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Characterisation of the mushroom-like flavour of *Melittis melissophyllum* L. subsp. *melissophyllum* by headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography (GC–FID) and gas chromatography–mass spectrometry (GC–MS)

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ABSTRACT

1-Octen-3-ol is an eight-carbon alcohol responsible for the unique fungal aroma and flavour of edible mushrooms. Among Lamiaceae plants, the highest concentration of this molecule was detected in *Melittis melissophyllum* subsp. *melissophyllum* growing in central Italy. On this basis in the present study a HS-SPME coupled with GC–FID and GC–MS was performed to check the influence of several analytical parameters on the amount of 1-octen-3-ol from the plant matrix. Results showed that 1-octen-3-ol is produced in the plant matrix independently from the harvesting time by an enzyme reaction that is enhanced by the optimisation of the extraction conditions (extraction temperature, 40 °C; water addition, 20 μl; extraction time, 30 min; particle size, 1 mm, sample amount, 30 mg). These findings revealed that *M. melissophyllum* is the first example of a plant, that under appropriate conditions, may be used as a mushroom-like flavouring agent in food products.

1. Introduction

Consumers highly appreciate the characteristic flavour of mushrooms, which consists of hundreds of odourous compounds such as derivatives of octane and octenes, lower terpenes, derivatives of benzaldehyde, sulphur compounds and others (Kalač, 2009). Among them, the main role in producing the unique fungal aroma and flavour has to be ascribed to the eight-carbon alcohol 1-octen-3-ol, discovered for the first time in *Tricholoma matsutake* and first called "matsutake alcohol" (Murahashi, 1938). It occurs with two optically active isomers, the naturally occurring (−) form having a stronger flavour than the (+) form (Zawirska-Wojtasiak, 2004). Its odour could be described as mushroom-like, musty or earthy, while it also possesses a sweet taste; this could be due to the hydroxyl group, which is close to the electronegative double bond (Shallenberger & Acree, 1967).

This alcohol is produced mainly in the cap and gills rather than in the stipe of many edible mushrooms, and its characteristic flavour is obtained during the physical disruption of the edible mushroom tissues (Wurzenberger & Grosch, 1983).

As for many eight-carbon volatiles emitted by mushroom sporophores, 1-octen-3-ol plays an ecological role in attracting flies and mosquitoes, which are helpful in the reproduction process to disseminate spores, or as a defense mechanism against predator or fungal insect pests (Combet, Henderson, Eastwood, & Burton, 2006).

Like many molecules contributing to the aroma of food products, the volatile flavour threshold of 1-octen-3-ol is very low: 1 ppb in water, 0.1 ppm in skim milk and butter fat, 0.5–1.0 ppm in soy milk (Badenhop & Wilkens, 1969; Evans, Moser, & List, 1971; Stark & Forss, 1964). Because of this, it is possible to detect 1-octen-3ol, apart from in edible mushrooms, in the aroma of many food products and beverages, as reported in Table 1.

1-Octen-3-ol has been included in the food additive database of the Food and Drug Administration (FDA) (US FDAs Center for Food Safety and Applied Nutrition, 2008), and in the Codex Alimentarius of FAO and WHO (http://www.codexalimentarius.net/download/standards/9/CXA_006e.pdf) to be used as a flavouring agent in the food industry.

The biosynthesis of 1-octen-3-ol in mushrooms is due to the aerobic oxidation of linoleic acid by lipoxygenase (LOX) into regio- and stereo-specific hydroperoxides (HPODs), followed by an enzymic cleavage by hydroperoxide lyase (HPL) of the corresponding HPODs to produce 1-octen-3-ol (Assaf, Hadar, & Dosoretz, 1997).

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Table 1
Food products and beverages whose aroma contains 1-octen-3-ol (data obtained from literature survey).

Food	Apricot, asparagus, avocado, bacon, beef, bread, bullfrog legs, caviar, cheese, cherry, chestnuts, chicken, crabs, cuttlefish, dairy products, duck, fish, French fries, frying oils, gilthead sea bream fish, goat, grana cheese, grape berries, ham, honey, lamb, linseed oil, loin, melon, milk, mussels, oysters, parma ham, pasta, peaches, pesto Genovese, porcine liver, pork, potato snacks, prawns, red bell peppers, rice, salami, salmon, sardine, sausages, sea scallop, shea butter, soy milk, soybean, sunflower oil, trout, truffles, vanilla beans, walnut, wheat semolina
Beverages	Black tea, brandy, coffee, red wines, orange essence oil, rum, whiskeys, white wines

Lipoxygenase is widely distributed in animals and plants and the LOX products have a wide range of biological functions, such as diverse signal molecules, oxidants, and modifiers of membrane structures. They present an economic interest for the food industry in that they are at the basis of the biochemical pathway of flavour volatiles formation.

The presence of 1-octen-3-ol in plants was observed for the first time in the 40s in various kinds of mint and lavender essential oils (Crabalona, 1944; Naves, 1943). Since then, many Lamiaceae essential oils were found to contain this molecule. However, to the best of our knowledge the plant essential oil which contains the highest percentage of 1-octen-3-ol is that of *Melittis melissophyllum* L. subsp. *melissophyllum* from central Italy, that was studied in previous investigations (Maggi et al., 2009). These, high concentrations, obtained by hydrodistillation, were probably not a true representation of the organism volatile profile, since 1-octen-3-ol was not detected as such in the plant material headspace; this suggests that high temperatures and moist environment are responsible for favouring some hydrolytic/oxidative reactions, which yield a high level of this volatile.

On this basis, we decided to investigate the occurrence of 1-octen-3-ol in the headspace of the plant material by SPME. This technique, developed by Pawliszyn and co-workers (Arthur & Pawliszyn, 1990), is a sample preparation technique incorporating extraction, concentration and sample introduction in a single step, using a fused silica fibre that is coated on the outside with an appropriate stationary phase. The method saves preparation time, solvent purchase and disposal costs, and can allow the analysis of very small amounts of plant material avoiding collection of the large amounts needed for extractions. It is a powerful tool when combined with gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) analysis, which yields high accuracy and precision on the volatile species released at a given time, without disrupting the sample.

Therefore, the goal of this work was the use of SPME technique, coupled with GC–FID and GC–MS, to study the effect of several parameters, (extraction temperature, water addition, extraction time, particle size, sample amount, and collection time), on the production of 1-octen-3-ol from *M. melissophyllum* subsp. *melissophyllum* plant material, in order to maximise the yield, and to emphasise the possible application of the plant as a mushroom flavouring additive in food processing. So far, the present work represents the only comprehensive study of the extraction of 1-octen-3-ol from the headspace of a plant.

2. Materials and methods

2.1. Plant material

Aerial parts of *M. melissophyllum* subsp. *melissophyllum* (450 g) were collected at flowering in May 2008 and after flowering in June 2008 in a woody place sited in Piedilapiaggia (central Italy, N 43°09′10″ E 13°07′18″, 590 m above sea level). Part of plant material was air-dried for 1 week (50 g), and protected from the light, while the remaining (400 g) was used as fresh. A voucher specimen was identified by Dr. Maggi using available literature and deposited in the Herbarium Camerinensis (acknowledge in the Index Herbariorum) of Dept. of Environmental Sciences, Sect. of Botany and Ecology, University of Camerino (Italy), under the accession code CAME 13430; it is also available at the following website: http://erbariitaliani.unipg.it. For HS-SPME analysis, dry material was grounded with a blender MFC model DCFH 48 IKA-WERK (Staufen, Germany) using sieves of 1, 1.5 and 2 mm size in diameter, respectively, whilst for hydrodistillation fresh material was used as such.

2.2. Chemicals

The following standards, purchased from Sigma–Aldrich (Milan, Italy), were used for identification: α-pinene, benzaldehyde, β-pinene, 1-octen-3-ol, p-cymene, limonene, terpinolene, linalool, verbenone, (E)-caryophyllene, coumarin, α-humulene, (E)-β-ionone, caryophyllene oxide, hexahydrofarnesyl acetone. For retention index determination, a mix of hydrocarbons ranging from octane (C_8) to triacontane (C_{30}) (Supelco, Bellefonte, PA, USA) was used and run at the experimental conditions reported below. For the determination of GC–FID response factors, the following standards purchased from Sigma–Aldrich (Milan, Italy) were used as well: 1-octen-3-ol for aliphatic alcohols, octanal and dodecanal for aliphatic aldehydes, bornyl acetate and bornyl isovalerate for esters, octane and octadecane for alkanes, benzaldehyde and p-vinylanisole for aromatics, β-pinene, limonene, γ-terpinene and p-cymene for monoterpene hydrocarbons, 1,8-cineole, linalool, camphor, terpinen-4-ol, verbenone, nerol and carvone for oxygenated monoterpenes, (E)-caryophyllene and α-humulene for sesquiterpene hydrocarbons, caryophyllene oxide for oxygenated sesquiterpenes. All compounds were of analytical standard grade. Analytical grade hexane solvent was purchased from Carlo Erba (Milan, Italy); it was distilled by a Vigreux column before use.

2.3. SPME fibre screening

A preliminary screening of several types of coating fibres of various polarity and retention capability was carried out in order to select the best type in terms of extraction efficiency and reproducibility for 1-octen-3-ol. The screening was conducted using the following analytical conditions: extraction temperature, 60 °C; water addition, 60 μl; extraction time, 30 min; particle size, 1 mm; amount of plant material, 30 mg; desorption time, 3 min. Five fibres of increasing polarity were tested and compared for 1-octen-3-ol headspace amount: polydimethylsiloxane (PDMS, 100 μm), carboxen™-polydimethylsiloxane (CAR-PDMS, 75 μm), Stableflex™ divinylbenzene-carboxen™-polydimethylsiloxane (DVB-CAR-PDMS, 50/30 μm), polydimethylsiloxane-divinylbenzene (PDMS-DVB, 65 μm) and carbowax®-divinylbenzene (CW-DVB, 65 μm). The silica fibres and the manual SPME holder were purchased from Supelco (Bellefonte, PA, USA). The coating of all fibres was 1 cm long. Before GC–FID and GC–MS analysis, each fibre was conditioned in the injector of the GC system, according to the instructions provided by the manufacturer.

2.4. Headspace solid-phase microextraction (HS-SPME)

Dry plant material (flowering aerial parts) was hermetically sealed in a 4 ml vial with a polypropylene hole cap and PTFE/silicone septa (Supelco, Bellefonte, PA, USA). Then, the SPME device

was inserted into the sealed vial by manually penetrating the septum and the fibre was exposed to the plant material headspace during the extraction time. In order to enhance the mushroom-like flavour from plant material, the following experimental parameters were optimised by a single-factor SPME analysis: extraction temperature (20–80 °C), water addition (0–200 μl), extraction time (1–60 min), particle size (1–2 mm), amount of plant material (5–60 mg). In addition, to verify the influence of the phenological cycle on the amount of 1-octen-3-ol released from the matrix, a collection of the no flowering aerial parts was performed as well in June and analysed by using the previously optimised conditions. For each investigated parameter, analysis was conducted in triplicate. For all measurements RSD% (relative standard deviation) values for 1-octen-3-ol ranged from 1% to 19%. After sampling, the SPME device was immediately inserted into the GC injector. A desorption time of 3 min at 250 °C was sufficient to desorb most of the analytes from the fibre. No reconditioning was needed for each fibre before sampling.

2.5. Hydrodistillation (HD)

Fresh aerial parts (400 g) were subjected to hydrodistillation for 3 h in a Clevenger-type apparatus, adding 3 ml of hexane to entrap volatiles. The obtained essential oil was dried over anhydrous sodium sulphate, then the solvent was evaporated under N_2 flow. Afterwards, it was stored in sealed vials protected from the light at −20 °C before GC–FID and GC–MS analyses. Hydrodistilled oil had a typical mushroom-like smell and yellow colour. Three oils were obtained by HD of the starting material and subsequently analysed by GC–FID and GC–MS. The oil yield (w/w, 0.02%) was estimated on a dry weight basis, drying the plant material in a stove at 110 °C for 24 h.

2.6. GC–FID and GC–MS analysis

GC–FID analysis of the volatile components was carried out using an Agilent 4890D instrument coupled to an ionisation flame detector (FID). Compounds were separated on a HP-5 capillary column (5% phenylmethylpolysiloxane, 25 m, 0.32 mm i.d.; 0.17 μm film thickness) (J&W Scientific, Folsom, CA, USA), with the following temperature program: 5 min at 60 °C, subsequently 10 °C/min (4 °C/min for essential oil) up to 220 °C, then 20 °C/min (11 °C/min) up to 280 °C, held for 15 min; injector and detector temperatures, 250 °C (280 °C for essential oil); carrier gas, helium (1.4 ml/min); injection volume of 1 μl; splitless mode for SPME, split mode for essential oil with a ratio of 1:34; run time: 42 min (SPME analysis), 65 min (essential oil analysis). A mixture of aliphatic hydrocarbons (C_8–C_{30}) (Sigma, Milan, Italy) diluted in hexane, was directly injected into the GC injector. For SPME analysis the same mixture was introduced in a 4 ml vial and then the solvent was removed under a N_2 flow. The mixture of alkanes was then injected using the above temperature program in order to calculate the retention indices (as Kovats indices) of each extracted compound.

GC–MS analysis was performed using an Agilent 6890N gas chromatograph coupled to a 5973N mass spectrometer equipped with a HP-5MS capillary column (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 μm film thickness) (J&W Scientific, Folsom, CA, USA), using the same program reported above; carrier gas: helium; flow rate: 1.0 ml/min; injector and transfer line temperatures: 250 °C (280 °C for essential oil); injection volume: 2 μl; splitless mode for SPME, split mode for essential oil with a ratio of 1:50; scan time: 42 min (SPME analysis), 65 min (essential oil analysis); acquisition mass range: 29–400 *m/z*. All mass spectra were acquired in electron-impact (EI) mode with an ionisation voltage of 70 eV.

2.7. Identification of 1-octen-3-ol and other volatiles

1-Octen-3-ol was identified by comparing the retention time, retention index and mass spectrum of the chromatographic peak with that of standard. Twenty components out of 60 in the headspace and 27 components out of 55 in the essential oil were identified by means of standards as well. Otherwise the peak assignment was based on computer matching with the WILEY275, NIST 08, ADAMS and a home-made (based on the analyses of reference oils and commercially available standards) library, taking into account the coherence of the retention indices of the analysed compounds with those reported by Adams (2007) and NIST 08 library (2008).

2.8. Quantification of volatiles

The relative amounts of volatile components, expressed as percentages, were obtained by FID peak-area internal normalisation, by calculating the response factor (RF) of the FID for different classes of volatiles occurring in the headspace and essential oil of *M. melissophyllum*, according to a method adopted in a previous work (Maggi et al., 2010).

2.9. Statistical analysis

Analysis of variance (ANOVA) was performed by SPSS (v. 13.0) software package for Windows (SPSS Inc., Chicago, IL, USA). Values of $p \leqslant 0.05$ were considered as statistically significant.

3. Results and discussion

3.1. SPME fibre screening

In this study, five fibres, i.e. polydimethylsiloxane (PDMS, 100 μm), carboxen™-polydimethylsiloxane (CAR-PDMS, 75 μm), Stableflex™ divinylbenzene-carboxen™-polydimethylsiloxane (DVB-CAR-PDMS, 50/30 μm), polydimethylsiloxane-divinylbenzene (PDMS-DVB, 65 μm) and carbowax®-divinylbenzene (CW-DVB, 65 μm) were evaluated for the analysis of the mushroom-like aroma of *M. melissophyllum* subsp. *melissophyllum*. The fibre screening, conducted under the cited experimental conditions (extraction temperature, 60 °C; water addition, 60 μl; extraction time, 30 min; particle size, 1 mm; amount of plant material, 30 mg; desorption time, 3 min), was based on sensitivity and reproducibility for 1-octen-3-ol. The results, reported in Fig. 1, showed that the PDMS and CAR-PDMS fibres produced the best results in terms of relative percentage (11.4% and 32.0%, respectively) and RSD% (7.9% and 4.9%, respectively) for the investigated compound. In particular, the 75 μm CAR-PDMS fibre showed better extraction efficiency for 1-octen-3-ol than the 100 μm PDMS owing to its higher polarity and stronger retention capability (Kataoka, Lord, & Pawliszyn, 2000). In fact, the CAR-PDMS fibre has a porous, bipolar coating with a higher relative signal response to low relative molecular mass volatiles in the C_2–C_{12} range (Perera, Marriot, & Galbally, 2002). However, this fibre had significant carryover from the prior extraction, due to larger molecules (>C_{12}) retained on the surface of the Carboxen particle and imbedded in the coating, therefore difficult to desorb. To avoid an extra-cleaning step before each sample injection that is time-consuming for routine analysis, the PDMS fibre, characterised by a shorter desorption time and without carryover from prior extraction, was chosen for the analysis. In fact, although PDMS is a non-polar fibre, it can be applied successfully to more polar compounds, particularly after optimising extraction conditions (Pawliszyn, 1997).

3.2. Optimisation of HS-SPME method

3.2.1. Extraction temperature

Temperature is a very important parameter to be optimised. In general, an increase in extraction temperature translates to an increase of analytes concentration in the headspace. However, in the case of 1-octen-3-ol, the enzymatic origin of the volatile has to be taken into account. In fact, it is known that lipoxygenase catalysing the oxidation of linoleic acid to 1-octen-3-ol is heat-sensitive (Assaf et al., 1997). Therefore, a high temperature could led to a decline in enzyme activity decreasing the formation of 1-octen-3-ol. On the other hand, higher temperatures could shift the equilibrium towards headspace leading to a decrease of adsorbed volatile by fibre. In the experiments six different temperatures were tested: 20, 30, 40, 50, 60 and 80 °C. The results, reported in Fig. 2a, indicated that 40 °C (with other parameters set as: water addition, 60 μl; extraction time, 30 min; particle size, 1 mm; amount of plant material, 30 mg; desorption time, 3 min) is the best extraction temperature for 1-octen-3-ol. This result was in line with that reported in literature for *Agaricus bisporus* (Liu, Zhou, Zeng, & Ouyang, 2004). In fact, it is known that the mushroom flavour is kept at a high level as the temperature increases in the range of 20–40 °C but it is weakened if the temperature is higher than 40 °C because of the decline in enzyme activity.

3.2.2. Water addition

Water has proven to be a very effective additive to facilitate the release of analytes from the matrix and it is often used to accelerate extraction (Zhang & Pawliszyn, 1995). For this reason, experiments were conducted exposing the PDMS 100 μm fibre to 30 mg of sample at 40 °C for 30 min, adding increasing quantities of water (0, 5, 20, 40, 100 and 200 μl). As shown in Fig. 2b, the added water influenced the production of 1-octen-3-ol, that was greatly enhanced by adding 20 μl of water (with other parameters set as: extraction temperature, 40 °C; extraction time, 30 min; particle size, 1 mm; amount of plant material, 30 mg; desorption time, 3 min). Also in this case our results are in accordance with those reported in literature (Ram & Seitz, 1999), where it is reported that added water enhances the recovery from grains of polar compounds such as alcohols and aldehydes, notably increasing the extraction of 1-octen-3-ol. 1-Octen-3-ol was not produced without water, meaning that a moist environment is strictly needed for the enzyme activity. Finally, it is interesting to note that the amount of 1-octen-3-ol strongly decreased as the added water exceeded 100 μl. This could be explained by a decrease of evaporation of volatiles from the plant matrix as the water increased (MacGillivray, 1999).

3.2.3. Extraction time

To investigate the time required for reaching the equilibrium between the stationary phase (fibre), the headspace of the vial and the studied analyte, six extraction times, i.e. 1, 5, 15, 30, 45 and 60 min, were tested at 40 °C adding 20 μl of water. The results are reported in Fig. 2c. To reach the right equilibration time, 30 min (with other parameters set as: extraction temperature, 40 °C; water addition, 20 μl; particle size, 1 mm; amount of plant material, 30 mg; desorption time, 3 min) were needed. In this time it is possible to extract the highest quantity of 1-octen-3-ol. These results are in line with those reported in literature (Husson, Bompas, Kermasha, & Belin, 2001; Liu et al., 2004), which have demonstrated that the maximum production of 1-octen-3-ol in *A. bisporus* was obtained within the first 40 min, during which the enzyme activity is the highest.

3.2.4. Particle size

When SPME is applied to the analysis of the solid sample, sample particle size often plays an important role in the extraction process. In general, as the material decreases in particle size, the more efficient the extraction of analytes from the matrix is. We studied three different sample particle sizes, i.e. 1, 1.5 and 2 mm, using 30 mg of plant material, with 20 μl of water heating at 40 °C for 30 min. The results are reported in Fig. 2d. From the profile of curve referred to 1-octen-3-ol, the minimum particle size, 1 mm (with the other parameters set as: extraction temperature, 40 °C; water addition, 20 μl; extraction time, 30 min; amount of plant material, 30 mg; desorption time, 3 min), allowed us to obtain the best re-

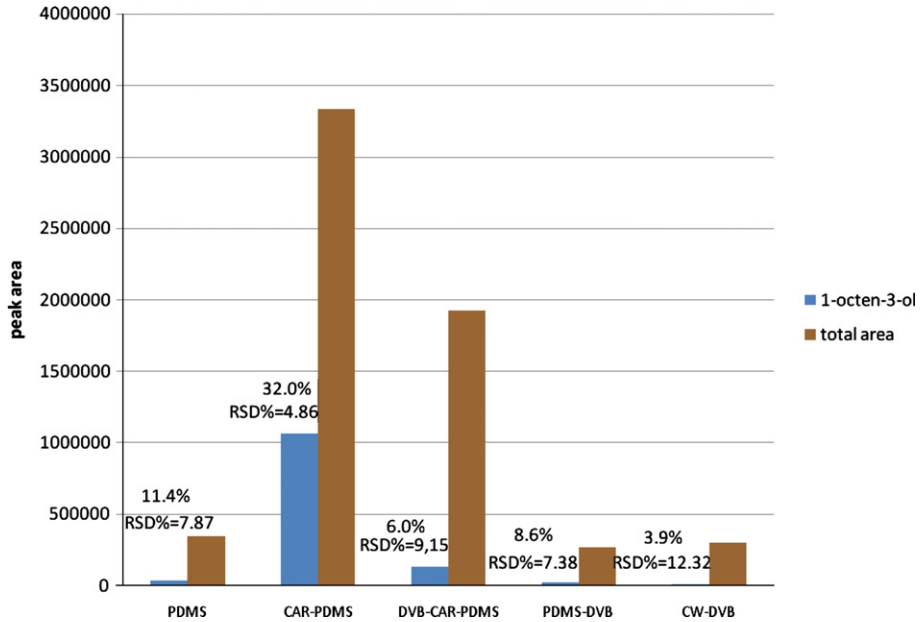

Fig. 1. Uptake of 1-octen-3-ol and total volatiles by five types of SPME fibre coating (with relative peak area % and RSD% for 1-octen-3-ol) under the following analytical conditions: extraction temperature, 60 °C; water addition, 60 μl; extraction time, 30 min; particle size, 1 mm; sample amount, 30 mg; desorption time, 3 min. Data, obtained by GC–FID analysis, are means of three determinations with the following standard deviations: 0.7 (PDMS), 7.7 (CAR-PDMS), 2.9 (DVB-CAR-PDMS), 1.5 (PDMS-DVB), 0.4 (CW-DVB).

sults in terms of extracted analyte, while larger sizes (especially 2 mm) exhibited worse results. There was no significant difference in the amount of 1-octen-3-ol obtained with 1 and 1.5 particle size ($p > 0.05$). However, we preferred to choose material of 1 mm in particle size because of the higher reproducibility of the results (RSD for 1-octen-3-ol of 1.17% instead of 6.61%).

3.2.5. Sample amount

In this work we tested four different amounts of plant material, i.e. 5, 10, 30 and 60 mg grounded with 1 mm mesh filter, at 40 °C for 30 min, adding 20 μl of water. The results are summarised in Fig 2e. As expected, the peak area of 1-octen-3-ol slightly increased with sample amount up to 30 mg (with the other parameters set as: extraction temperature, 40 °C; water addition, 20 μl; extraction time, 30 min; particle size, 1 mm; desorption time, 3 min), while it decreased by using 60 mg. This decrease, by using 60 mg of material, can be explained with the fact that water added to the matrix (20 μl) is not sufficient to efficiently extract analyte from the increased material. There was no significant difference in the amount of 1-octen-3-ol obtained with 10 and 30 mg ($p > 0.05$). However,

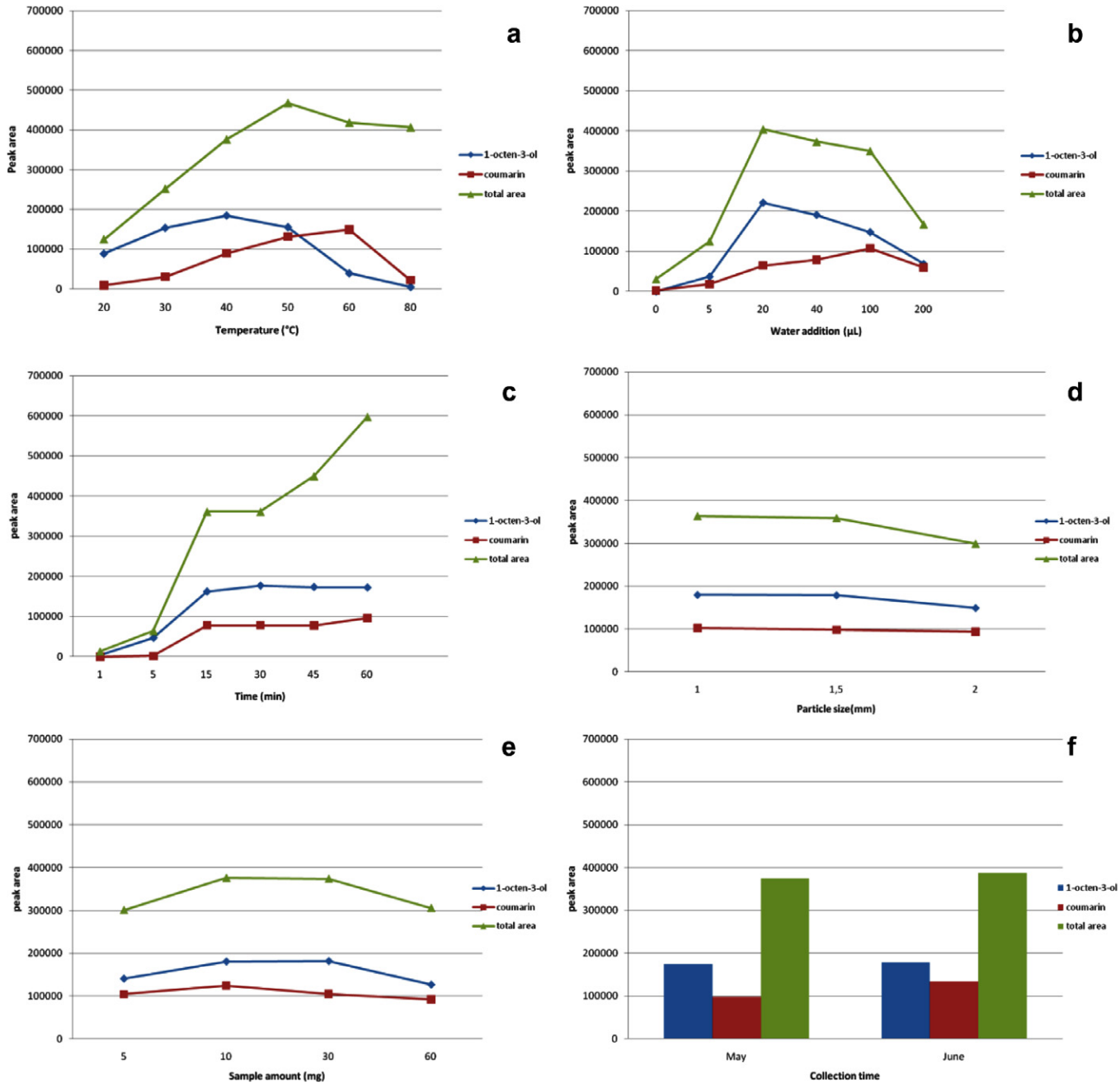

Fig. 2. Effect of temperature, extraction time, water addition, particle size, sample amount and harvesting time on the peak area of 1-octen-3-ol captured by PDMS fibre. (a) Extraction time 30 min, water addition 60 μl, particle size 1 mm, sample amount 30 mg, desorption time 3 min; (b) extraction time 30 min, extraction temperature 40 °C, particle size 1 mm, sample amount 30 mg, desorption time 3 min; (c) extraction temperature 40 °C, water addition 20 μl, particle size 1 mm, sample amount 30 mg, desorption time 3 min; (d) extraction time 30 min, extraction temperature 40 °C, water addition 20 μl, sample amount 30 mg, desorption time 3 min; (e) extraction time 30 min, extraction temperature 40 °C, water addition 20 μl, particle size 1 mm, desorption time 3 min; (f) extraction time 30 min, extraction temperature 40 °C, water addition 20 μl, particle size 1 mm, sample amount 30 mg, desorption time 3 min. All the experiments were performed in triplicate with RSD% values in the following ranges: 8–19% (a), 5–19% (b), 5–19% (c), 1–15% (d), 7–12% (e), and 7–14% (f). For sake of clarity, the curve referred to coumarin and total volatiles is also reported.

Table 2
Headspace composition of *Melittis melissophyllum* subsp. *melissophyllum*.

No.	Compounds[a]	RF[d]	RI HP-5[b]	RI LIT[c]	Peak area (%) ± SD[e]	Peak area (%) ± SD[f]	ID[g]
	Aliphatics				16.5	62.5	
	Alcohols	1.5			12.4	58.2	
1	1-Octen-3-ol		983	979	12.1 ± 0.6	56.3 ± 3.4	MS,RI,std
2	3-Octanol		998	991	0.2 ± 0.0	1.9 ± 0.0	MS,RI
3	Undecanol		1364	1370	0.1 ± 0.0	tr[h] ± 0.0	MS,RI
	Aldehydes and chetons	1.7			1.7	4.2	
4	(2E)-hexenal		856	855	0.2 ± 0.0	0.2 ± 0.0	MS,RI
5	(2E,4E)-heptadienal		1006	1007	tr ± 0.0	2 ± 0.6	MS,RI
6	(2E)-octen-1-al		1067	1054	tr ± 0.0	0.2 ± 0.0	MS,RI
7	Nonanal		1112	1100	0.1 ± 0.0	0.3 ± 0.0	MS,RI
8	(2E,6Z)-nonadienal		1159	1154	0.5 ± 0.1	0.3 ± 0.1	MS,RI
9	Decanal		1205	1199	0.2 ± 0.0	0.4 ± 0.0	MS,RI
10	Undecanal		1305	1306	tr ± 0.0	tr ± 0.0	MS,RI
11	Dodecanal		1404	1408	0.3 ± 0.0	0.1 ± 0.0	MS,RI
12	3,5-Octadien-2-one		1078	1078	0.4 ± 0.1	0.8 ± 0.1	MS,RI
	Alkanes	1.0			2.4	0.1	
13	Tridecane		1300	1300	0.1 ± 0.0	tr ± 0.0	MS,RI,std
14	Octadecane		1800	1800	0.1 ± 0.0	tr ± 0.0	MS,RI,std
15	Heneicosane		2100	2100	0.3 ± 0.0	0.1 ± 0.0	MS,RI,std
16	Docosane		2200	2200	0.1 ± 0.0	tr ± 0.0	MS,RI,std
17	Tricosane		2300	2300	0.5 ± 0.4	tr ± 0.0	MS,RI,std
18	Pentacosane		2485	2500	0.3 ± 0.2	tr ± 0.0	MS,RI,std
	Aromatics	1.7			0.3	0.5	
19	Benzaldehyde		966	960	0.1 ± 0.0	0.3 ± 0.0	MS,RI
20	Benzene acetaldehyde		1054	1042	0.2 ± 0.1	0.2 ± 0.0	MS,RI
	Phenols	1.7			50.9	18.7	
21	Methyl salicylate		1196	1191	tr ± 0.0	0.1 ± 0.0	MS,RI
22	Coumarin		1447	1434	50.9 ± 3.4	18.6 ± 3.0	MS,RI,std
	Terpenoids				57.0	13.4	
	Monoterpene hydrocarbons	1.1			6.4	4.8	
23	α-Thujene		928	930	tr ± 0.0	tr ± 0.0	MS,RI
24	α-Pinene		934	939	5.9 ± 3.9	4.1 ± 2.3	MS,RI,std
25	β-Pinene		977	979	0.4 ± 0.2	0.3 ± 0.1	MS,RI,std
26	p-Cymene		1027	1024	tr ± 0.0	0.1 ± 0.1	MS,RI,,std
27	Limonene		1031	1029	0.1 ± 0.0	0.2 ± 0.1	MS,RI,std
28	Terpinolene		1090	1088	tr ± 0.0	0.1 ± 0.1	MS,RI
	Oxygenated monoterpenes	1.5			4.9	4.3	
29	Linalool		1106	1096	0.2 ± 0.0	0.5 ± 0.0	MS,RI,std
30	α-Campholenal		1130	1126	0.1 ± 0.0	0.1 ± 0.0	MS,RI
31	Trans-pinocarveol		1143	1139	0.2 ± 0.1	0.5 ± 0.0	MS,RI
32	Trans-verbenol		1150	1144	0.4 ± 0.2	0.3 ± 0.0	MS,RI
33	Pinocarvone		1167	1164	0.3 ± 0.0	0.4 ± 0.0	MS,RI
34	p-Cymen-8-ol		1190	1182	0.1 ± 0.0	0.1 ± 0.0	MS,RI
35	Myrtenol		1198	1195	0.1 ± 0.0	0.2 ± 0.0	MS,RI,std
36	Safranal		1202	1196	0.1 ± 0.0	0.1 ± 0.0	MS,RI
37	Verbenone		1213	1205	0.2 ± 0.0	0.2 ± 0.0	MS,RI,std
38	Cis-chrysanthenyl acetate		1263	1265	3.1 ± 0.3	1.9 ± 0.1	MS,RI
39	Isobornyl acetate		1287	1285	0.1 ± 0.0	tr ± 0.0	MS,RI
	Sesquiterpene hydrocarbons	1.1			4.4	2.1	
40	α-Cubebene		1349	1348	0.2 ± 0.0	0.1 ± 0.0	MS,RI
41	α-Copaene		1375	1376	0.7 ± 0.1	0.3 ± 0.0	MS,RI,std
42	β-Bourbonene		1384	1388	0.1 ± 0.0	tr ± 0.0	MS,RI
43	β-Cubebene		1389	1388	0.1 ± 0.0	tr ± 0.0	MS,RI
44	(E)-caryophyllene		1419	1419	0.9 ± 0.3	0.2 ± 0.l	MS,RI,std
45	α-Trans-bergamotene		1435	1434	0.1 ± 0.0	tr ± 0.0	MS,RI
46	α-Humulene		1455	1454	0.3 ± 0.5	0.7 ± 0.1	MS,RI,std
47	γ-Gurjunene		1474	1477	0.7 ± 0.0	0.3 ± 0.0	MS,RI
48	γ-Muurolene		1478	1479	0.5 ± 0.0	0.2 ± 0.0	MS,RI
49	Germacrene D		1483	1485	0.2 ± 0.0	0.2 ± 0.0	MS,RI
50	Cis-calamenene		1526	1529	0.6 ± 0.5	0.1 ± 0.0	MS,RI
	Oxygenated sesquiterpenes	1.3			5.2	0.9	
51	Caryophyllene oxide		1585	1583	2.6 ± 0.1	0.1 ± 0.0	MS,RI,std
52	Caryophylla-4(12),8(13)-dien-5α-ol		1642	1640	0.6 ± 0.1	0.4 ± 0.1	MS,RI
53	14-Hydroxy-(Z)-caryophyllene		1664	1669	0.6 ± 0.1	0.2 ± 0.0	MS,RI
54	2-Pentadecanone, 6,10,14-trimethyl		1845	1846	1.4 ± 1.2	0.2 ± 0.1	MS,RI
	Oxygenated diterpenes	1.2			0.1	tr ± 0.0	
55	13-Epi-manool oxide		2011	2010	0.1 ± 0.1	tr ± 0.0	MS,RI
	C_{13}-Norisoprenoids	1.1			3.6	1.3	
56	β-Cyclocitral		1223	1219	0.2 ± 0.0	0.2 ± 0.0	MS,RI
57	2-Undecanone, 6,10-dimethyl		1400	1400	0.1 ± 0.0	tr ± 0.0	MS,RI

Table 2 (*continued*)

No.	Compounds[a]	RF[d]	RI HP-5[b]	RI LIT[c]	Peak area (%) ± SD[e]	Peak area (%) ± SD[f]	ID[g]
58	(E)-α-ionone		1429	1430	0.1 ± 0.0	tr ± 0.0	MS,RI
59	(E)-β-ionone		1487	1488	1.9 ± 0.4	0.6 ± 0.0	MS,RI,std
60	Dihydroactinidiolide		1536	1532	1.3 ± 0.2	0.5 ± 0.0	MS,RI
	Total identified (%)				92.5 ± 0.5	95.7 ± 0.6	

[a] Compounds belonging to each class are listed in order of their elution from a HP-5 column; percentage values are means of three determinations ± standard deviation; they were obtained at FID by peak-area normalisation calculating the relative response factor.
[b] Retention index on HP-5 column, experimentally determined using homologous series of C_8–C_{30} alkanes.
[c] Relative retention index taken from Adams (2007) and/or NIST 08 (2008).
[d] Relative response factor of FID detector.
[e] Initial conditions: extraction time 30 min, extraction temperature 60 °C, water addition 60 μl, particle size 1 mm, sample amount 30 mg, desorption time 3 min.
[f] Optimised conditions: extraction time 30 min, extraction temperature 40 °C, water addition 20 μl, particle size 1 mm, sample amount 30 mg, desorption time 3 min.
[g] Identification methods: MS, by comparison of the mass spectrum with those of the computer mass libraries Wiley, Adams (2007) and NIST 08 (2008); RI, by comparison of RI with those reported in literature (Adams, 2007; NIST, 2008); std, by comparison of the retention time and mass spectrum of available authentic standard.
[h] tr, traces (mean value below 0.1%).

we preferred to choose 30 mg because of the higher reproducibility of the results (RSD for 1-octen-3-ol of 6.61% instead of 7.99%).

3.2.6. Collection time

To investigate the influence of the phenological cycle on the amount of 1-octen-3-ol released from the plant matrix, a collection of the plant material after flowering period (June) was performed as well. Analysis was conducted with the following analytical conditions: extraction temperature, 40 °C; water addition, 20 μl; extraction time, 30 min; particle size, 1 mm; amount of plant material, 30 mg; desorption time, 3 min. As reported in Fig. 2f, the harvesting time did not influence at all the amount of 1-octen-3-ol giving similar response ($p > 0.05$) in May and June, supporting the hypothesis that in the plant this molecule does not play a role in attracting insects or animals useful for pollination. The results proved that the precursor molecule from which the mushroom-like alcohol is produced is still occurring in the plant matrix after the flowering period.

3.3. Analysis of the headspace volatiles of M. melissophyllum subsp. melissophyllum

As shown in Table 2, 60 components, accounting for 95.7% of total volatiles, were identified in the headspace by SPME-GC–MS using the optimised method described above (extraction temperature, 40 °C; water addition, 20 μl; extraction time, 30 min; particle size, 1 mm; sample amount, 30 mg; desorption time, 3 min). Quantitation of volatiles, expressed as percentages, were obtained at FID by calculating the GC correction factor for each class of volatiles occurring in the plant headspace. The obtained values, reported in Table 2, were the following: 1.49 for aliphatic alcohols, 1.73 for aldehydes, 1.45 for esters, 1.19 for alkanes, 1.68 for aromatics, 1.09 for monoterpene hydrocarbons, 1.48 for oxygenated monoterpenes, 1.05 for sesquiterpene hydrocarbons and 1.30 for oxygenated sesquiterpenes.

Adopted experimental conditions allowed the highest amount of the mushroom-like alcohol 1-octen-3-ol in the headspace of

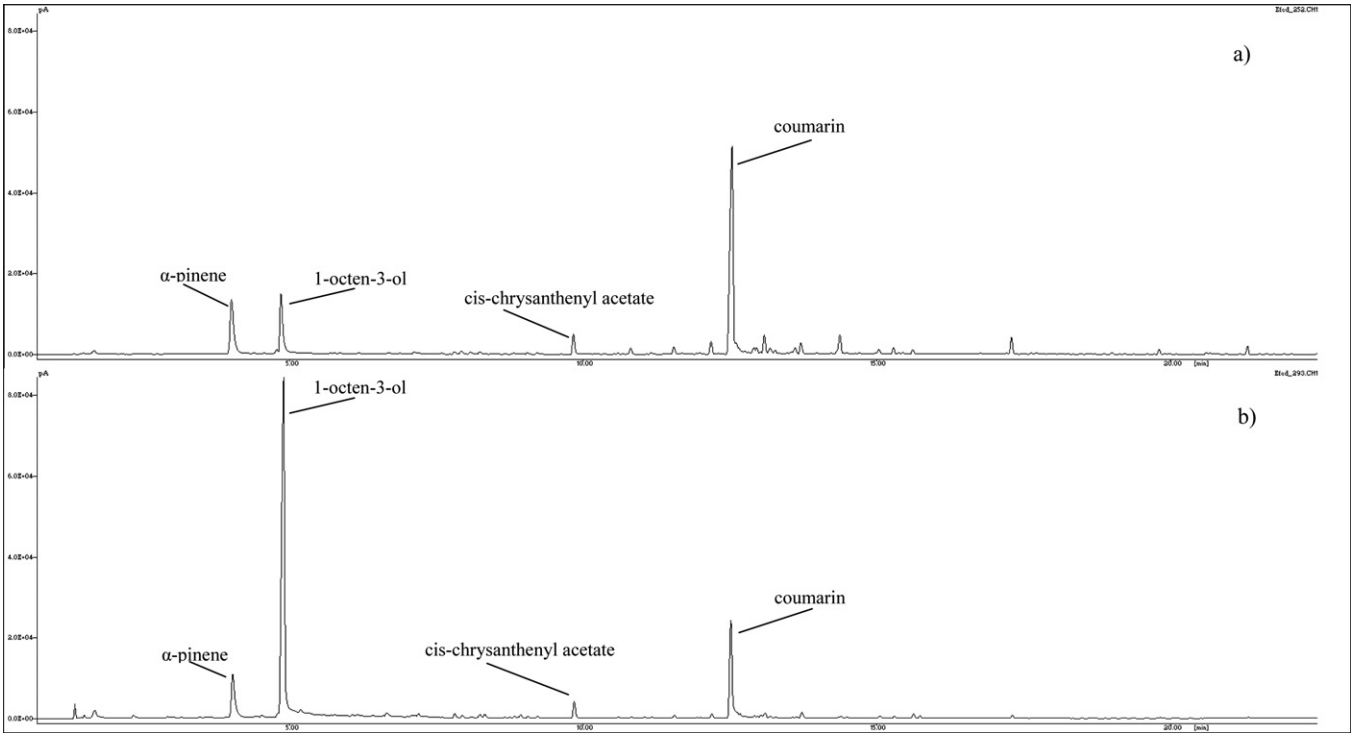

Fig. 3. HS-SPME-GC–FID chromatograms of *Melittis melissophyllum* subsp. *melissophyllum* using two different extraction conditions: (a) extraction temperature 60 °C, water addition 60 μl, extraction time 30 min, particle size 1 mm, sample amount 30 mg, desorption time 3 min; (b) extraction temperature 40 °C, water addition 20 μl, extraction time 30 min, particle size 1 mm, sample amount 30 mg, desorption time 3 min.

Table 3
Chemical composition of the essential oil of *Melittis melissophyllum* subsp. *melissophyllum*.

No.	Compounds[a]	RF[b]	RI HP-5[c]	RI LIT[d]	Peak area (%) ± SD	ID[e]
	Aliphatics				72.5	
	Alcohols	1.5			56.8	
1	Hexanol		872	870	0.1 ± 0.1	MS,RI
2	1-Octen-3-ol		983	979	56.3 ± 2.5	MS,RI,Std
3	3-Octanol		1000	991	0.4 ± 0.4	MS,RI
	Aldehydes and chetons	1.7			0.9	
4	(2E)-hexenal		854	855	0.5 ± 0.3	MS,RI
5	Heptanal		906	902	0.1 ± 0.0	MS,RI
6	Nonanal		1107	1100	0.2 ± 0.1	MS,RI
7	Decanal		1207	1201	0.1 ± 0.0	MS,RI
	Acids	1.4			0.4	
8	Tetradecanoic acid		1776	1767	0.1 ± 0.1	MS,RI
9	Hexadecanoic acid		1976	1960	0.3 ± 0.1	MS,RI,Std
	Esters	1.5			1.1	
10	Hexadeanoic acid methyl ester		1937	1938	tr[f] ± 0.0	MS,RI
11	Methyl linoleate		2103	2095	1.1 ± 0.7	MS,RI
	Alkanes	1.2			13.4	
12	Heneicosane		2107	2100	06 ± 0.3	MS,RI,Std
13	Docosane		2202	2200	0.8 ± 0.7	MS,RI,Std
14	Tricosane		2300	2300	2.1 ± 0.1	MS,RI,Std
15	Tetracosane		2399	2400	0.5 ± 0.4	MS,RI,Std
16	Pentacosane		2501	2500	2.1 ± 0.8	MS,RI,Std
17	Hexacosane		2599	2600	0.6 ± 0.4	MS,RI,Std
18	Heptacosane		2700	2700	3.3 ± 0.2	MS,RI,Std
19	Octacosane		2800	2800	0.7 ± 0.5	MS,RI,Std
20	Triacontane		3000	3000	2.6 ± 0.2	MS,RI,Std
	Aromatics	1.7			0.6	
21	(3Z)-hexenyl benzoate		1575	1566	0.2 ± 0.1	MS,RI
22	Benzyl benzoate		1765	1760	0.4 ± 0.2	MS,RI
	Phenols	1.7			0.7	
23	Coumarin		1440	1434	0.7 ± 0.3	MS,RI,Std
	Terpenoids				19.2	
	Monoterpene hydrocarbons	1.1			0.4	
24	α-Pinene		933	939	0.3 ± 0.3	MS,RI,Std
25	Sabinene		974	975	0.1 ± 0.1	MS,RI,Std
26	β-Pinene		977	979	tr ± 0.0	MS,RI,Std
27	Limonene		1031	1029	tr ± 0.0	MS,RI,Std
28	γ-Terpinene		1073	1059	tr ± 0.0	MS,RI,Std
	Oxygenated monoterpenes	1.5			2.6	
29	Linalool		1104	1096	1.9 ± 0.4	MS,RI,Std
30	Terpinen-4-ol		1179	1177	0.3 ± 0.2	MS,RI,Std
31	α-Terpineol		1191	1188	0.4 ± 0.2	MS,RI,Std
	Sesquiterpene hydrocarbons	1.1			7.6	
32	α-Copaene		1375	1376	0.1 ± 0.0	MS,RI,Std
33	β-Bourbonene		1384	1388	0.1 ± 0.1	MS,RI
34	β-Cubebene		1388	1388	tr ± 0.0	MS,RI
35	β-Elemene		1390	1390	0.1 ± 0.0	MS,RI
36	(E)-caryophyllene		1414	1424	3.4 ± 0.4	MS,RI,Std
37	β-Copaene		1428	1432	tr ± 0.0	MS,RI
38	α-Humulene		1450	1454	0.2 ± 0.1	MS,RI,Std
39	Germacrene D		1478	1485	2.1 ± 0.1	MS,RI
40	β-Selinene		1483	1490	0.1 ± 0.0	MS,RI
41	Bicyclogermacrene		1494	1493	0.2 ± 0.1	MS,RI
42	α-Muurolene		1498	1500	0.1 ± 0.0	MS,RI
43	(E,E)-α-farnesene		1511	1505	1.1 ± 0.2	MS,RI
44	δ-Cadinene		1523	1523	0.2 ± 0.0	MS,RI
	Oxygenated sesquiterpenes	1.3			1.7	
45	(E)-Nerolidol		1566	1563	0.3 ± 0.1	MS,RI
46	Caryophyllene oxide		1580	1583	0.7 ± 0.0	MS,RI,Std
47	α-Bisabolol		1685	1685	1 ± 0.8	MS,RI,Std
48	Hexahydrofarnesyl acetone		1848	1846	0.6 ± 0.3	MS,RI
	Diterpene hydrocarbons	1.4			0.3	
49	Abietatriene		2056	2056	0.3 ± 0.3	MS,RI
	Oxygenated diterpenes	1.4			5.6	
50	13-Epi-manool oxide		2008	2010	0.7 ± 0.6	MS,RI
51	Phytol		2119	2112	4.9 ± 1.1	MS,RI,Std
	Norisoprenoids	1.2			0.1	
52	β-Cyclocitral		1222	1219	tr ± 0.0	MS,RI
53	(E)-β-ionone		1487	1488	0.1 ± 0.0	MS,RI,Std

Table 3 (*continued*)

No.	Compounds[a]	RF[b]	RI HP-5[c]	RI LIT[d]	Peak area (%) ± SD	ID[e]
	Others				0.1	
54	Cis-jasmone	1.0	1398	1392	tr ± 0.0	MS,RI
55	Mint sulphide	1.4	1731	1741	0.1 ± 0.0	MS,RI
	Total identified (%)				93.3 ± 0.7	

[a] Compounds belonging to each class are listed in order of their elution from a HP-5 column; percentage values are means of three determinations ± standard deviation; they were obtained at FID by peak-area normalisation calculating the relative response factor.
[b] Relative response factor of FID detector.
[c] Retention index on HP-5 column, experimentally determined using a homologous series of C_8–C_{30} alkanes.
[d] Relative retention index taken from Adams (2007) and/or NIST 08 (2008) for DB-5 and HP-5 capillary column, respectively.
[e] Identification methods: MS, by comparison of the mass spectrum with those of the computer mass libraries Wiley, Adams (2007) and NIST 08 (2008); RI, by comparison of RI with those reported in literature (Adams, 2007; NIST, 2008); std, by comparison of the retention time and mass spectrum of available authentic standard.
[f] tr, traces (mean value below 0.1%).

plant material (56.3%) to be obtained. A low contribution to the headspace, was given by terpenoids (13.4%), with the monoterpene hydrocarbon α-pinene (4.1%) and the monoterpene ester cis-chrysanthenyl acetate (1.9%) as the most representative. Noteworthy is the occurrence in the headspace (Fig. 2e and f) of coumarin (1,2-benzopyrone) (18.7%), a phenolic compound occurring in many plants used as flavouring ingredients in foods as cinnamom, cassia, lavender, peppermint and *Melilotus alba*, because of its sweet, aromatic, creamy vanilla bean odour with nut-like tones (Sproll, Ruge, Andlauer, Godelmann, & Lachenmeier, 2008). Based on reports of hepatotoxicity and suspected carcinogenicity and mutagenicity (Lake, 1999), the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food of European Food Safety Authority (EFSA) has recently established a tolerable daily intake (TDI) for coumarin of 0–0.01 mg/kg body weight based on the no observed adverse effect level (NOAEL) for hepatotoxicity (EFSA, 2004). On the other hand, Directive 88/388/EEC lays down maximum levels for coumarins in foodstuffs (European Council, 1988). In particular, the amount of coumarin is limited to 2 mg/kg for foodstuffs and beverages with the exception of alcoholic beverages, certain types of caramels and chewing gums (10, 10 and 50 mg/kg, respectively).

In Fig. 3, is reported the comparison of SPME-GC–FID chromatograms relative to volatiles captured by PDMS fibre with two different experimental conditions (standard conditions cited above adopted for the fibre screening, and the optimised conditions). By optimising the extraction parameters the levels of 1-octen-3-ol in the headspace were significantly increased (from 12.1% to 56.3%). Vice versa the amount of coumarin decreased from 50.9% with the initial conditions to 18.7% after optimising the SPME parameters. It can be observed that higher temperature and larger amounts of water extract more terpenes and coumarins (owing to its water solubility), rather than aliphatics such as 1-octen-3-ol that need lower extraction temperatures and small amounts of water.

3.4. Analysis of the essential oil of M. melissophyllum subsp. melissophyllum

The composition of the essential oil of *M. melissophyllum* subsp. *melissophyllum* is given in Table 3. A total of 55 components were identified, representing 93.3% of the essential oils under study. The major component was the mushroom-like aromatic compound 1-octen-3-ol, which represented 56.3% of the total oil. Aliphatic compounds accounted for 72.5% of the oil, with alcohols being the major fraction (56.8%). Terpenoids constituted a minor fraction of the essential oils (19.2%) with sesquiterpenes (7.6%) and diterpenes (5.6%) as the most prevalent; (E)-caryophyllene (3.4%), germacrene D (2.1%) and phytol (4.9%) were the most representatives of these classes of volatiles. By comparing the chromatographic profile of essential oil with respect to that of headspace we can notice that coumarin is very scant in essential oil (0.7%) because of its hydrophilicity, while in the headspace is the main compound along with 1-octen-3-ol; in addition higher molecular weight sesquiterpenes, diterpenes and cuticular waxes-derived alkanes were represented mostly in essential oil owing to higher temperatures and deeper hydrolytic reactions occurring during hydrodistillation.

4. Conclusions

To the best of our knowledge, the present work represents the only comprehensive study of the extraction of the mushroom-like alcohol 1-octen-3-ol from the headspace of a plant. Following the optimisation of the SPME method, the flavour of the plant material was strengthened owing to an increase of the 1-octen-3-ol yield. In fact, the SPME method allowed the same 1-octen-3-ol levels occurring in essential oil to be obtained in the headspace, underlining the potentiality of the plant as mushroom flavouring or a mushroom-like flavour enhancer in food industry. Finally, with this study we highlighted that eight-carbon volatile formation is not unique to fungi and is likely to involve also a plant-specific pathway. Plants, as do fungi, utilise their fatty acid resources to produce volatile compounds; the fatty acid molecules are first oxidised and then cleaved to produce the short-chain volatiles. Both lipoxygenase and hydroperoxide lyase are enzymes present in plants and involved in short-chain volatile synthesis. For this reason, further works on the enzymic process leading to the production of 1-octen-3-ol from *M. melissophyllum* are needed. The potentiality of this plant to produce economically interesting natural flavour has to be deeply investigated.

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