of the island for sample 3), in different growth phases (flowering for samples 1 and 3, vegetative for sample 2) in order to elucidate the chemical character of this species on the island and to investigate the relationship between essential oil composition, developmental stage and geographic locations. Analysis by GC-FID and GC-MS enabled us to identify and quantify a total of 39 constituents accounting for 97.1–98.0 % of the oils. The three essential oil samples, all obtained by hydrodistillation, showed a high percentage of the aromatic compound thymohydroquinone dimethyl ether (89.9–92.8 %). All other minor components remained more or less unchanged both qualitatively and quantitatively with respect to the stage of growth. On the contrary, variations were observed with geographic distribution on the island.

The composition of the oils isolated from the three *A. triplinervis* specimens investigated here by us was then compared to those obtained from the same species collected in different geographical sites in the world (Brazil and India). For comparison purposes, only the main components were considered. It appears then a clear geographical diversity in *A. triplinervis* with respect to the chemical composition and the main components of the essential oils. Two chemotaxonomic groups may be established:

Group 1: Species that mainly synthesize the aromatic ether thymohydroquinone dimethyl ether. This was described for species from Brazil and India (Saugar).

Group 2: Species producing principally the oxygenated sesquiterpene selina-4 (15),7(11)-dien-8-one. This is the case of *A. triplinervis* from India (Lucknow).

On this basis, our *A. triplinervis* samples from Reunion Island considerably dominated by thymohydroquinone dimethyl ether (average: 91.2 %) may be included in the group 1. However, in this group, by looking at the other main components, three chemotypes may tentatively be considered: chemotype thymohydroquinone dimethyl ether/α-phellandrene/borneol (India/Saugar); chemotype thymohydroquinone dimethyl ether/β-caryophyllene (Brazil); chemotype thymohydroquinone dimethyl ether (Reunion Island).

Toddalia asiatica (Rutaceae) is an evergreen woody liana native of tropical Asia from India and Sri Lanka to Malaysia; it is also found in temperate Asia, tropical Africa, Madagascar and Mascarene Islands. This species is widely used in folk medicine as remedies for a human variety of ailments such as malaria, stomach complaints and coughs. As a consequence of the ethnobotanical uses, this species has been investigated chemically. So far, alkaloids, coumarins, benzopyranones, terpenoids and cyclohexylamides are the most characteristic compounds. Most of them as well as crude extracts and essential oils of the plant were studied for their pharmacological activities revealing important biological properties: anti-HIV, antimalarial, antiplatelet aggregation, antipyretic, anti-inflammatory, analgesic, antiviral, antimicrobial, spasmolytic, antifeedant, anticancer and skin whitening. Despite this extensive investigation little is known about its essential oil composition. The objective of our study was then to characterize *Toddalia asiatica*, growing wild in Reunion Island, through the composition of its essential oil in order to observe the homogeneity of the composition or, conversely, to evidence a chemical variability among specimens from different geographical regions of the world [43].

Thus, comparing the results obtained for our plant material with those reported for the same species from India, profound differences were revealed in the composition of essential oils: monoterpenes such as linalool (12.4 %), α-borneol (9.4 %), *p*-cymene (8.8 %), α-terpineol (8.6 %) were reported as the characteristic components of Indian specimen, while the two aldehydes, prenal (24.6 %) and 2-hydroxy-3-methyl-but-2-enal (22.8 %) were the most important volatiles of Reunion Island specimen. Moreover, it is well known that aldehydes usually play a significant role in odor composition. So, these two major constituents could be putatively considered as key odorants of *Toddalia asiatica* essential oil principally characterized by choking, pungent, powerful, aldehydic and fruity notes.

Headspace

Floral perfumes continue to inspire perfumers and the headspace technology offers perfumers the option to clone the essence of flowers from which no oils can be extracted such as orchids and has led to a surge of new scents. So, in order to discover new fragrances, which may become a source of inspiration for a new generation of perfumes or home fragrances, we decided to investigate by micro-extraction on solid phase, the headspace of around 100 flowers *in vivo*, from wild or ornamental, endemic or indigenous plants of La Réunion. The results of 60 flowers were selected and given in a book for perfumers (Book ongoing).

2.7 Conclusions

Several practical approaches to plant volatiles analysis are described (isolation techniques, separation and detection techniques, compound identification procedure). More precisely, a selection of sampling methods for volatile compounds that range from conventional, inexpensive, solvent-free, quick sampling methods to innovative methods, as well as an overview of detection and identification methods of volatiles including GC-FID and GC-MS are presented.

Some examples of research projects conducted in LCSNSA are highlighted. In particular, research of bioactive molecules and chemotaxonomic studies concerning volatile compounds generated by essential oils extracted from endemic or indigenous plants of Reunion, Mauritius and Madagascar islands are developed as well as applications in perfume industry and plant-insect interactions dedicated to volatiles found in the airspace (headspace) surrounding flowers.

Such research topics underline the engagement of the LCSNSA to enhance volatile compounds (essential oils and headspace) and to fulfil environment protection and biodiversity conservation.

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