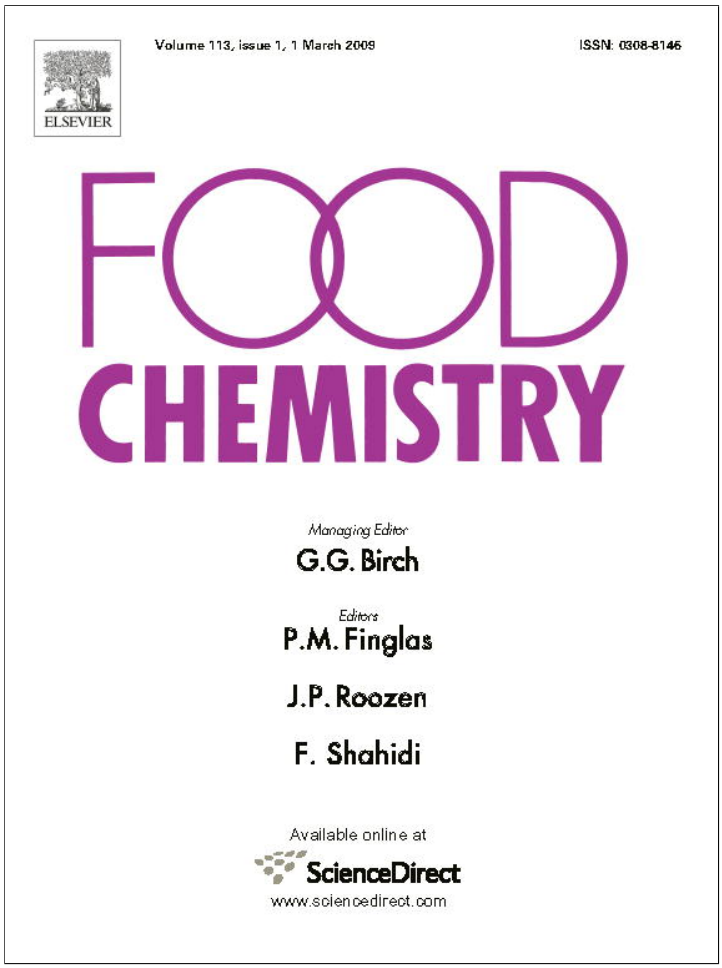

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Melittis melissophyllum L. subsp. *melissophyllum* (Lamiaceae) from central Italy: A new source of a mushroom-like flavour

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ABSTRACT

1-Octen-3-ol is the most important C8 mushroom aromatic compound produced by many species of edible fungi and is also an aroma component in several food and beverages products. Under this view, the essential oil of flowering aerial parts of *Melittis melissophyllum* subsp. *melissophyllum* (Lamiaceae) growing in central Italy, obtained by hydrodistillation was characterised by GC–FID and GC–MS. This oil contained extremely high amount of the mushroom-like aroma component 1-octen-3-ol (43.6–54.2%), and could be considered as a new natural product for the use as flavouring agent in the food industry. Furthermore, headspace analysis suggested that this aromatic compound is only present in low concentration in the plant part, and is primarily formed in higher amount during hydrodistillation of this material.

1. Introduction

1-Octen-3-ol is a volatile alcohol produced by many species of edible fungi (Combet, Henderson, Eastwood, & Burton, 2006). The odour of this alcohol is "mushroom like", and nowadays it is considered the most important C8 mushroom aromatic compound (Zawirska-Wojtasiak, 2004). It was discovered by Murahashi in *Tricholoma matsutake* (S. Ito & S. Imai) Singer and first called matsutake alcohol (Murahashi, 1938); it is formed in fruiting bodies of higher fungi by enzymatic oxidative degradation of linoleic acid (Badings, 1970); it has two optically active isomers, the naturally occurring (−) form having a stronger flavour than the (+) form (Combet et al., 2006). 1-Octen-3-ol is produced by fungi as repulsive agent against predators (Wood, Archer, & Largent, 2001), but it can also act as a fungal hormone, since an increase of its level recruits insects to disseminate fungal spores (Chitarra, Abee, Rombouts, & Dijksterhuis, 2005).

Besides edible mushrooms, 1-octen-3-ol is reported to be an aroma component in several food products and beverages such as truffles (Zeppa et al., 2004), sausages (Marco, Navarro, & Flores, 2007), ham (Carrapiso, Jurado, Timon, & García, 2002), soy milk (Lozano, Drake, Benitez, & Cadwallader, 2007), dairy products (Friedrich & Acree, 1998), salmon (Wierda, Fletcher, Xu, & Dufour, 2006), oysters (Piveteau et al., 2000), wines (Genovese, Dimaggio, Lisanti, Piombino, & Moio, 2005; Genovese, Gambuti, Piombino, & Moio, 2007), orange essential oil (Högnadóttir & Rouseff, 2003). 1-Octen-3-ol is a widely used food flavouring agent; it has been included in the food additive database of Food and Drug Administration (FDA) (US FDAs Center for Food Safety, 2008), and in the FAO and WHO Codex Alimentarius (www.codexalimentarius.net/download/standards/9/CXA_006e.pdf) as flavouring agent in food industry.

1-Octen-3-ol has been found also in plants, especially in the Lamiaceae family, where it is a component of the essential oil and plays an important role in the particular odourous profile of these plants (Tomei, Uncini Manganelli, Flamini, Cioni, & Morelli, 2003). In particular, it was detected in significant amounts (>10%) in some species: 12.4–20.7% in *Prasium majus* L. (Basta, Tzakou, Couladis, & Yannitsaros, 2007), 19.7% in *Stachys recta* L. (Chalchat, Petrovic, Maksimovic, & Gorunovic, 2000), and 12.62% in *Sideritis romana* L. subsp. *romana* (Kirimer, Tabanca, Özek, Tümen, & Başer, 2000).

On the above, a cheap and readily available vegetal source of 1-octen-3-ol would be of great interest for the food industry, since a flavouring agent of natural origin is much better accepted by

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consumers than synthetic equivalent, not forgetting the risk, for artificial flavours, of potentially toxic impurities deriving from synthesis.

Melittis melissophyllum L. (Lamiaceae) is a perennial herb with big axillary flowers inhabiting shady places in Western, Southern and Central Europe (Ball, 1972). It occurs in Italy with two subspecies: subsp. *albida* (Guss) P.W. Ball, occurring in the South and Islands; subsp. *melissophyllum*, occurring mainly in the North-Western and central Italy (Conti, Abbate, Alessandrini, & Blasi, 2005). In the folk medicine of central Italy inflorescences of this plant, called "Erba Lupa", were used under infusion as antispasmodic, against insomnia and eyes inflammations (Guarrera, 2005). Previously, the essential oil of *M. melissophyllum* subsp. *albida* from Greece was analysed by Skaltsa-Diamantidis, Tsitsa-Tzardi, Tzakou, and Argiriadou (1991) who found α-terpineol (17.1%), chrysanthenyl acetate (12.3%) and caryophyllene oxide (10.8%) as main components, whilst the subsp. *melissophyllum* growing in Spain was investigated by Velasco-Negueruela, Sanz, Pérez-Alonso, and Palá-Paúl (2004) who found 8,11,14-eicosatrienoic acid (29.1–31.4%) and hexadecanoic acid (10.9–21.4%) as the main components both in fresh and dried samples, whereas 1-octen-3-ol was detected in minor amounts (3.9%) only in fresh samples.

In this study we report the essential oil analysis of *M. melissophyllum* subsp. *melissophyllum* growing in central Italy, performed by GC–FID and GC–MS. The essential oil of this plant revealed unexpectedly to be an important source of this mushroom-like aroma component. Finally, in order to detect 1-octen-3-ol in plant material, a headspace analysis (HS–GC) was performed as well.

2. Materials and methods

2.1. Plant material

Aerial parts of *M. melissophyllum* subsp. *melissophyllum*, were collected at flowering in May 2007 in two sites in central Italy (Site 1: Piedilapiaggia, near the town of Camerino, 600 m above sea level, coordinates N43°09′10″ E13°07′18″; Site 2: Crispiero, near the town of Castelraimondo, 400 m above sea level, coordinates N43°11′19″ E13°06′27″), on woody environment. Voucher specimens were identified and deposited in the Herbarium Camerinensis (Department of Environmental Sciences, Section of Botany and Ecology, University of Camerino, Italy), under the accession codes CAME13429 and CAME13430; they are also available at the following website: http://erbariitaliani.unipg.it. For headspace analysis (HS), dried plant material was grounded with a blender MFC model DCFH48 IKA-WERK using 2 mm diameter filters.

2.2. Chemicals

1-Octen-3-ol, hexadecanoic acid, octadecane, heneicosane, tricosane, tetracosane, pentacosane, heptacosane, nonacosane, benzene acetaldehyde, coumarin, α-pinene, sabinene, γ-terpinene, terpinolene, linalool, terpinen-4-ol, α-terpineol, (*E*)-β-ionone, (*E*)-caryophyllene, α-humulene, caryophyllene oxide, α-bisabolol, globulol, phytol were purchased from Sigma-Aldrich (Milan, Italy). All compounds were of analytical standard grade. Analytical grade hexane solvent, distilled before use, was from Carlo Erba (Milan, Italy). Na_2SO_4 was of analytical reagent grade from J.T. Baker (Deventer, Holland).

2.3. Extraction of essential oil

Aerial parts (120–193 g) were dried protected from light at room temperature for one week. Then, they were cut in small pieces and placed in a flask containing 2.4–3.8 l of water and hydrodistilled for 4 h in a Clevenger-type apparatus, adding 10 ml of hexane to entrap volatiles. After distillation, solvent was eliminated under a N_2 flow, the oil was dried over anhydrous Na_2SO_4 and stored in sealed vials protected from the light at −20 °C for GC and GC–MS analyses. Each sample was divided into 3 portions, and every portion was hydrodistilled and analysed by GC–FID and GC–MS. The oil yield (w/w, 0.02–0.04%) was estimated on a dry weight basis. Hydrodistilled oils had a typical mushroom-like smell and yellow colour.

2.4. GC–FID and GC–MS analysis

2.4.1. GC–FID

GC–FID analysis of volatile components was carried out using Agilent 4890D with FID detector and HP-5 capillary column (5% phenylmethylpolysiloxane, 25 m, 0.32 mm i.d.; 0.17 μm film thickness) (J&W Scientific, Folsom, CA), with the following temperature program: 5 min at 60 °C, then at 4 °C/min to 220 °C, then 11 °C/min to 280 °C, held for 15 min; injector and detector temperatures, 280 °C; carrier gas, helium (1.4 ml/min); injection volume of 1 μl, split ratio, 1:34.

2.4.2. GC–MS

GC–MS analysis was performed using an Agilent 6890N-5973N GC–MS system operating in the EI mode at 70 eV, using a HP-5MS capillary column (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 μm film thickness) (J&W Scientific, Folsom), which was programmed at 60 °C for 5 min, then ramp at 4 °C/min to 220 °C, then 11 °C/min to 280 °C held for 15 min, finally 11 °C/min to 300 °C, held for 5 min; carrier gas: helium; flow rate: 1.0 ml/min; injector and transfer line temperatures: 280 °C; injection volume: 2 μl; split ratio: 1:50; scan time: 75 min; acquisition mass range: 29–400 amu.

2.4.3. Identification and quantification of volatiles

The identification of volatiles was based on computer matching with the WILEY275, NIST05, and ADAMS libraries, as well as by comparison of the mass spectra and retention indices (RI) according to Kovats (1958), with those reported in the literature (Adams, 2007). In addition, a home-made library, based on the analyses of reference oils and commercially available standards, was used as well. Whenever possible, components were identified by comparison of their retention times, mass spectra, and retention indices related to *n*-alkanes with those of authentic standards available in author's laboratory. Percentage compositions of the oil components were obtained from electronic integration using flame ionisation detection (FID, 280 °C), dividing the area of each component by the total area of all components isolated under these conditions. The percentage values for volatile components were the mean of three determinations.

2.4.4. Quantification of 1-octen-3-ol in essential oil

The amount of 1-octen-3-ol in the essential oil was quantified by multiple standard addition method (Kolb & Ettre, 1997) (Table 2). Known amounts of analyte (0.05, 0.1, 0.2 mg) were added to the oil sample, and the peak area measured versus the amount added was plotted. Each addition was performed in triplicate. The intercept of the regression line on the amount added axis equaled the amount of analyte (mg) in the original essential oil sample.

2.5. HS–GC analysis

In order to verify the presence of 1-octen-3-ol in plant material, an headspace analysis (HS) using an Agilent 7694E headspace sampler coupled with the gas chromatograph (Agilent 4890D) de-

Table 1
Essential oil composition of *Melittis melissophyllum* subsp. *melissophyllum* from central Italy

No.	Compounds[a]	RI[b]	Peak area (%)[c]		Identification methods[d]
			Site 1	Site 2	
	Alcohols				
1	(3*Z*)-Hexenol	860	0.2	0.4	MS, RI
2	(2*Z*)-Hexenol	870	0.1	0.7	MS, RI
3	Hexanol	872	Tr[f]	Tr	MS, RI
4	1-Octen-3-ol[e]	983	43.6	54.2	MS, RI, std
5	3-Octanol	1000	0.5	0.6	MS, RI
	Aldehydes and chetons				
6	(2*E*)-hexenal	854	1.0	0.5	MS, RI
7	3-Octanone	992	0.3	0.3	MS, RI
8	2-Pentadecanone, 6,10,14-trimethyl-	1843	0.7	0.4	MS, RI
	Acids				
9	Hexadecanoic acid	1973	11.3	9.0	MS, RI, std
	Esters				
10	Methyl hexadecanoate	1922	0.3	0.2	MS, RI
11	Isopropyl hexadecanoate	2026	–	0.2	MS, RI
12	Methyl linolenate	2095	0.6	0.7	MS, RI
13	Ethyl linoleate	2146	3.0	1.0	MS, RI
	Alkanes				
14	Octadecane	1800	Tr	0.2	MS, RI, std
15	Heneicosane	2100	0.7	0.6	MS, RI, std
16	Tricosane	2300	2.5	1.5	MS, RI, std
17	Tetracosane	2400	0.3	0.9	MS, RI, std
18	Pentacosane	2500	1.6	1.4	MS, RI, std
19	Heptacosane	2700	2.9	1.5	MS, RI, std
20	Nonacosane	2900	2.2	1.1	MS, RI, std
	Aromatics				
21	Benzene acetaldehyde	1052	0.6	1.2	MS, RI, std
22	Acetophenone	1075	–	0.3	MS, RI
	Phenols				
23	Coumarin	1439	1.6	1.6	MS, RI, std
	Monoterpene hydrocarbons				
24	α-Pinene	933	1.1	0.7	MS, RI, std
25	Sabinene	974	0.4	0.1	MS, RI, std
26	γ-Terpinene	1063	–	0.2	MS, RI, std
27	Terpinolene	1089	0.1	0.5	MS, RI, std
	Oxygenated monoterpenes				
28	Linalool	1102	1.9	2.2	MS, RI, std
29	Terpinen-4-ol	1179	0.3	1.1	MS, RI, std
30	α-Terpineol	1192	0.3	0.5	MS, RI, std
31	*cis*-Chrysanthenyl acetate	1265	0.4	–	MS, RI
32	(*E*)-β-Ionone	1487	0.8	0.7	MS, RI, std
	Sesquiterpene hydrocarbons				
33	β-Bourbonene	1381	0.1	–	MS, RI
34	β-Elemene	1390	0.4	-	MS, RI
35	(*E*)-Caryophyllene	1414	3.0	1.3	MS, RI, std
36	α-Humulene	1450	0.4	0.3	MS, RI, std
37	Germacrene D	1477	1.8	0.7	MS, RI, std
38	Bicyclogermacrene	1492	0.3	0.2	MS, RI
39	(*E*,*E*)-α-Farnesene	1509	0.5	0.2	MS, RI
40	δ-Cadinene	1522	0.3	0.2	MS, RI
	Oxygenated sesquiterpenes				
41	Spathulenol	1575	0.4	0.3	MS, RI, std
42	Caryophyllene oxide	1579	1.7	0.9	MS, RI, std
43	Globulol	1588	–	0.4	MS, RI, std
44	α-Bisabolol	1684	0.9	0.5	MS, RI, std
	Diterpene hydrocarbons				
45	Abietatriene	2055	0.1	Tr	MS, RI
	Oxygenated diterpenes				
46	Manool oxide	1997	0.2	0.6	MS, RI
47	Phytol	2111	4.6	3.8	MS, RI, std
48	2-Keto-manool oxide	2213	0.9	1.1	MS, RI
	Total identified		94.7	95.0	

[a] Compounds belonging of each classes are listed in order of their elution from a HP-5 column.
[b] RI, retention indices as determined on HP-5 column using homologous series of C8-C30 alkanes.
[c] Percentage values were the mean of three determinations with a RSD in the range of 2–10%.
[d] Identification methods: MS, by comparison of the mass spectrum with those of the computer mass libraries Wiley, NIST 05 and ADAMS; RI, by comparison of RI with those reported from Adams (2007) and Davies (1990); std, by comparison of the retention time, mass spectrum and retention index of authentic standard.
[e] Quantified by the multiple standard addition method: 260–323 mg analyte/g of essential oil.
[f] Tr, traces (mean value below 0.1%).

Table 2
Analysis of *Melittis melissophyllum* subsp. *melissophyllum* essential oil (Site 1) for 1-octen-3-ol, using the multiple standard addition method

Amount of 1-octen-3-ol added (mg)	Peak area	Average area	SD	CV%
0	22912.0000 22602.0000 25543.0000	23685.6667	1615.9487	6.8225
0.05	37100.0000 38591.0000 42974.0000	39555.0000	3053.3491	7.7192
0.1	65341.0000 63067.0000 71038.0000	66482.0000	4106.1687	6.1764
0.2	113614.0000 102910.0000 100881.0000	105801.6667	6841.3174	6.4662
Linear regression				
Correlation coefficient *r*	0.997			
Slope *a*	420,545			
Intercept *b*	21,856			

scribed above was performed. Three plant samples (0.5 g each) were placed in 10-ml headspace vials and analysed as follows (temperatures in °C): incubation time, 30 min; pressurisation time, 20 s; loop fill time, 20 s; loop equilibration time, 5 s; injection time, 1 min; pressurisation gas, Helium; incubation temperatures, 40, 60, and 80; loop temperatures, 50, 70, 90; transfer line temperatures, 70, 90, 110. Each analysis was conducted in triplicate. A capillary column Innowax (30 m, 0.32 mm i.d., 0.25 μm film thickness) (J&W Scientific, Folsom) was used; it was programmed at 60 °C for 5 min, then ramp at 3 °C/min to 200 °C, then 10 °C/min to 240 °C for 10 min; injector and detector temperatures, 280 °C; carrier gas, helium (1.4 ml/min); split-less mode. Identity of 1-octen-3-ol was confirmed by co-injection of an authentic standard, while its percentage was obtained from electronic integration using FID. A positive control was performed using Site 1essential oil sample obtained by hydrodistillation and incubated at 40 °C.

3. Results and discussion

Composition of the essential oil of *M. melissophyllum* L. subsp. *melissophyllum* is given in Table 1. Forty-eight components were identified, representing 94.7–95.0% of the oil under study. The major component was the mushroom-like aromatic compound 1-octen-3-ol, which represented 43.6–54.2% (calculated on the basis of the 1-octen-3-ol peak area/total peak area) of the oil (Fig. 1), and which mass spectrum is reported in Fig. 2. To our knowledge, these 1-octen-3-ol percentages are the highest detected in essential oils up to now. Other contribution to the characteristic mushroom flavour came from 3-octanol and 3-octanone, which were present in the oil in scant amounts (0.5–0.6% and 0.3%, respectively). Aliphatic compounds accounted for more than two third (71.9–75.4%) of the oil components, with alcohols being the major fraction (44.4–56.0%). The second most abundant compound was the aliphatic hexadecanoic acid (9.0–11.3%). Terpenes constituted a minor fraction (16.6–20.7%), with linalool (1.9–2.3%), (*E*)-caryophyllene (1.3–3.0%), and phytol (3.8–4.6%) being the most important representatives of monoterpenes, sesquiterpenes, and diterpenes, respectively.

Comparing the results with previously published ones (Velasco-Negueruela et al., 2004) related to the same entity from a different country (Spain), one may notice qualitative and quantitative differences. The oil preparation shared only 28/48 components with samples from Spain. 8,11,14-Eicosatrienoic acid, which was the most abundant component (29.1–31.4%) in Spanish samples, was not detected in the oil sample, whilst 1-octen-3-ol, predominant here (43.6–54.2%), was present only in scanting amounts (3.9%) and in traces (<0.1%) in fresh and dried Spanish samples, respectively. Phytol and spathulenol, two main components in dried Spanish samples (16.0% and 5.2%, respectively), were present in minor amounts (3.8–4.6% and 0.3%, respectively).

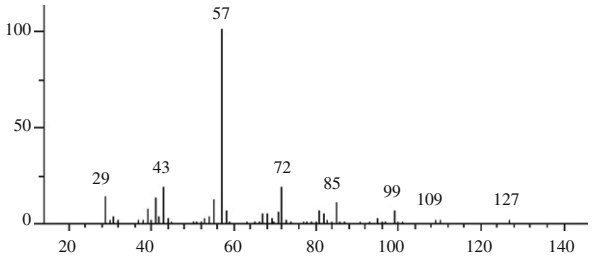

Fig. 2. Mass spectrum of 1-octen-3-ol.

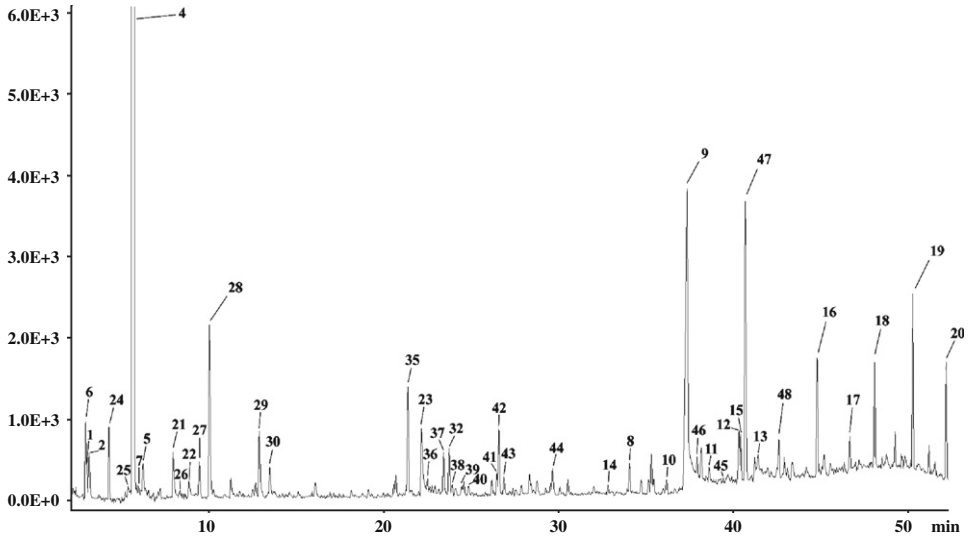

Fig. 1. GC–FID chromatogram of the hydrodistilled volatiles of *Melittis melissophyllum* L. subsp. *melissophyllum* from central Italy (sample from Site 2). Peak numbers corresponding to the identification of the compounds are cited in Table 1.

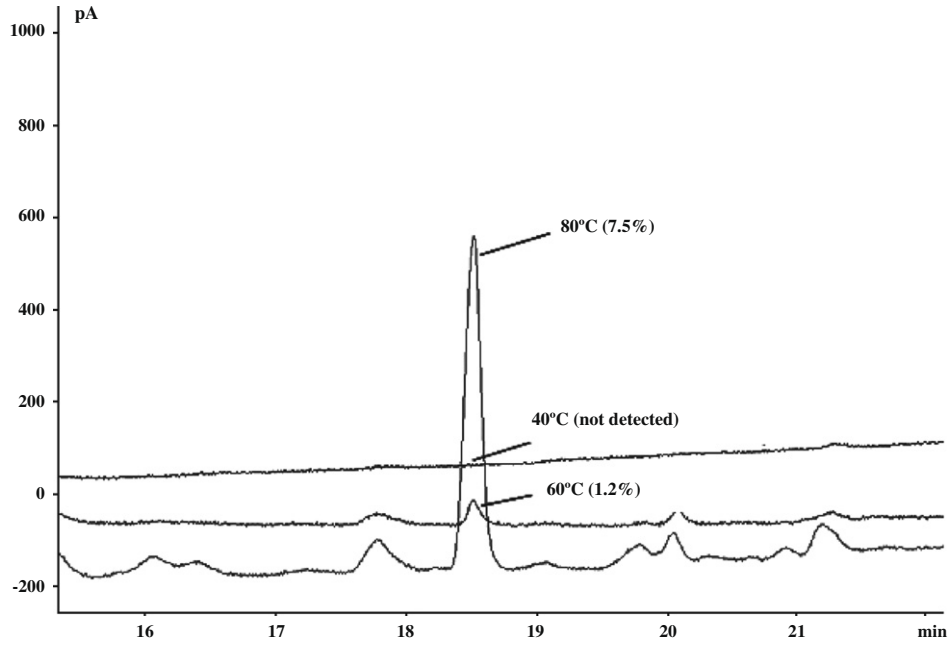

Fig. 3. HS–GC profile of 1-octen-3-ol obtained at three different extraction conditions on Innowax column (sample from Site 1); percentages from electronic integration using FID.

For the control of the concentration of 1-octen-3-ol was used the multiple standard addition method: the results showed a concentration in the range of 260–323 mg analyte/g of essential oil (quite lower than that calculated from peak area ratios in the full chromatogram). A good relationship between the amount (x) and peak area (y) of 1-octen-3-ol was obtained (Table 2). The linear regression equation was $y = 420{,}545x + 21{,}856$ ($r = 0.997$); the relative standard deviation (RSD) ranged from 6.17% to 7.71% ($n = 3$).

To determine whether the detected high concentration of 1-octen-3-ol was present as such in the plant, or was formed during extraction processes and procedures, we decided to determine 1-octen-3-ol level in plants; Fig. 3 reports a portion of the chromatographic profiles of the dried plant material analysed by HS–GC at three different incubation temperatures. It is possible to observe that 1-octen-3-ol is not detected at 40 °C; its percentage at 60 °C was 1.3%, while at 80 °C reached 7.5%. However, those percentages were much lower than those in the hydrodistilled oils (43.6–54.2%), and to the 28.0% of positive control (essential oil, incubated at 40 °C and analysed by HS–GC). This data suggest that high temperatures (about 100 °C) and moist environment are responsible for favoring, in the plant material, those hydrolytic/oxidative reactions, which yield high amount of 1-octen-3-ol.

In conclusion, the high amount of 1-octen-3-ol measured in our investigation makes the essential oil hydrodistilled from this plant an important source of this molecule, and may suggest that *M. melissophyllum* subsp. *melissophyllum* could be an important natural source of mushroom like food and beverages flavouring agent.

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