Chapter 23
The Smell of Truffles: From Aroma Biosynthesis to Product Quality

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23.1 Introduction

Truffle fungi (*Tuber* spp.) along with caviar and champagne stand on top of the list of luxury food items. Indeed, in Europe, the retail price of the Périgord truffle *Tuber melanosporum* Vittad. can reach up to 2,000 € per kg, while the Piedmont truffle, *Tuber magnatum* Pico, can reach up to 5,000 € per kg. Additionally, especially large specimens of the latter species have even been sold at auctions for 50,000 € per kg, rendering truffles as expansive as gold. In the USA, local truffle species (i.e., *Tuber lyonii* Butters, *Tuber oregonense* Trappe, Bonito, & P. Rawl.) also sell for thousands of € per kg. These high prices are the result of an undersupplied market and the limited seasonal availability of fresh truffles. Indeed, in Europe and the USA, the most requested truffle sorts are harvested during a few months in autumn and in winter.

What makes truffles so famous, besides their high prices and rarity, is their unique smell. *Tuber melanosporum* can be considered as one of the most aromatic species and it has been defined as a "black diamond of cuisine" due to its potent and complex aroma. It is endemic to Spain, France, and Italy and has been introduced in numerous countries of the southern and northern hemispheres (i.e., in some

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European countries as well the USA, New Zealand, Australia, and Chile (Jeandroz et al. 2008; Bonito et al. 2010; Hall and Haslam 2012; Lefevre 2012; see also Chap. 2). This species is very vulnerable to fraud given that the Chinese truffle *Tuber indicum* Cooke & Massee looks very similar (dark gleba and black peridium) but has a much lower market value. Indeed it is difficult to tell both species apart by traditional morphological observations. Additionally, the aroma of the Chinese species is less intense and complex than the characteristic aroma of *T. melanosporum* (Culleré et al. 2013a). *Tuber brumale* Vittad. is another black truffle with a characteristic musky odor, with "earthy" notes. *Tuber aestivum* Vittad. is a black truffle which can be collected all over Europe (Jeandroz et al. 2008; Splivallo et al. 2012a; see also Chap. 4). It is less aromatic than *T. melanosporum*, although it has a good aroma quality and it is very appreciated by end consumers. Lastly, *T. magnatum*, the most expensive truffle on the market, is often considered as the finest species and has a complex aroma reminiscent of garlic and cheese. It is essentially collected in Italy and in the Carpathian Basin of Central Eastern Europe. Besides the five species briefly described above, about 5–10 additional ones are traded commercially throughout the world.

Truffle aromas are made of a mixture of hydrocarbons containing alcohol, ketone, aldehyde, and ester functional groups but also sulfur atoms. To date, more than 300 volatiles have been described from about eleven species (Splivallo et al. 2011). The aromatic profile of a single species typically contains 30–60 volatile constituents (Bellesia et al. 1998; Díaz et al. 2003; Mauriello et al. 2004; Gioacchini et al. 2005; March et al. 2006; Splivallo et al. 2007). Some of these compounds are not only widespread among truffles but also in other fungi and even plants, while others are much more specific and occur in a few or one truffle sort only. Common truffle volatiles include, for example, 2-methyl-1-butanol and 3-methyl-1-butanol and their respective aldehydes, as well as 1-octen-3-ol (Fig. 23.1), an eight carbon-containing volatile with typical fungal flavor (Combet et al. 2006). Sulfur-containing volatiles such as dimethyl sulfide (Fig. 23.1) and dimethyl disulfide also commonly occur in truffles (Splivallo et al. 2011). By contrast to common truffle volatiles, other compounds are restricted to a few truffle species. This is the case, for example, for the thiophene derivatives 3-methyl-4,5-dihydrothiophene (Fig. 23.1) and 2-methyl-4,5-dihydrothiophene which are only found in *Tuber borchii* Vittad. (Bellesia et al. 2001; Splivallo et al. 2011, 2014). Similarly, the other sulfur volatile 2,4-dithiapentane (Fig. 23.1) occurs in a few species only: it is the dominant constituent of *T. magnatum* and has been reported once in *Tuber panniferum* Tul. & C. Tul. (Mauriello et al. 2004; Splivallo et al. 2011). Yet other volatiles such as 1-methoxy-3-methylbenzene have been only described in the black truffle species *T. aestivum*, *T. brumale*, *Tuber mesentericum* Vittad., and *T. melanosporum* (Díaz et al. 2003; Mauriello et al. 2004; March et al. 2006).

Overall truffles are stand-alone gourmet products on their own rights. This is essentially due to their rarity and to their unique fragrances. Considering that only 10–15 truffle sorts are traded commercially out of the 180 existing species (Bonito

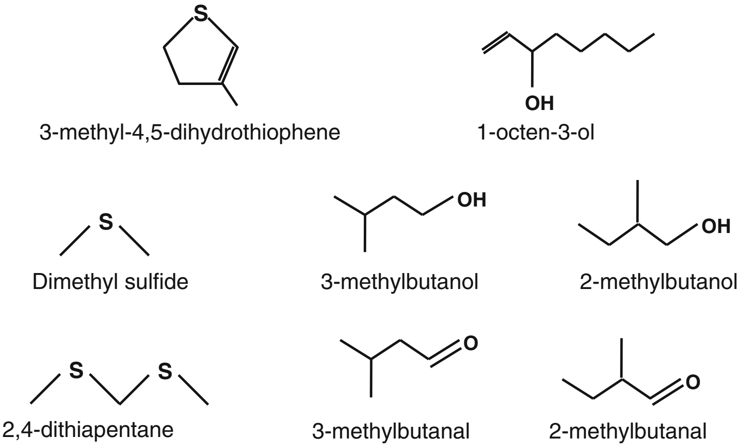

Fig. 23.1 Characteristic volatiles from various truffle species

et al. 2010), truffles represent a valuable and largely unexplored source of aroma compounds.

23.2 Do Truffles or Microbes Produce Truffle Aromas?

Truffles form underground following sexual reproduction between two individuals of opposite mating type (Martin et al. 2010; Rubini et al. 2011). During this process, numerous microbes might end up trapped inside fruiting bodies. As a matter of fact, truffle-fruiting bodies are a hotspot for microbial diversity and host bacteria (Barbieri et al. 2005, 2007; Antony-Babu et al. 2013; Splivallo et al. 2014; see also Chap. 18), yeasts (Buzzini et al. 2005), filamentous fungi (Pacioni et al. 2007; see also Chap. 17), and possibly even viruses (Stielow and Menzel 2010; see also Chap. 20). Bacteria are the dominant group of truffle's microbiomes, the community composition of which have been described in details for *T. melanosporum*, *T. magnatum*, *T. aestivum*, and *T. borchii* in a recent review (Vahdatzadeh et al. 2015). The role of this microbiome in truffle aroma formation has for long been speculative. A historical perspective reveals that Buzzini et al. (2005) demonstrated that yeasts isolated from *T. magnatum* and *T. melanosporum* and grown in pure culture in the presence of L-methionine had the ability to produce some key aroma constituents characteristic of the latter species. The authors therefore hypothesized that yeasts present at the surface of fruiting bodies might fulfill the same role. Indirect evidence further supporting the role of microbes in aroma formation came from the observation that some volatiles characteristic of *T. borchii* fruiting bodies were not found in mycelium grown in pure culture (Tirillini et al. 2000; Splivallo et al. 2007). By contrast, the genome of *T. melanosporum* suggested that truffles might possess all the molecular toolkits necessary to synthesize their volatiles on their own (Martin et al. 2010). This observation was nevertheless speculative since it was based on the presence of genes which function had not been demonstrated (Martin et al. 2010; Splivallo et al. 2011). In 2014, using *T. borchii* as a model organism, the role of the microbiome in truffle aroma formation was finally demonstrated for a group of volatiles (thiophene derivatives) characteristic of the latter species (Splivallo et al. 2014) (Fig. 23.2). The results indicated that inside *T. borchii* fruiting bodies, an unidentified precursor is metabolized into volatile thiophene derivatives only by bacteria and not by truffle mycelium or yeasts. Whether other volatiles characteristic of truffle aromas are produced by truffles or their microbiomes remains to be demonstrated. Based on literature data, common truffle volatiles (i.e., as dimethyl sulfide, dimethyl disulfide) are emitted by mycelial pure cultures (Splivallo and Maier 2011; Liu et al. 2013) but also by numerous yeasts and bacteria (Lemfack et al. 2014). Hence they might hypothetically be produced by both truffles and their microbiomes inside fruiting bodies (Vahdatzadeh et al. 2015). Demonstrating their exact origins will be a challenge considering that microbe-free truffles cannot be currently obtained. A recent meta-analysis about truffle aroma and their microbiomes nevertheless suggests that microbes might play a central role in aroma formation for most, if not all truffle species (Vahdatzadeh et al. 2015).

Regardless of the organismic origin of truffle volatiles, their biosynthesis might follow a scheme which is rather conserved in microbes. Sulfur volatiles such as dimethyl sulfide and dimethyl disulfide and dimethyl trisulfide could be derived from the catabolism of L-methionine (Martin et al. 2010; Splivallo et al. 2011). Two routes might possibly exist in truffles (Splivallo et al. 2011). Methionine could either be converted to methanethiol (MTL) by a C-S lyase or transaminated to 4-methylthio-2-oxobutyric acid, which can be transformed in a series of intermediates eventually leading to MTL. The latter volatile can spontaneously decompose into dimethyl sulfide and dimethyl disulfide. The biosynthetic pathways leading to more complex truffle volatiles (i.e., thiophene derivatives or 2,4-dithiapentane, respectively, characteristic of *T. borchii* and *T. magnatum*) have not been elucidated. L-Methionine remains their hypothetical precursor; however feeding this amino acid to bacteria either grown alone or in the presence of *T. borchii* mycelium did not induce the thiophene derivatives typical of fruiting bodies but only derivatives with related structures (Splivallo et al. 2014). This indicates that a piece of the puzzle is missing and that the biosynthesis of thiophene derivatives might possibly proceed through more complex organismic interactions (i.e., yeasts, bacteria, and truffle).

Other volatiles common to numerous truffle species include branched chain and aromatic hydrocarbons such as 2-methylbutanal, 3-methylbutanal, 2-methylpropanal,, and 2-phenylethanol. Similarly to sulfur volatiles, these might be derived from the catabolism of the specific amino acids through the Ehrlich pathway (Hazelwood et al. 2008; Martin et al. 2010; Splivallo et al. 2011). Indeed feeding isoleucine to *T. borchii* and *T. melanosporum* mycelia induces the production of 2-methylbutanal and 2-methylbutanol; similarly feeding leucine induces 3-methylbutanal and 3-methylbutanol (Splivallo and Maier 2011). Clearly the

Fig. 23.2 Illustration of bacteria eating truffles and emitting thiophene derivatives

identity of the precursors and intermediates should be confirmed by feeding labeled compounds.

The last group of volatiles which are extremely important in truffles and fungi in general is eight carbon-containing volatiles. The most well known is 1-octen-3-ol which occurs as two stereoisomers (R and S forms). Fungi predominantly produce the R form which has also a typical fungal smell compared to the S form which had a moldy, grassy smell (Combet et al. 2006). It is likely that truffles similarly produce the R form even if this has not yet been demonstrated. In mushrooms 1-octen-3-ol is synthesized from linoleic acid which is also the dominant fatty acid in *T. melanosporum* (Harki et al. 2006). Ascomycete fungi might synthesize these volatiles from linoleic acid through a single enzyme known as Ppo (Brodhun et al. 2010). Indeed in the ascomycete mold *Aspergillus nidulans* (Eidam) G. Winter, linoleic acid is transformed through the dioxygenase activity of the Ppo enzyme to 10-hydroperoxyoctadecadienoic acid which might decompose nonenzymatically to 1-octen-3-ol (Garscha and Oliw 2009; Brodhun et al. 2010). The genome of the Périgord truffle *T. melanosporum* suggests that two copies of the Ppo enzymes might exist in truffles. Characterizing the exact pathway will require combining molecular and chemical techniques to fully identify enzymes and precursors.

23.3 Which Factors Influence Truffle Aroma?

As for any other food products of vegetal or fungal origin, truffle aroma is influenced by a series of biotic and abiotic factors. These might, for example, not only include fruiting body maturity (Zeppa et al. 2004), species identity (Mauriello et al. 2004), genotype (Splivallo et al. 2012b; Molinier et al. 2015), and microbial community (Splivallo et al. 2014; Splivallo and Ebeler 2015; Vahdatzadeh et al. 2015) but also soil nutritional content or the identity of the host tree. Unlike with culture intensive crops for which clones are available and for which the influence of these factors can be systematically determined, it is much more difficult to address these questions with truffles. Indeed, trees infested with truffle clones are not yet available commercially, and even within the same orchard, the same genotypes (clones) rarely yield fruiting bodies from one season to the next (Murat et al. 2013; Molinier et al. 2015). This greatly complicates the study of the influence of a single factor on the aroma, since these investigations should be conducted under controlled conditions with the guarantee to collect sufficient truffles for sound statistical analysis. Despite these limitations, studies have been conducted by various groups on truffles collected in natural and artificial truffle orchards and highlighted the possible influence of specific factors. For example, fruiting body maturation has been reported to influence the aroma of *T. borchii*. In fresh fruiting bodies, specific thiophene derivatives were suggested to occur only in fully mature truffles (Zeppa et al. 2004). Recently it has been demonstrated that thiophene derivatives were produced by bacteria colonizing truffle-fruiting bodies

(Splivallo et al. 2014). The observation that the structure of the bacterial communities evolves during maturation in *T. melanosporum* (Antony-Babu et al. 2013) indicates that maturity and microbial community are linked. Furthermore in *T. borchii*, a correlation between the concentration of thiophene derivatives and microbial density has been observed during storage at room temperature (Splivallo et al. 2014). Therefore telling apart the contribution of maturity and bacterial community will only be achieved by studying both factors at the same time in fresh and stored truffles.

Other factors which possibly influence truffle aroma are geographical origin and genotype. Indeed fruiting bodies of *T. magnatum* collected from distinct geographical locations might differ in their aroma profile specifically for terpenoid volatiles (Gioacchini et al. 2008; Federico et al. 2015). Considering that genotypes of truffle-fruiting bodies might not only differ between geographical regions but also from one season to the next, it will be necessary to sample truffles over many seasons to know which factor (i.e., genotype, soil, bacterial community, maturation) determines the production of terpenoids in *T. magnatum*. Indeed in *T. aestivum*, genotype determines the concentration of eight carbon-containing volatiles (Splivallo et al. 2012b; Molinier et al. 2015); therefore this might also be the case in *T. magnatum*. Investigating many specimens for telling apart the contribution of each factor influencing truffle aroma formation will be necessary to understand whether specific aroma constituents can be used as markers of geographical origin.

23.4 Human-Sensed Truffle Aroma

Volatiles responsible for truffle characteristic aromatic blend are alcohols, ketones, aldehydes, aromatic, and sulfur compounds and were first described in the 1980s (Ney and Freitag 1980; Claus et al. 1981). Later, Talou and colleagues (Talou et al. 1987) identified a total of 14 volatiles as black Périgord truffle (*T. melanosporum*) aroma constituents, but none showed the same characteristic as bis(methylthio)methane, the single volatile responsible of the smell of the white truffle *T. magnatum* (Fiecchi et al. 1967). Moreover, a synthetic truffle aroma made up of less than ten volatiles was formulated in 1989 (Talou et al. 1989). According to both sniffing gas chromatography (GC) and sensory analyses carried out in the study with synthetic model mixtures, they concluded that (a) none of the identified compounds was uniquely responsible for the typical aroma of the Périgord truffle and (b) that dimethyl sulfide and 2-methylbutanal, respectively, responsible for sulfurous and pungent notes, had the greatest importance in the final aroma impression.

Volatile sulfur compounds can be naturally generated in oceans, marshes, grounds, and vegetation areas. They can also have an anthropogenic origin in processes such as residual water treatments, composting installations, and recovery plants of organic matter (Smet et al. 1999). For example, the Kraft process used to degrade lignin and produce wood pulp emits as wastes methanethiol, dimethyl sulfide, carbonyl sulfide, and hydrogen sulfide (de Zwart and Kuenen 1992). These sulfur compounds are relevant to food science, since they are present in products such as cheese (Burbank and Qian 2005), beer (Gerbersmann et al. 1995), and wine (Segurel et al. 2004; López et al. 2007; Franco-Luesma and Ferreira 2014). Volatile sulfur compounds can communicate unpleasant smells and tastes often described as rotten eggs (hydrogen sulfide), putrefaction (methanethiol), asparagus (dimethyl sulfide), garlic (diethyl sulfide), cauliflower (dimethyl disulfide), or onion (diethyl disulfide). At small concentrations, however, they are normal components of several food and beverages and can even exert a decisive role on sensory descriptors (Escudero et al. 2007; Mottram and Madruga 1994).

In recent years, some studies have analyzed the volatile compounds of different species of truffles using mainly headspace–solid-phase microextraction (HS–SPME) technique (Pelusio et al. 1995; Bellesia et al. 1998; Díaz et al. 2003, 2009; Mauriello et al. 2004; Gioacchini et al. 2005) and direct headspace analysis (March et al. 2006). The information obtained by gas chromatography–mass spectrometry (GC–MS) analysis is interesting but incomplete since it does not reveal if specific constituents play a role in human-sensed aroma. For this purpose it is indeed necessary to evaluate which of the compounds are in fact important odorants. This can be achieved through sensory analysis using a gas chromatography–olfactometry (GC–O) approach, which essentially relies on the human nose as a detector. On one side GC–O is more time consuming than GC–MS since it requires that a single sample be analyzed by numerous panelists to generate statistically sound results. On the other side GC–O can actually be even more sensitive than an MS detector essentially due to the irreplaceable ability of the human nose to detect specific odorants (i.e., sulfur compounds). Hence GC–MS and GC–O should be regarded as complementary methods.

Techniques in sensory science (i.e., GC–O) have been applied essentially to commercially relevant truffle species. Jansen et al. (2003) characterized and compared the odor profile of a by-product of the black truffle industry to the one of a reference product (truffle, cooking juice). The chemical nature of the compounds responsible for these odors has nevertheless not been identified. Piloni et al. (2005) characterized white truffle aroma by GC–MS and by GC–O analysis and demonstrated that besides bis(methylthio)methane, other sulfur volatiles contributed to the smell of that species. The aromatic compositions of two truffle species (*T. melanosporum* and *T. aestivum*) were compared by GC–O (Culleré et al. 2010). As expected, the olfactogram of *T. melanosporum* was more intense and complex than the one of *T. aestivum*. The aroma emitted by *T. melanosporum* was due to at least 17 different aroma molecules, six of which were reported for the first time: 1-hexen-3-one, 2-methyl-3-furanthiol, furaneol, 3-ethylphenol, 3-propylphenol, and 5-methyl-2-propylphenol. The most important aroma compounds of *T. melanosporum* were 2,3-butanedione, dimethyl disulfide, ethyl butyrate, dimethyl sulfide, 3-methyl-1-butanol, and 3-ethyl-5-ethylphenol. In addition, relevant differences between the aroma profiles from both species were observed. While dimethyl sulfide and dimethyl disulfide and 3-methyl-1-butanol were in both cases among the five most important aroma compounds, the species differed in their content of 2,3-butanedione and ethyl butyrate (higher in *T. melanosporum*) and of methional (higher in *T. aestivum*).

An olfactometric comparison (GC–O) of *T. indicum* with *T. melanosporum* was carried out for the first time by Culleré et al. (2013a). *Tuber melanosporum* aroma was much more intense than the one of *T. indicum*. This might partially explain the higher gastronomical value of *T. melanosporum*. Nevertheless, eight important odorants were identified in *T. indicum*. In order of aromatic significance, these were 1-octen-3-one and 1-octen-3-ol, followed by two ethyl esters (ethyl isobutyrate and ethyl 2-methylbutyrate), 3-methyl-1-butanol, isopropyl acetate, and finally the two sulfides dimethyl disulfide and dimethyl sulfide. Some of these odorants have also been reported in another study on *T. indicum* and on two other Asian truffle species (Liu et al. 2012). Interestingly 1-octen-3-one and 1-octen-3-ol contributed more to the aroma of *T. indicum* than of *T. melanosporum*. By contrast the opposite was true for dimethyl sulfide and dimethyl disulfide. The volatile profiles of both species were also studied by means of headspace–solid-phase microextraction (HS–SPME/GC–MS). This confirmed that the family of C8 compounds (3-octanone, octanal, 1-octen-3-one, 3-octanol, and 1-octen-3-ol) was present at much higher levels in *T. indicum* than in *T. melanosporum*. Therefore either of the two chromatographic methods (GC–O or HS–SPME/GC–MS) could be used as a screening technique to distinguish between *T. indicum* and *T. melanosporum* and thus avoid possible fraud. However, as genotype might influence truffle aroma (Molinier et al. 2015; Splivallo et al. 2012b) and since at least two genetic groups exist for *T. indicum* (see Chap. 2), these results should be validated with *T. indicum* samples belonging to defined genetic groups.

Besides the truffle species described above, sensory techniques were also applied to the whitish truffle *T. borchii*. Interestingly, some of the most important contributors of *T. borchii* aroma were thiophene derivatives and sulfur cyclical compounds, which are derived from truffle's microbiome (Splivallo et al. 2014; Splivallo and Ebeler 2015). This highlights the central role of microbes in human-sensed truffle aroma.

23.5 Truffle Conservation Methods and Substitutes

Distinct preservation methods might affect aroma differently and it is therefore important to evaluate their impact on product quality. Some studies have been carried out in order to examine the influence of the freezing process on the volatile composition of several products, but only a few of them investigated mushrooms (Al-Ruqaie 2006; Jaworska and Bernaś 2009). Al-Ruqaie revealed that freezing might be more effective than drying to conserve the desert truffles *Terfezia claveryi* Chatin and *Terfezia hafizi* Chatin and concluded that blanching in a 4 % NaCl solution and storage at −18 °C proved to be the best preservation method in terms of quality (Al-Ruqaie 2006). Jaworska and Bernaś (2009) proposed a maximum storage period of 4 months for the frozen product in the case of unblanched *Boletus edulis* Bull.

Freezing and frozen storage might affect truffle aroma as well, and this has indeed been recently demonstrated for *T. melanosporum* (Culleré et al. 2013b). Volatile composition data revealed that *T. melanosporum* aromatic profile was deeply modified as a consequence of freezing. These aromatic changes could explain the loss of freshness observed in all frozen truffles. Methional and some phenols appeared as markers of freezing time. Interestingly, 1-octen-3-one was as a general marker of freezing process. After only 24 h of freezing, there was a significant loss of the "characteristic truffle aroma," although intensity was unaffected. Indeed all panelists agreed that none of the frozen samples retained the aroma of fresh truffles. However, the descriptive term "sulfur," usually attributed to the characteristic aroma of truffles, was unaffected by freezing (irrespective of the duration or intensity, −20 or −80 °C). As a matter of fact, the concentration of sulfur volatiles such as dimethyl sulfide and dimethyl disulfide and dimethyl sulfoxide was not affected by freezing, corroborating the sensory results. Overall besides the sulfur attribute, other notes contributed substantially to the typical truffle aroma.

One downside of freezing is that it often destroys food texture. Other "softer" methods have consequently been developed for preservation. Irradiation is recognized as a safe and effective preservation method and it is used to extend the shelf life of raw and processed foods worldwide. The main benefit of irradiation is widely accepted as eliminating microorganisms, insects, or parasites capable of inflicting food spoilage and toxicity, thus replacing chemical fumigants. One downside of food irradiation is that it might decompose fatty acids and subsequently generate off-flavors. Irradiation is nevertheless interesting with truffles since their fruiting bodies are colonized by many microbes (bacteria, yeasts, filamentous fungi, and viruses) and occasionally by insects. Some of these microbes might produce part of truffle aroma; however their uncontrolled growth might also result in off-flavors and spoilage. Indeed the average shelf life of untreated fresh truffles is 10–15 days. In the case of *T. melanosporum*, two irradiation methods were tested for shelf life extension. An electron beam treatment induced important changes in aromatic profile but γ-irradiation at 2.5 kGy did not result in any significant changes. γ-Irradiation therefore constitutes a technique which could potentially be used to increase the shelf life of fresh truffles (Culleré et al. 2012). An alternative method has recently been proposed for *T. magnatum*. It consists of a transparent edible thin film described in a patent application and which is said to double shelf life of truffle-fruiting bodies (Pacioni et al. 2011). But interestingly, traditional methods for conserving fresh truffles are also not equivalent. Indeed simply warping truffles in paper seems more efficient than storing them in rice or under vacuum (Costa et al. 2015).

Overall understanding how truffle aroma is formed through the intimate interactions of truffles with their microbiome will help in developing preservation techniques for shelf-life extension. These techniques should consider the fragile balance between the truffle microbiome and microbes responsible of spoilage/off-flavors and possibly preserve truffle's microbiome but limit the spread of foreign microbes.

23.6 Conclusions

Truffle-fruiting bodies offer a unique niche to investigate from simple to rather complex pathways leading to aroma formation. Techniques in sensory science have complemented traditional profiling approaches by revealing the most important truffle odorants. These studies established the central role of sulfur volatiles in truffle aroma.

Elucidating the pathways leading to characteristic sulfur volatiles as well as the involvement of the different players of truffles' microbiome will require creative thinking. Characterizing these pathways might eventually lead to the biotechnological production of truffle aroma.

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