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Discrimination of truffle fruiting body versus mycelial aromas by stir bar sorptive extraction

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Abstract

Stir bar sorptive extraction (SBSE) was applied in head space mode (HS), coupled with GC/MS, to compare the aroma profile of three truffle species. A total of 119 volatile organic compounds (VOCs) were identified from the fruiting bodies, of which 70 were not yet described in truffles and 60 in fungi. VOCs profile showed a high intra- and inter-specific variability, with alcohols and sulfur compounds dominating the HS of *Tuber borchii* and, alcohols, aldehydes and aromatic compounds the HS of *T. melanosporum* and *T. indicum*. Despite these variations, eight VOCs markers could be identified allowing the discrimination of the three species. Additionally, *T. borchii* and *T. melanosporum* both distinguished themselves from *T. indicum* due to higher aroma content and larger variety of sulfur containing compounds. Mycelial VOCs production was also investigated under two cultural conditions and led to the identification of eight VOCs. On one side, seven of them were also detected in the fruiting body, confirming their mycelial origin. On the other side, the total absence of some class of compounds (i.e. sulfur) in the mycelium raises questions about their origins in the fruiting bodies and confirms deep metabolic changes between the reproductive (fruiting body) and vegetative (mycelium) stages.

Keywords: Truffle; *Tuber borchii*; *T. melanosporum*; *T. indicum*; Fruiting body; Mycelium; Volatile organic compounds; Stir bar sorptive extraction

1. Introduction

Truffles, hypogeous fungi that live in symbiosis with trees and some shrubs, are widely appreciated for their organoleptic properties (Mello et al., 2006). Since the pioneer work on the aroma of the black truffle *Tuber melanosporum* in the 80' (Ney and Freytag, 1980; Claus et al., 1981), several papers have been published identifying more than 200 volatile organic compounds (VOCs) produced by various truffle species of commercial interest, including the French "black diamond" *T. melanopsorum*, the notorious white Italian truffle *T. magnatum* and other less expensive species (Talou et al., 1987a,b, 1989a–d; Flament et al., 1990, and references cited therein). Truffle VOCs, which derive from various metabolic pathways such as fatty acid catabolism, polyketidic and isoprenoid pathways, are small hydrocarbons containing alcohol, ester, ketone and aromatic groups as well as sulfur atoms.

The number of truffle VOCs has rocketed in the past five years, mainly due to an increasingly sensitive and versatile extraction technique (e.g. solid phase micro-extraction, SPME) (Díaz et al., 2003; Mauriello et al., 2004; Zeppa et al., 2004). SPME is a non-destructive technique that has been applied to truffle fruiting bodies for the study of sulfur compounds (Pelusio et al., 1995), qualitative comparison of the aromatic profile among different species (Díaz et al., 2003; Mauriello et al., 2004), influence of storage on aroma (Falasconi et al., 2005), aromatic fingerprinting at different maturity stages (Zeppa et al., 2004), discrimination among species based on total mass spectra (Gioacchini et al., 2005) and also to the characterization

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of VOCs produced by yeasts isolated from truffle fruiting bodies (Buzzini et al., 2005).

Based on the same SPME principle, stir bar sorptive extraction (SBSE) was developed more recently by Baltussen et al. (1999). Its design includes a central magnet permitting either stirring of the sample to extract or suspension into the headspace through magnetic force (Demyttenaere et al., 2004). With detection limits 10–25 times lower than SPME (Pfannkoch et al., 2002), SBSE is typically used for trace and ultra-trace analysis. Additionally, because of the much larger fiber surface area than in SPME, competition among analytes at the fiber surface is strongly reduced; making quantification feasible even with a limited knowledge of the matrix (SUPELCO, 2001; Pfannkoch et al., 2002). The drawback of the technique is the need of additional equipment for GC/MS analyses such as a thermal desorption device and a cold trap necessary to introduce the analytes in the GC, leading also to an overall longer analysis time than with SPME.

Here we show the use of SBSE to characterize the aromatic profile of truffles.We analyzed the fruiting bodies of two species that are difficult to tell apart with traditional morphological observations (*T. melanosporum* and *T. indicum*) and the fruiting bodies and mycelium of *T. borchii*, because of the relatively good growth of the latter compared to other species (Iotti et al., 2002). More precisely, we (1) evaluated the potential of HS-SBSE to identify VOCs not yet described in truffles or fungi; (2) compared the VOC profile of each species and identified some species-specific VOCs (markers) allowing the discrimination of the truffle species; and (3) differentiated the major VOCs produced during the vegetative phase (mycelium) from those produced at the reproductive stage (fruiting body).

2. Results and discussion

2.1. Search for undescribed VOCs

The first aim of this paper was to evaluate the potential of HS-SBSE/GC–MS to identify VOCs not yet described in truffles. Indeed, identification of such flavor compounds is of primary importance for the food industry, always in search for new compounds with undescribed smell and taste. By using SBSE as depicted in Fig. 1, we could detect 166 VOCs from the fruiting bodies of three species (*T. melanosporum*, *T. borchii* and *T. indicum*). The identity of 119 of those VOCs was determined (without chirality determination) as described in Table 1. Comparing this list to the most recent publications on the VOCs of two white species (*T. magnatum* and *T. borchii*) and four black ones (*T. melanosporum*, *T. uncinatum*, *T. indicum*, and *T. aestivum*), we estimated that 70 of the VOCs were first described for the six species cited above. Similarly, based on a recent review on fungal VOCs (Chiron and Michelot, 2005) and on the study of the VOCs emitted by yeasts isolated from truffle

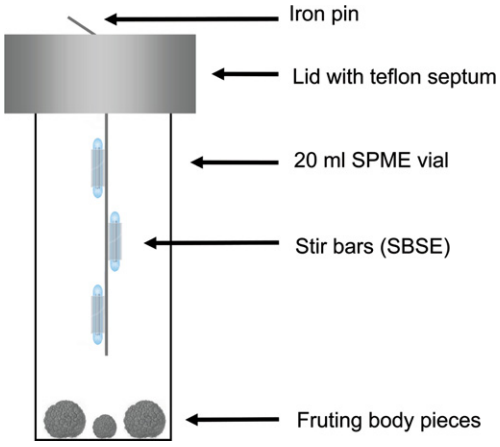

Fig. 1. VOCs were extracted in head-space mode with two or three stir bars suspended to an iron pin over the sample (fruiting body or mycelium) for 63 h.

fruiting bodies (Buzzini et al., 2005), 60 of the compounds identified had not been described in fungi. Some of the VOCs might be derived from the fermentation processes of microorganisms inhabiting the fruiting bodies, while others might be related to specific ascoma developmental stages. However, our goal was not to discriminate among different maturity/shelf-life stages, rather to provide an overview of the aromatic profile of the samples. The "newly" described VOCs included alcohols, aldehydes, aromatic compounds, esters, furans, hydrocarbons, ketones, and nitrogen- and sulfur-containing compounds (Table 1). The alcohols, aldehydes and ketones were simple linear chained or branched molecules resulting, respectively, from lipid oxidation or Strecker degradation of amino acids (Bellesia et al., 2001). One aldehyde, 2-octenal, was observed in all the three truffle species investigated in this study and interestingly in three other mycorrhizal mushrooms, *Boletus edulis*, *Craterellus cornucopioides* and *Lactarius trivialis* (Table 1). Of the aromatic compounds, phenylmethanol (benzylalcohol) was first described in truffles, however, its presence is not surprising because, as in the case of 2-phenylethanol and benzaldehyde, it is known to derive from the biotransformation of L-phenylalanine by fungi (Lomascolo et al., 1999). Methylphenyl ketone (acetophenone) was also identified in all samples of *T. borchii* and *T. melanosporum* investigated here. This VOC has been reported, along with the identified compound veratrole (1,2-dimethoxybenzene), to be an oviposition aggregation pheromone to female desert locust (*Schistocerca gregaria*) (Rai et al., 1997). Interestingly, both acetophenone and veratrole were present in *T. melanosporum*, and might be involved in the chemotropism observed with the flies of the genus *Suillia*, known to deposit their eggs on mature fruiting bodies of *T. melanosporum* (Talou et al., 1990). Many of the esters identified are new to truffles and fungi, adding up to a list of 17 esters recently identified from six truffle species by GC/MS at high mass resolution (March et al., 2006). The biological role of these compounds or

Table 1
VOCs produced by the fruiting bodies of three truffle species

Ref	RI	Identity	Mode of Identification[a]	*T. borchii* (TIC peak area)			*T. melanosporum* (TIC peak area)			*T. indicum* (TIC peak area)	Identified in truffle fruiting bodies[b]	Identified in other fungi[c]
				#1	# 2	#1-5	#1	#2	#1-13	#1-5		
Alcohols												
1	603	1-Propanol	RI, MS	6.E+07	–	–	2.E + 07	–	5.E + 07	–	*T. mel* (7), *T. unc.* (5), *T. aest*. (7),	–
2	617	2-Methyl-1-propanol	RI, MS	–	–	–	5.E + 07	2.E+08	2.E+08	–	*T. borc* (5), *T. mel.* (4,5,7), *T. unc*.(5), *T. ind.* (3), *T. aest.* (4,7)	*Lentinellus cochleatus, Pleurotus cornucopiae, Lentinula edodes, Armillaria ostoyae*. . .etc. . . (9)
3	737	3-Methyl-1-butanol	RI, MS, std	2.E+08	3.E+08	6.E+08	1.E + 09	7.E + 08	2.E + 09	4.E + 08	*T. borc.* (1,2,5), *T. mel.* (1,2,4), *T. unc.* (1,5), *T.ind.* (3), *T. aest*. (4)	*Agaricus bisporus, Boletus edulis*. . .*etc*. . . (9)+ *yeasts isolated from truffle fruiting bodies* (8)
4	765	Pentanol	RI, MS	–	–	–	–	–	–	2.E + 07	–	*Marasmius oreades, Ischnoderma benzoinum, Armillaria mellea*. . .*etc*. . .(9)
5	788	2,3-Butanediol	RI, MS	7.E + 06	–	1.E + 07	–	–	–	–	*T. unc.* (5)	*Pleurotus sajor-caju, Gloephyllum odoratum, Micromphale perforans* (9)
6	836	2-Methyl-1-pentanol	RI, MS	–	–	–	–	–	9.E + 07	–	–	–
7	864	1-Hexanol	RI, MS, std	8.E + 06	8.E + 06	3.E + 07	5.E + 07	–	1.E + 08	2.E + 08	*T. mel*.(4), *T. aest.* (4)	*Agaricus bisporus, Boletus edulis, Cantharellus cibarius, Lactarius rufus*. . .etc. . . (9)
8	876	Cyclohexanol	RI, MS	–	–	–	–	–	–	8.E + 06	–	–
9	930	5-Methyl-1-hexanol	MS	–	–	–	2.E + 07	–	1.E + 08	–	–	–
10	964	1-Heptanol	RI, MS	4.E + 06	5.E + 06	1.E + 07	7.E + 06	–	6.E + 07	2.E + 07	*T. aest.* (4)	*Clitocybe nebularis, Marasmius oreades*. . .etc. . . (9)
11	979	1-Octen-3-ol	RI, MS, std	–	–	9.E + 08	1.E + 08	2.E + 08	4.E + 07	7.E + 08	*T. borc.* (1,2,6), *T. mel*.(4), *T.unci* (1), *T.aest*. (4)	*Agaricus bisporus, Boletus edulis, Gyromirta esculenta, Cantharellus cibarius*. . . etc. . .(9)
12	989	6-Methyl-1-heptanol	MS	–	–	–	5.E + 07	–	–	–	–	–
13	994	3-Octanol	RI, MS, std	–	–	7.E + 08	–	–	–	–	*T. mel.* (4), *T. aest.* (4)	*Piptoporus betulinus, Pleurotus ostreatus*. . .etc. . .(9)
14	1019	2-Ethyl-1-hexanol	RI, MS	7.E + 06	3.E + 06	–	–	–	–	–	*T. mel.* (4), *T. aest.* (4)	–
15	1058	*trans*-(2-Ethylcycl-opentyl)methanol	MS	–	–	5.E + 07	–	–	8.E + 07	–	–	–
16	1069	1-Octanol	RI, MS	–	3.E + 06	2.E + 07	–	–	–	2.E + 07	–	Also described in a long list of higher fungi (9) as the other C8 molecules
17	1069	*E*-2-Octen-1-ol	RI, MS	–	–	3.E + 07	–	–	–	8.E + 07	–	Also described in a long list of higher fungi (9) as the other C8 molecules
18	1073	(5-Ethylcyclopent-1-enyl)methanol	MS	2.E + 06	–	8.E + 06	1.E + 07	–	1.E + 07	2.E + 07	–	–

Aldehydes												
19	671	3-Methylbutanal	RI, MS	–	–	–	9.E + 07	6.E + 08	1.E + 08	–	*T. magn.* (1,5), *T. borc.* (1,2,5), *T. mel.* (1,4,5), *T. unc.* (1,5), *T. ind.* (3), *T. aest.* (4)	*Agaricus bisporus, Boletus edulis, Marasmius alliaceus*. . .etc. . . (9)
20	681	2-Methylbutanal	RI, MS	–	–	–	–	2.E + 08	–	–	*T. magn.* (1,5), *T. borc.* (2,5), *T. mel.* (1,4,5), *T. unc.* (1,5), *T. ind.* (3), *T. aest.* (4)	*Marasmius alliaceus* (9)
21	749	*E*-2-Methyl-2-butenal	RI, MS	–	–	–	–	1.E + 08	–	–	*T. borc* (6), *T. mel.* (4), *T. aest.* (4)	–
22	755	*E*-2-Pentenal	RI, MS	–	–	–	–	1.E + 07	–	–	*T. borc.* (1,2)	–
23	760	2-Methylpentanal	MS	–	–	–	–	3.E + 07	–	–	–	–
24	799	Hexanal	RI, MS	–	–	–	1.E + 07	4.E + 07	2.E + 07	–	*T. magn.*(1), *T. borc.* (1,2), *T. mel.* (1,4,5), *T. ind.* (3), *T. aest*. (4)	*Agaricus bisporus, Marasmius oreades, Clitocybe nebularis*. . .etc. . . (9)
25	828	2-Methyl-2-pentenal	MS	–	–	–	3.E + 07	4.E + 08	–	–	*T. magn (1), T. borc.* (1)	–
26	891	Heptanal	RI, MS	–	–	–	–	5.E + 07	–	1.E + 07	*T. magn.*(1), *T. borc.* (1,2), *T. mel.* (1,4), *T. aest.* (4)	*Marasmius oreades, Fomitopsis pinicola*. . .etc. . .(9)
27	917	2-Methylheptanal	MS	–	–	–	4.E + 07	–	7.E + 07	–	–	–
28	943	2-Ethyl-2-hexenal	MS	–	–	–	–	4.E + 07	9.E + 06	–	–	–
29	947	*Z*-2-Heptenal	MS	–	–	–	–	–	–	3.E + 07	–	–
30	964	2-Ethyl-2-hexenal	MS	–	–	–	–	1.E + 08	–	–	–	–
31	996	Octanal	RI, MS	–	–	–	–	–	–	1.E + 07	*T. aest.*(4)	*Pleurotus ostreatus*. . .etc. . . (9)
32	1026	(5-Ethylcyclopent-1-enyl)-methanal	MS	–	–	TRACE	8.E + 07	6.E + 07	4.E + 07	2.E + 08	–	–
33	1056	*trans*-2-Octenal	RI, MS, std	–	–	ND	ND	ND	ND	2.E + 08	2-octenal identified in *T. mel.*(4), *T. aest.* (4)	*Boletus edulis, Lactarius trivialis, Craterellus cornucopioides, Pleurotus ostreatus* (9)
34	1106	2-Isopropyl-5-methylhex-2-enal	MS	–	–	–	4.E + 07	–	–	–	–	–
35	1119	2-Isopropyl-5-methylhex-2-enal	MS	–	–	–	2.E + 07	–	1.E + 07	–	–	–
36	1165	*E*-2-Nonenal	RI, MS	–	–	–	–	–	–	2.E + 07	–	–
37	1223	*E*,*E*-2,4-Nonadienal	RI, MS	–	–	–	–	–	–	2.E + 07	–	–
Aromatic compounds									–			
38	850	Phenylethane (or ethylbenzene)	RI, MS	9.E + 06	5.E + 06	–	–	–	–	–	*T. aest.* (4)	*Agrocybe aegerita* (9)
39	949	Phenylmethanal (or benzaldehyde)	RI, MS	–	–	TRACE	1.E + 08	1.E + 08	3.E + 07	3.E + 07	*T. mel.* (4), *T. unc.*(5), *T. ind* (3), *T. aest.* (4)	*Agaricus bisporus, Boletus edilus, Lactarius rufus, Pleurotus euosmus*. . .etc. . . (9)
40	1006	1-Methoxy-3-methylbenzene (or 3-methyl anisole)	MS	2.E + 07	2.E + 07	–	–	–	–	–	*T. borc.* (6), *T. mel.* (4), *T. unc* (5)	–
41	1030	Phenylmethanol (or benzyl alcohol)	RI, MS	–	TRACE	8.E + 06	5.E + 07	–	4.E + 07	3.E + 07	–	*Agaricus bisporus, Phellinus igniarius, Phallus impudicus, Polyporus tuberaster*. . .etc. . .(9)

(*continued on next page*)

Table 1 (*continued*)

Ref	RI	Identity	Mode of Identification[a]	*T. borchii* (TIC peak area)			*T. melanosporum* (TIC peak area)			*T. indicum* (TIC peak area)	Identified in truffle fruiting bodies[b]	Identified in other fungi[c]
				#1	# 2	#1-5	#1	#2	#1-13	#1-5		
42	1038	2-Phenylethanal (or phenylacetaldehyde)	RI, MS	–	–	–	–	6.E + 07	1.E + 07	2.E + 07	*T. mel.*(4), *T. aest.* (4)	–
43	1064	Methylphenyl ketone (or acetophenone)	RI, MS	3.E + 06	4.E + 06	2.E + 07	3.E + 07	1.E + 07	7.E + 07	–	*T.borc.* (6)	*Agaricus bisporus, Morchella crassipes, Pleurotus euosmus* (9)
44	1104	1-Ethyl-4-methoxybenzene	MS	–	4.E + 06	–	–	–	–	–	–	–
45	1115	2-Phenylethanol	RI, MS, std	5.E + 07	8.E + 07	7.E + 08	4.E + 07	4.E + 07	3.E + 08	8.E + 07	*T. mel.* (4), *T. unci.* (5), *T. aest.* (4)	*Phellinus igniarius, Pleurotus euosmus, Phallus impudicus, Marasmius oreades*. . .etc. . . (9)
46	1152	1,2-Dimethoxybenzene (or veratrole)	RI, MS	–	–	–	1.E + 08	2.E + 08	6.E + 07	–	*T. mel.* (4)	*Phallus impudicus* (9)
47	1179	2-Phenyl-1-propanol	MS	–	–	3.E + 08	–	–	–	–	–	–
48	1190	(1-Ethylpropyl)-benzene	MS	–	–	–	–	1.E + 08	7.E + 07	–	–	–
49	1259	1-Phenyl-1-butanone	RI, MS	–	–	–	2.E + 07	–	–	–	–	–
50	1261	β-Ethylphenethyl alcohol	MS	–	–	–	–	–	7.E + 07	–	–	–
51	1283	2-Phenyl-2-buten-1-al (or α-ethylidene-benzene-acetaldehyde)	MS	–	–	–	2.E + 08	5.E + 08	2.E + 08	7.E + 07	*T. mel.* (4), *T. aest.* (4)	*Phallus impudicus, Boletus edilus, Agrocybe aegerita, Clitocybe nebularis* (9)
52	1287	(1-Propylbutyl)-benzene	MS	–	–	–	–	5.E + 07	3.E + 07	–	–	–
53	1296	1-Methoxy-4-(1-propenyl)-benzene (or *trans*-anethol)	RI, MS	–	–	–	–	–	1.E + 07	–	*T. mel.* (4)	–
54	1310	2-Phenyl-2-buten-1-al (or see no 51)	MS	–	–	–	5.E + 07	9.E + 07	–	–	*T. mel.* (4), *T. aest.* (4)	*Phallus impudicus, Boletus edilus, Agrocybe aegerita, Clitocybe nebularis* (9)
55	1383	2-Phenyl-4-methyl-2-pentenal (or α -2-methylpropylidene-benzeneacetaldehyde)	MS	–	–	–	–	2.E + 07	8.E + 06	–	–	–
56	1387	3-Methyl-*N*-(2-phenylethylidene)-1-butanamine	MS	–	–	–	3.E + 07	–	–	–	–	–
57	1435	1,2,3-Trimethoxy-5-methylbenzene	MS	–	–	–	–	4.E + 07	5.E + 07	–	–	–
58	1484	2-Hydroxy-4-isopropylnaphthalene	MS	–	–	–	–	2.E + 07	8.E + 07	–	–	–
59	1488	5-Methyl-2-phenyl-2-hexenal	RI, MS	–	–	–	2.E + 08	3.E + 08	3.E + 08	–	–	–
60	1521	5-Methyl-2-phenyl-2-hexenal	RI, MS	–	–	–	–	6.E + 07	1.E + 08	–	–	–

Esters												
61	634	Ethyl ethanoate (or ethyl acetate)	RI, MS	4.E + 07	7.E + 07	–	–	–	–	–	*T. borc.* (5), *T. mel.* (4,7), *T. unc.* (5), *T. ind.* (3), *T. aest.* (4,7)	*Pleurotus ostreatus* (9)
62	708	Ethyl propanoate	MS	–	1.E + 07	–	–	–	–	–	*T.mel.* (7), *T. aest* (7)	*Marasmius oreades* (9)
63	755	Ethyl 2-methylpropanoate	RI, MS	4.E + 06	2.E + 07	–	–	–	–	–	–	–
64	839	Ethyl 2-methylbutanoate	RI, MS	1.E + 07	2.E + 07	–	–	–	–	–	*T. mel.* (5,7), *T. aest* (7)	–
65	844	Ethyl 3-methylbutanoate	RI, MS	1.E + 07	4.E + 07	–	–	–	–	–	*T. mel.* (4), *T. aest.* (4)	*Marasmius alliaceus* (9)
66	867	3-Methylbutyl ethanoate	MS	2.E + 07	2.E + 07	–	–	–	–	–	–	–
67	926	Ethyl 2-methyl-but-2-enoate	MS	–	3.E + 07	–	–	–	–	–	–	–
68	966	3-Methylbutyl propanoate	RI, MS	–	–	–	–	–	4.E + 07	–	–	–
69	994	Ethyl hexanoate	RI, MS	–	2.E + 06	–	–	–	–	1.E + 07	–	*Laetiporus sulphureus* (9)
70	1004	2-Methylpropyl 3-methylbutanoate	RI, MS	–	–	–	–	–	2.E + 07	–	*T. mel.* (4)	–
71	1011	3-Methylbutyl 2-methylpropanoate	RI, MS	–	–	–	–	–	4.E + 07	–	*T. mel.* (4)	–
72	1015	2-Methylbutyl 2-methylpropanoate	RI, MS	–	–	–	–	–	2.E + 07	–	*T. mel.* (5)	–
73	1104	3-Methylbutyl 2-methylbutanoate	RI, MS	–	–	5.E + 07	–	–	5.E + 07	1.E + 07	–	–
74	1110	3-Methylbutyl 3-methylbutanoate	MS	–	–	2.E + 08	–	–	1.E + 08	7.E + 07	–	–
75	1113	2-Methylbutyl 3-methylbutanoate	RI, MS	–	–	–	–	–	–	2.E + 07	–	–
76	1572	2,4,4-Trimethylpentane-1,3-diyl bis(2-methylpropanoate)	MS	–	2.E + 07	–	–	–	6.E + 08	–	–	–
77	1746	1-Methylethylte-tradecanoate	MS	–	8.E + 06	–	–	–	–	–	–	–
Furanes and furanones												
78	904	5*H*-Furan-2-one	MS	3.E + 06	5.E + 06	9.E + 06	–	–	–	–	–	*Clitocybe odora* (9)
79	945	Dihydro-4-methyl-3*H*-furan-2-one (or 4-methyloxolan-2-one)	MS	2.E + 06	2.E + 06	–	–	–	–	–	–	C4 and C6-Lactones described in *Polyporus durus, Tyromyces sambuceus, Ischnoderma benzoinum* (9)
80	1036	4-Methyl-5*H*-furan-2-one	MS	2.E + 07	1.E + 07	3.E + 07	–	–	–	–	–	–
81	1225	3-Phenyl-furan	RI, MS	–	–	TRACE	8.E + 06	1.E + 07	2.E + 07	–	–	–
82	1372	Dihydro-5-pentyl-3*H*-furan-2-one	MS	–	–	–	–	–	1.E + 07	–	–	C4 and C6-Lactones described in *Polysporus durus, Tyromyces sambuceus, Ischnoderma benzoinum* (9)

(*continued on next page*)

Table 1 (*continued*)

Ref	RI	Identity	Mode of Identification[a]	*T. borchii* (TIC peak area)			*T. melanosporum* (TIC peak area)			*T. indicum* (TIC peak area)	Identified in truffle fruiting bodies[b]	Identified in other fungi[c]
				#1	# 2	#1-5	#1	#2	#1-13	#1-5		
Hydrocarbons											–	
83	566	2-Methyl-1,3-butadiene	MS	1.E + 08	9.E + 07	–	–	–	–	–	*T. borc* (5)	–
84	619	Hexane	RI, MS	2.E + 07	9.E + 07	–	2.E + 07	6.E + 07	–	–	*T. borc.* (1,2), *T. ind* (3)	–
85	991	4-Methyl-1,3-heptadiene	MS	–	–	–	–	5.E + 07	–	–	–	–
86	1043	3,7-Dimethyl- 1,3,7-octatriene (or ocimene)	RI, MS	2.E + 06	4.E + 06	2.E + 07	–	–	–	–	*T. borc.* (5,6)	*Phallus impudicus, Clathrus ruber* (9)
87	1400	Tetradecane	RI, MS	1.0 E7	2.E + 06	–	–	–	–	–	*T. borc* (5)	–
Ketones												
88	634	2-Butanone	RI, MS	–	–	–	2.E + 07	–	4.E + 07	–	*T. mel.* (4), *T. aest.* (7)	–
89	700	2-Pentanone	MS	TRACE	–	–	3.E + 07	7.E + 07	4.E + 07	–	–	–
90	716	3-Hydroxy-2-butanone	RI, MS	–	–	–	–	–	–	2.E + 07	*T. mel.* (4)	*Hericium erinaceus; Pleurotus sajor-caju; Agrocybe aegerita* (9)
91	788	2-Hexanone	RI, MS	–	–	–	1.E + 07	8.E + 06	–	–	–	*Tricholoma caligatum* (9)
92	834	3-Hexen-2-one	MS	–	–	–	–	6.E + 07	–	–	–	–
93	883	2-Heptanone	RI, MS	–	–	–	–	–	–	1.E + 07	–	*Boletus edulis*. . .etc. . .(9)
94	930	2,2,4,4-Tetramethyl-3-pentanone	MS	–	–	–	–	–	1.E + 07	–	–	–
95	949	6-Methyl-2-heptanone	MS	–	–	–	–	–	3.E + 07	–	–	–
96	970	1-Hepten-3-one	RI, MS	–	–	–	–	–	–	3.E + 07	–	–
97	977	2-Undecanone	MS	–	3.E + 06	–	–	–	–	–	*T. mel.* (4), *T. aest.* (4)	–
98	979	3-Octanone	RI, MS, std	5.E + 06	6.E + 06	8.E + 08	5.E + 07	–	4.E + 08	1.E + 08	*T. borc.* (5), *T. mel.* (4), *T. unci. (*1,5), *T. aest.* (4)	*Agaricus bisporus, Boletus edulis, Cantharellus cibarius, Gyromirta esculenta*. . .etc. . . (9)
99	987	2-Octanone	RI, MS	–	–	9.E + 06	–	–	–	4.E + 07	*T. aest.* (4)	–
100	1036	*E*-3-Octen-2-one	RI, MS	–	–	–	–	–	–	2.E + 07	3-octen-2-one identified in *T. mel.* (4)	*Marasmius oreades, Lepista nuda* (9)
101	1045	4,6-Dimethyl-2-heptanone	MS	–	–	–	–	–	–	6.E + 06	–	–
102	1093	2-Nonanone	RI, MS	5.E + 07	–	6.E + 07	–	–	2.E + 07	–	–	–
103	1305	2-Undecanone	RI, MS	–	–	–	–	–	–	2.E + 07	*T. mel.* (4), *T. aest.* (4)	–
104	1455	6,10-Dimethyl-5,9-undecadien-2-one (or geranylacetone)	RI, MS	–	–	–	–	–	1.E + 07	–	–	*Chroogomphus rutilus, Suillus granulatus, Cantharellus tubaeformis* (9)
105	1479	4-(2,6,6-Trimethyl 2-cyclohexen-1-yl)-3-methyl-3-buten-2-one (or α-isomethyl ionone)	RI, MS	–	–	2.E + 07	–	–	–	–	–	–

Nitrogen													
106	755	Pyridine	RI, MS	–	–	–	7.E + 07	–	–	–	–	–	
107	1194	2-Nonenenitrile	MS	–	–	–	9.E + 06	–	8.E + 06	–	–	–	
Sulfur													
108	743	Methyldisulfanyl-methane (or dimethyldisulfide)	RI, MS	2.E + 07	–	–	–	–	–	–	*T. magn.* (1,5), *T. mel.* (1,4), *T. unc.* (1), *T. aest.* (4)	*Marasmius alliaceus* (9) + yeasts isolated from truffle fruiting bodies (8)	
109	716	3-Methylthiophene	MS	1.E + 08	4.E + 07	–	–	–	–	–	–	–	
110	896	2-Methyl-4,5-dihydrothiophene	MS	9.E + 08	3.E + 08	1.E + 08	–	–	–	–	*T. borc.* (1,2,6)	–	
111	896	3-Methylsulfanyl-propanal (or 3-methylthiopropanal)	RI, MS	–	–	–	–	3.E + 07	–	4.E + 06	–	*Laetiporus sulphureus* (9)	
112	917	1-Isothiocyanato-butane	RI, MS	3.E + 06	2.E + 06	–	–	–	–	–	–	–	
113	928	4-Ethyl-5-methylthiazole	MS	8.E + 06	4.E + 06	–	–	–	–	–	–	–	
114	953	Methylsulfanyldi-sulfanylmethane (or dimethyltrisulfide)	RI, MS	–	–	–	1.E + 07	2.E + 07	–	–	*T. magn.* (1,5), *T. mel.* (1,4), *T. aest.*(4)	*Lentinula edodes, Phallus impudicus*...etc... (9) + yeats isolated from truffle fruiting bodies (8)	
115	972	3-Methyl-sulfanylpropanol	RI, MS	1.E + 07	2.E + 07	–	–	–	5.E + 07	–	–	*Laetiporus sulphureus* (9) + yeasts isolated from truffle fruiting bodies (8)	
116	1047	Containing 1-methylsulfanylbutane (or 1-methylthiobutane)	MS	–	–	–	–	3.E + 07	–	–	–	–	
117	1106	Methylsulfanyl-cyclohexane	MS	–	–	–	4.E + 07	1.E + 08	–	–	–	–	
118	1141	5-Methyl-3*H*-1,2-dithiol-3-one	MS	9.E + 07	9.E + 07	2.E + 08	–	–	–	–	*T. borc.* (6)	–	
119	1201	Methylsulfanyl-cyclopentane	MS	2.E + 07	3.E + 06	6.E + 07	–	–	–	–	–	–	

VOCs produced by three samples of *T. borchii*, three of *T. melanosporum* and one of *T. indicum*. Values (TIC peak areas) are the average of two or three stir bars per sample and are reported with their Kovats indices (RI). Volatiles were identified by one or more method mentioned under column (a) comparison of their Kovàts-indices (HP-5 column) to literature data (RI); comparison of their MS data to the NIST'98 database (MS), and when available with authentic standards run under similar GC-MS conditions (std). The data was compared to recent literature on truffle volatiles (b) (for *T. magn.*= *T. magnatum; T. borc.* = *T. borchii; T. mel.* = *T. melanosporum, T. unc.* = *T. uncinatum; T. ind.* = *T. indicum; T. aest.* = *T. aestivum*) and fungal volatiles (c) [literature on truffles: (1) Bellesia et al., 1996; (2) Bellesia et al., 2001; (3) Bellesia et al., 2002; (4) Díaz et al., 2003; (5) Mauriello et al., 2004; (6) Zeppa et al., 2004; (7) March et al., 2006. Literature on yeasts and higher fungi: (7) Buzzini et al., 2005; (8) Chiron and Michelot, 2005]. For each VOC, generally only 3 species were listed in column (c). Refer to (8) for an exhaustive list. ND= present in the sample but not determined cause co-eluted with another compound. TRACE = present at trace levels.

their involvement in the final aroma of truffles remains unknown. Of the furanones, four new lactones: 5*H*-furan-2-one, dihydro-4-methyl-3*H*-furan-2-one; 4-methyl-5*H*-furan-2-one and dihydro-5-pentyl-3*H*-furan-2-one were identified from *T. borchii* and *T. melanosporum.* Microbial lactones are generally present in trace amounts in plants, making them a prime research target of the food industry (Krings and Berger, 1998). Of the ketones, two isoprenoids, geranylacetone (6,10-dimethyl-5,9-undecadien-2-one) and α-isomethyl ionone (4-(2,6,6-trimethyl 2-cyclohexen-1-yl)-3-methyl-3-buten-2-one) were identified for the first time in one sample of *T. melanosporum* and one sample of *T. borchii*, respectively. Zeppa et al. (2004) demonstrated that the presence of some isoprenoid VOCs in the fruiting bodies of *T. borchii* depends on their maturity. Additionally, Gabella et al. (2005) observed in mature *T. borchii* fruiting bodies an increase in the expression of three genes involved in isoprenoid synthesis. Such a difference in maturity could consequently explain why geranylacetone and α-isomethyl ionone were detected in one sample out of three. Finally, a total of 12 sulfur compounds could be identified (Table 1). Out of them, five were described for the first time in truffles: 3-methyl-sulfanyl-propanal; 1-isothiocyanatobutane; 5-ethyl-4-methyl-1,3-thiazole; methylsulfanylcyclohexane and methylsulfanyl-cyclopentane. They certainly are major contributors to the final bouquet of truffles as sulfur containing compounds are known for their very low olfactory threshold.

With 70 newly described VOCs in truffles belonging to nine different groups (Table 1), HS-SBSE is certainly a technique of choice to hunt for new compounds, even for organisms such as truffles which aroma has been already extensively studied.

2.2. VOCs profile variation and species markers

The fruiting body VOCs identified in this study were classified in groups according to Table 1, and each group was expressed as the percentage of the total VOC content (Figs. 2 and 3). The VOC profile of *T. melanosporum* and *T.indicum* was dominated by alcohols (48–57%), aldehydes (4–27%) and aromatic compounds (9–30%) (Fig. 2), whereas the profile of *T. borchii* was dominated by alcohols (16–47%) and sulfur compounds (7–59%) (Fig. 3). Only trace amounts of aldehydes could be detected in *T. borchii* (Table 1 and Fig. 3).

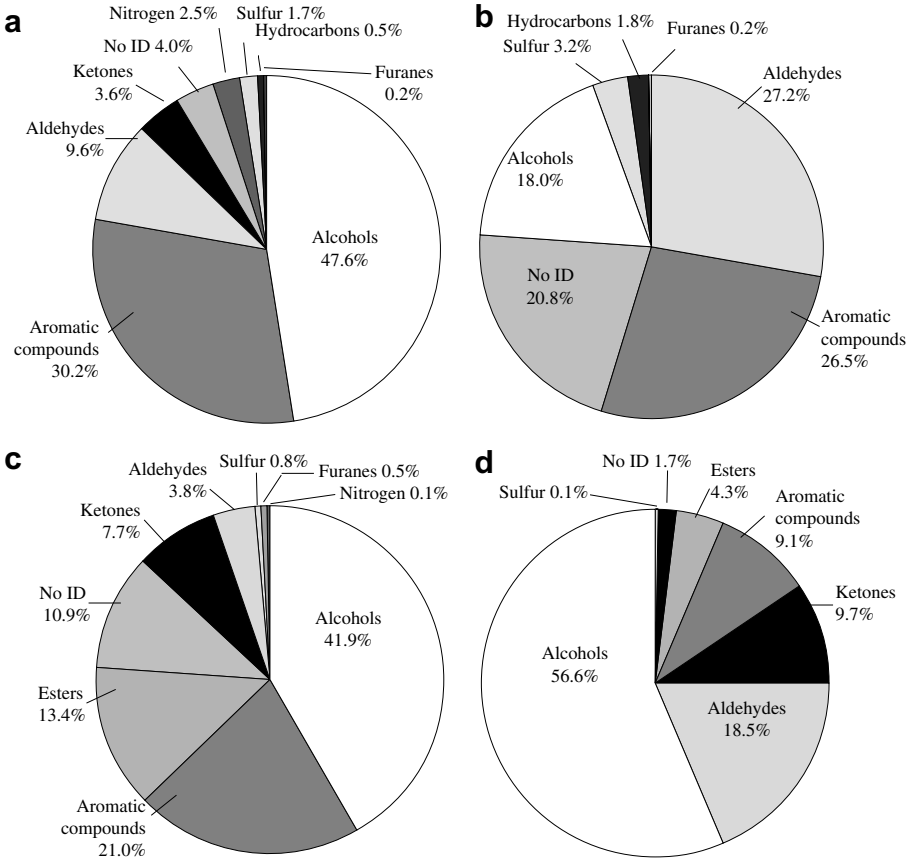

Fig. 2. Variability of VOCs profile in fruiting bodies of *T. melanosporum* and *T. indicum*. The VOC profile of *T. melanosporum* (a = #1, b = #2, c = #1–13) and *T. indicum* (d = #1–5) was dominated by alcohols, aldehydes and aromatic compounds. Note the relatively low amount of sulfur compounds in *T. indicum* #1–5 (d) as compared to the other *T. melanosporum* samples (a–c). No ID = not identified compounds.

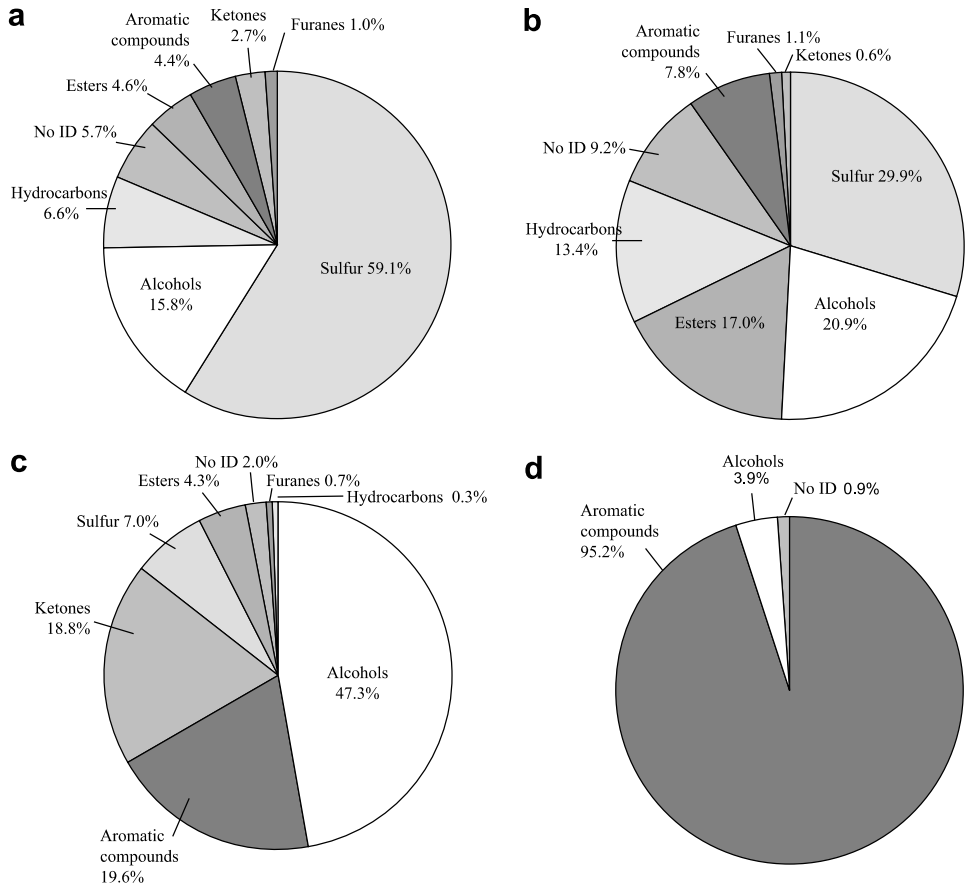

Fig. 3. VOCs profile of fruiting bodies and mycelium of *T. borchii*. The VOC profile of all three samples of fruiting bodies of *T. borchii* (a = #1, b = #2, c = #1–5) was dominated by alcohols and sulfur compounds. In contrast, aromatic compounds and alcohols made up the major volatiles produced by the mycelium of *T. borchii* (strain ATCC 96540) when grown on PDA (d). No ID = not identified compounds.

The high inter-specific variation in VOCs profiles observed in this study can be attributed to four factors. (i) The influence of the geographical origin as suggested by Bertault et al. (1998) for *T. melanosporum*, and exemplified by Díaz et al. (2003) who demonstrated that such variation could be observed between two samples of *T. aestivum*, collected from different geographical regions in Spain. (ii) The genetic variability documented for *T. melanosporum* (Murat et al., 2004) and *T. magnatum* (Mello et al., 2006). (iii) The third factor, fruiting body maturity, plays a major role in the qualitative composition of the aroma (Zeppa et al., 2004). (iv) Finally, storage conditions such as temperature influence the aroma evolution of truffle. Bellesia et al. (2001) indeed observed that the two sulfur compounds: 3-methyl-1-(3-methylbutyldisulfanyl)butane and 2-methyl-4,5-dihydrothiophene where evolved upon storage of *T. borchii* at room temperature. The large amount of sulfur compounds in our samples collected in 2006 (59% in *T. borchii* #1, Fig. 3a and 30% in *T. borchii* #2, Fig. 3b) compared to those collected in 2004 (7% in *T. borchii* #1–5) suggests that those samples spent more time at room temperature before freezing (i.e. due to shipping).

If the aromatic profiles of the three truffle species investigated here presented a high intraspecific and interspecific variation, they also shared common features. Some VOCs such as 3-methyl-1-butanol and 2-phenylethanol were identified in every single sample analyzed of *T. melanosporum*, *T. borchii* and *T. indicum* (Table 2). On the other hand, four VOCs: 1,2-dimethoxybenzene; 2-phenyl-2-buten-1-al; 5-methyl-2-phenyl-2-hexenal and one unidentified compound where specific to *T. melanosporum* or *T. indicum* and four other VOCs were specific to *T. borchii* [5*H*-furan-2-one; 4-methyl-5*H*-furan-2-one; ocimene (3,7-dimethyl-1,3,7-octatriene) and 2-methyl-4,5-dihydro-thiophene] (Table 2). If detected in a significant number of samples, those volatiles could serve as markers helpful to distinguish among the three truffle species in food products and could complement the DNA based method recently developed by Mabru et al. (2001) and Douet et al. (2004) to discriminate between the French *T. melanosporum* and the Chinese *T. indicum*, which are difficult to tell apart based solely on morphological criteria (Riousset et al., 2001; Douet et al., 2004). March et al. (2006), investigating the VOCs of six truffle species (one single sample per species), suggested that some esters could be

Table 2
Common and species-specific volatiles

Ref.	RI	Identity	*T. borchii*			*T. melanosporum*			*T. indicum*
			#1	#2	S#1–5	#1	#2	S#1–13	S#1–5
In all fruiting bodies									
3	737	3-Methyl-1-butanol	×	×	×	×	×	×	×
45	1115	2-Phenylethanol	×	×	×	×	×	×	×
In T. indicum and/or T. melanosporum only									
46	1152	1,2-Dimethoxybenzene				×	×	×	
51	1283	2-Phenyl-2-buten-1-al				×	×	×	×
59	1488	5-Methyl-2-phenyl-2-hexenal				×	×	×	
×××	965	No ID				×	×	×	
In T. borchii only									
78	904	5*H*-Furan-2-one	×	×	×				
80	1036	4-Methyl-5*H*-furan-2-one	×	×	×				
86	1043	3,7-Dimethyl-1,3,7-octatriene (ocimene)	×	×	×				
110	896	2-Methyl-4,5-dihydrothiophene	×	×	×				

Compounds such as 3-methyl-1-butanol and 2-phenylethanol where detected in every single sample analyzed (single fruiting body or pool of fruiting bodies from the same species), while other compounds where species specific and could serve as potential markers for species identification if truffle based foods (i.e. truffle oil, truffle juice) where morphological identification is no longer possible (RI = Kovàts-indices on the HP-5 column; Ref. = references to Table 1).

used to distinguish certain species from the others (i.e. ethyl 4-methylpentanoate for *T. melanosporum*). Yet none of the esters identified in this study were common to the fruiting bodies of a single species (Tables 1 and 2), suggesting that their presence/absence might be random and eventually due to other factors than the geographical origin.

For each pooled sample (*T. borchii* #1–5, *T. melanosporum* #1–13, *T. indicum* #1–5), we quantified the major VOCs with external standards (Table 3). For *T. borchii* they accounted for $79 \pm 20\%$ ($\pm$standard deviation) in terms of TIC peak area percentage and 157 ± 53 [$\mu g\ g^{-1}$ of fruiting body] in terms of quantity; for *T. melanosporum* $52 \pm 15\%$ and 228 ± 29 [$\mu g\ g^{-1}$ fruiting body], respectively, and $71 \pm 17\%$ and 82 ± 17 [$\mu g\ g^{-1}$ fruiting body] for *T. indicum* (Table 3). Such a difference in aroma intensity between *T. melanosporum* and *T. indicum* confirms the observations of Bellesia et al. (2002), who furthermore stressed that no sulfur compounds was detectable in *T. indicum*. On the contrary we could identify one sulfur compound from *T. indicum* (3-methylsulfanylpropanal, Table 1); however, it only accounted for 0.1% of the total aroma, in other words 8–32 times less than the sulfur compounds of *T. melanosporum* (Fig. 2).

Table 3
Quantification of major VOCs produced by the fruiting bodies of three truffle species

Ref.	Identity	Quantity ($\mu g\ g^{-1}$ fruiting body)					
		T. borchii #1–5		*T. melanosporum* #1–13		*T. indicum* #1–5	
3	3-Methyl-1-butanol	59 ± 44	a	184 ± 23	b	33 ± 18	a
7	1-Hexanol	3.0 ± 1.4	a	14.3 ± 1.9	b	17 ± 3	b
11	1-Octen-3-ol	19.5 ± 2.4	a	0.81 ± 0.02	b	15.6 ± 1.6	c
13	3-Octanol	5.8 ± 0.5	a	–	b	–	b
32	(5-ethylcyclopent-1-enyl)-methanal	–	–	NQ	–	NQ	–
33	*trans*-2-Octenal	BD	–	BD	–	7 ± 4	–
45	2-Phenylethanol	70 ± 12	a	28 ± 5	b	8 ± 5	c
76	2,4,4-Trimethylpentane-1,3-diyl bis(2-methylpropanoate)	–	–	NQ	–	–	–
98	3-Octanone	16 ± 3	a	6.8 ± 1.0	b	1.84 ± 0.16	c
	Total quantity	157 ± 53	a	228 ± 29	a	82 ± 17	b

VOCs produced over a period of 63 h by 1.0 g of fruiting body. A VOC was defined as major when its TIC pick represented more than 5% of the Total TIC Pick Area at least in one species. Reference numbers on the left are according to Table 1. Of the three truffle species, *T. melanosporum* and *T. borchii* produced significantly more VOCs than *T. indicum*. Values in $\mu g\ g^{-1}$ of fruiting body +/− standard deviations ($n = 3$) were calculated with the specific calibration curved obtained for each VOCs. Different letters indicate statistically significant differences among the three species for a given VOC or for the total quantity of VOCs ($P \leqslant 0.05$; Mann–Whitney test). NQ = not quantified. BD = compound not quantified cause co-eluted with another VOC.

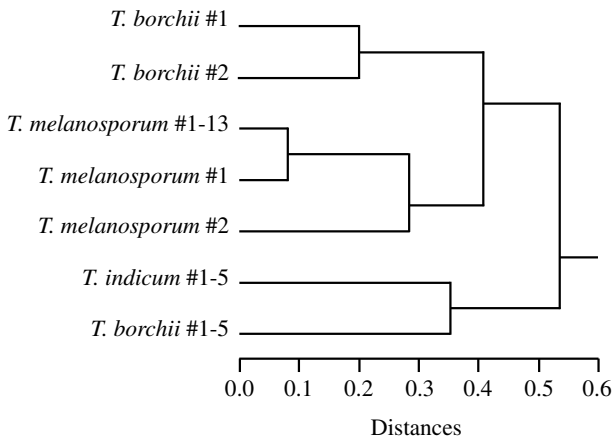

Fig. 4. Cluster analysis of VOCs emitted by *T. melanosporum*, *T. borchii* and *T. indicum*. A clear distinction is present between the VOC pattern of *T. melanosporum* and the other two truffles. *T. indicum* shows a close statistical linkage with *T. borchii* collected in 2004 (see text for explanations).

Investigation of the aromatic profile of three truffle species by HS-SBSE led to the identification of some species-specific markers and to the quantification of the major VOCs allowing to further distinguish/rank the aromatic intensity of those species.

Fig. 4 shows the cluster analysis (CA) calculated on the data matrix of Table 1 by using Pearson distances with single linkage method. Three clusters are evident: the first cluster is characterized by the two samples of *T. borchii* collected in 2006 (#1 and #2), the second cluster gathers all *T. melanosporum* samples, whereas the third cluster is made by *T. indicum* and the sample of *T. borchii* collected in 2004. The results of the CA clearly show the distinction between *T. melanosporum* and *T. indicum* based on VOC pattern. The fact that the sample of *T. borchii* collected in 2004 (#1–5) does not cluster with the two other sample of *T. borchii* collected in 2006 (#1 and #2) is due to important concentration differences in four VOCs (1-octen-3-ol, 3-octanone, 2-phenylethanol and 2-phenylpropanol). Indeed the three *T. borchii* samples cluster together when those volatiles are omitted for *T. borchii* #1–5 (data not shown).

In recent years many Phylograms based on ribosomal genes and particularly on ITS sequences have been constructed to illustrate the relationships existing among the truffle species. Roux et al. (1999) clearly demonstrated that *T. melanosporum* and *T. indicum* belong to the same clade, while *T. borchii* belongs to the "whitish truffles" clade. On one side, there is a good congruency between information originating from both genetics and phenetic (aroma CA) analyses, since the *T. borchii* and *T. melanosporum* samples are separated. On the other side, the linkage between *T. borchii* (#1–5) and *T. indicum* (#1–5) confirms that the genetic background is not the only factor determining the aroma.

2.3. Mycelium versus fruiting body VOCs

Truffle fruiting bodies are exclusively sampled in natural conditions, were bacteria, yeasts and also insects usually colonize hyphal structures. The third aim of this paper was therefore to discriminate the VOCs emitted by the fungus from those of microbial origin (yeasts and bacteria). Because the metabolites production of a given organism is highly dependant on cultural conditions such as media composition, physico-chemical parameters such as light, O_2 and temperature (Bode et al., 2002; Sunesson et al., 1995), we investigated which VOCs the mycelium of *T. borchii* (strain ATCC 96540) would produce under two cultural conditions. A total of eight compounds were detected (Table 4), six from the mycelium grown on the solid media PDA and two from liquid Media A. Those VOCs included a C_5 alcohol, 3-methyl-1-butanol; a C_8 alcohol, 1-octen-3-ol, the latter being a fungal hormone known to regulate sporulation in *Penicillium paneum* (Chitarra et al., 2004); a C_8 ketone, 3-octanone which along with 1-octen-3-ol is responsible of the typical fungal smell of most fungi (Venkateshwarlu et al., 1999; Wnouk et al., 1983); a group of four aromatic compounds, including benzaldehyde, the bitter almond odor derived from L-phenylalanine oxidative degradation (Krings and Berger, 1998), and an unidentified compound. Of those eight VOCs, seven were also identified in the fruiting bodies of various *Tuber* species (Table 4), but interestingly only one (3-octanone) was identified in a previous study on the VOCs of *T. borchii* mycelium (Tirillini et al., 2000). The eight VOCs listed above were produced in pure culture, therefore confirming their truffle origin.

Table 4
VOCs produced by *T. borchii* mycelium

Ref.	RI	Identity	TIC peak area		Detected in *Tuber* ssp. fruiting bodies
3	737	3-Methyl-1-butanol	9.E + 06	a	x
xxx	939	No ID	2.E + 06	a	x
11	979	1-Octen-3-ol	9.E + 07	b	x
39	949	Phenylmethanal	4.E + 07	a	x
41	1030	Phenylmethanol	3.E + 06	a	x
45	1115	2-Phenylethanol	4.E + 07	a	x
98	979	3-Octanone	3.E + 07	b	x
xxx	1064	Benzyl methanoate	2.E + 08	a	–

VOCs produced over 63 hours by strain ATCC 96540 grown under two different conditions (a = Potato dextrose agar, 20 ml flask; b = Liquid media A, 100 ml Erlenmeyer). Note that the VOC profile is highly dependant on the cultural conditions. Values (peak areas) are the average of 3 biological replicates (2 stir bars per replicates). X indicates that the VOCs have been detected in the fruiting bodies from various truffle species according to Table 1. (RI = Kovàts-indices on the HP-5 column; Ref. = reference to Table 1).

The highest variety of VOCs found in the fruiting bodies has at least three causes. The first reason is purely technical as some of the VOCs produced by the mycelium might have been masked in the chromatogram by the background signal due to the agar/liquid media. Second, some metabolic pathways may only be activated in fruiting bodies as demonstrated by Gabella et al. (2005). Third, fruiting bodies contain a variety of organisms including bacteria (Barbieri et al., 2005) and yeasts (Buzzini et al., 2005), all able to produce VOCs or possibly to trigger fungal VOC biosynthesis. This "mixed" VOCs origin is furthermore supported by the presence of two indicators of bacterial activity, acetoin (3-hydroxy-2-butanone) and 2,3-butanediol, observed in the fruiting bodies of two samples of *T. borchii* and one of *T. indicum* (Table 1). Additionally, some VOCs produced by the mycelium such as 3-methyl-1-butanol were also synthesized in pure cultures by yeasts isolated from the fruitbodies of *T. melanosporum* and *T. magnatum* (Buzzini et al., 2005). 3-Methyl-1-butanol might consequently be of mixed origin in the fruiting body.

Comparing the VOCs production of *T. borchii* mycelium grown on PDA with the one of the fruiting body of the same species, it is striking to note that on PDA, the dominant VOCs are made up of 95% aromatic compounds and 4% alcohols, while in the fruiting bodies alcohols are generally more represented than aromatic compounds (Fig. 3). Another major difference is that no sulfur compounds were identified in pure mycelial cultures, while in fruiting bodies of *T. borchii* they represented from 7% to 59% of the total VOCs. Yet, Tirillini et al. (2000) identified one sulfur compound (dimethyltrisulfide) from the mycelium of *T. borchii*, confirming that VOCs emission highly depends on cultural conditions. Nevertheless, as for 3-methyl-1-butanol, dimethyltrisulfide (along with 3-methylsulfanylpropanol) have also been described in yeasts isolated from truffle fruiting bodies (Buzzini et al., 2005), and might consequently also be of mixed origin in the fruiting bodies.

In conclusion, it is reasonable to argue that in the fruiting body, the seven VOCs listed in Table 4 could be (at least partially) of mycelial origin.

3. Conclusions

Investigation of three truffle species by HS-SBSE/GC–MS has permitted to identify a large number of compounds to our knowledge never described before in the truffles species investigated here. The technique is therefore a powerful tool to identify new VOCs. The high intra- and inter-specific variability already documented in earlier studies was clearly exemplified here and raises concerns about the difficulties faced when developing an electronic nose or a truffle synthetic aroma. However, a clear distinction was obtained between *T. melanosporum* and *T. indicum* based on VOC pattern. Finally, by comparing VOCs emitted by the mycelium with those emitted from the fruiting bodies, a list of compounds of mycelial origin could be distinguished from those VOCs of mixed origin. Such an approach could be of special interest to the food industry in order to produce in pure culture truffle natural aroma from, i.e. mycelium and yeasts previously isolated from fruiting bodies.

4. Experimental

4.1. Tuber fruiting bodies identification and sample preparation

Fruiting bodies of *T. melanosporum* (13 fruiting bodies) and *T. borchii* (seven fruiting bodies) – both from northern Italy (Piedmont) – were collected in 2004, and 2004 and 2006, respectively; *T. indicum* (five fruiting bodies), from Yunnan and Sichuan Provinces (China) in 2004. All samples were washed free of soil and frozen until VOCs extraction.

Species identification was confirmed for every single fruiting body on the basis of carpophore morphology and spore shape, as well as PCR amplification (ITS 1F, ITS4) of two fruiting bodies taken at random for each species (Murat et al., 2004).

Small fruiting bodies pieces (~0.05–0.10 g of peridium and gleba) were cut off to reach 1.0 g (made of either one single fruiting body, or pooled from different fruiting bodies of the same species in order to minimize VOCs variations due to (i.e.) maturity or origin). In details, five fruiting bodies of *T. borchii* (2004) where pooled (#1–5), while two others (2006) were analyzed separately (#1 and #2); 13 fruiting bodies of *T. melanopsorum* (2004) were pooled (#1–13), while two (2004) were analyzed separately (#1 and #2); finally, five fruiting bodies of *T. indicum* (2004) where pooled (#1–5).

4.2. Fruiting bodies extraction method

The sample (1.0 g) was placed in a 20 ml SMPE Teflon sealed vial (SUPELCO), and left for 2 h at room temperature for equilibration. Two or three stir bars (Twister from Gerstel, 0.5 mm thick, 10 mm long, polydimethylsiloxane (PDMS) coating) were suspended in the headspace onto an iron pin (Fig. 1). Extraction was performed in the dark at 23 °C. Out of the different extraction procedures tested (static and dynamic head space, extraction times: 3 h and 63 h), 63 h (static head-space) resulted in the highest signal in terms of total ion chromatogram intensity. Besides in static head-space mode, the truffle aroma remained rather unaltered to the nose during the first 63 h, and this extraction procedure was consequently adopted. An empty SPME vial with two SB was used as the control for each experiment. Before VOCs extraction, all glassware was thoroughly washed and dried at 150 °C for 3 h to avoid any organic contamination.

4.3. Tuber mycelium and culture conditions

Tuber borchii mycelium (strain ATCC 96540, provided by Prof A. Zambonelli, University of Bologna, Italy) was cultured under two conditions:

Condition 1: As a 1.0 cm diameter mycelial plug grown on 2.0 ml of potato dextrose agar (PDA – Fluka – 30 g/l, pH 6.3) in a 20 ml cotton sealed SPME flask. The mycelium was grown for 12 days at 23 °C in the dark prior to VOCs extraction.

Condition 2: Ten mycelial plugs (0.8 cm diameter) grown in 40 ml liquid Media A (woody plant medium (Lloyd and McCown, 1980) modified for the quantity of sugar: D(+)-glucose: 5.0 g/l; saccharose: 5.0 g/l, and with no agar, solution pH 6.3) in a 100 ml cotton sealed Erlenmeyer. The mycelium was grown for 30 days at 23 °C in the dark prior to VOCs extraction.

4.4. Mycelial VOCs extraction methods

Condition 1: Extraction was performed with two SB for 63 h with the SPME vial sealed with a Teflon septum under the exact same conditions as for the fruiting bodies (Fig. 1). A SPME vial containing no mycelium (PDA only) was used as the control.

Condition 2: Extraction was performed at 23 °C in the dark for 15 days with two stir bars suspended by an iron pin into the headspace of the flask (sealed with cotton). An Erlenmeyer containing liquid Media A and 10 agarized plugs was used as a control.

4.5. GC/MS conditions

The stir bar was introduced in the Gerstel "Twister Desportion Unit" operating under the following conditions. He flux of 50 ml/min. Splitless mode. Temperature: start 36 °C – hold for 0.5 min, ramp of 25 °C/min to 260 °C – hold for 5 min. Transfer temperature to the "Cooled Injection System" (CIS): 260 °C. CIS conditions: initial temperature −50 °C – hold for 0.2 min, ramp of 12 °C/s to 290 °C – hold for 3 min. Splitless mode for the first 6 min, then split 1:20.

Column: HP 5 (HP 19091J-433) – 5% diphenyl, 95% dimethyl siloxane – oven conditions: starting temperature: 50 °C, ramp of 3 °C/min to 200 °C, hold for 10 min; then ramp of 10 °C/min to 290 °C, hold for 10 min. Helium (1.0 ml/min) was used as a carrier gas. Quadrupole detector in autotune mode, ion M+, operating at 70 eV (MS source 150 °C, MS quad 230 °C).

4.6. Authentic standards and calibration

The following authentic standards where purchased from Sigma. 3-Methyl-1-butanol, 99+%; 1-hexanol; *trans*-2-octenal, 94+%; phenylethyl alcohol, 99+%; 1-octen-3-ol; 3-octanone; 3-octanol, 99%.

For calibration, ~0.5 g cotton (cosmetic use) was placed in the bottom of a SPME vial, with two SB suspended in the headspace as described for the fruiting bodies extraction (Fig. 1). A volume of 10 µl of a mixtures of synthetic standards diluted in cold CH_2Cl_2 was applied to the cotton with a GC syringe (to reach for every single standard final concentrations between 0.002 and 5.0 ppm per volume in the 20 ml SPME vial). Extraction conditions where exactly the same as for the fruiting bodies. Calibration for each standard was preformed at least at three different concentrations, with two SB per concentration ($n \geqslant 6$).

4.7. Statistical analyses

VOCs identification was performed by comparison with spectra in mass spectra databases (NIST 98®), comparison of Kovàts retention indices to the literature data (http://www.pherobase.com/database/kovats/kovats-index.php; http://www.flavornet.org/flavornet.html, and an in-house database), and in some cases direct GC–MS comparison with authentic standards. At least three replicates were performed for each experiment. VOCs quantification data were statistically compared by the Mann–Whitney test (SPSS Software). Cluster analysis was calculated by using the data matrix of Table 1 with the Systat10 software. Pearson distance and single linkage method were used.

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