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Volatile compounds from the fruiting bodies of beefsteak fungus *Fistulina hepatica* (Schaeffer: Fr.) Fr.

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Abstract

The volatile compounds from the fruiting bodies of wild *Fistulina hepatica* (Schaeffer: Fr.) Fr. were isolated by continuous liquid liquid extraction (CLLE), and investigated by high resolution gas chromatography–mass spectrometry (HRGC–MS), GC-atomic emission detector (GC-AED), and gas chromatography-olfactometry (GC-O). Forty eight major volatile compounds were identified and semi-quantified. 11 odorous compounds significantly contributed to the overall flavour of *F. hepatica*: 1-octen-3-one, 1-octen-3-ol, linalool, phenylacetaldehyde, butanoic acid, an unidentified volatile compound with mouldy odour, (*E*)-2-methyl-2-butenoic acid, (*E*)-methyl cinnamate, (*Z*)-9-hexadecenoic acid methyl ester, bisabolol oxide B and phenylacetic acid.

Keywords: *Fistulina hepatica*; Volatile compounds; GC-O; HRGC–MS; Flavour

1. Introduction

Fistulina hepatica (Schaeffer: Fr.) Fr. is an annual edible fungus of the class basidiomycetes, seven to 30 cm wide and two to 8 cm thick, reddish to brown, fleshy and juicy, with a slightly sour tannic taste. It is distributed in temperate and subtropical hardwood forest ecosystems. It grows on numerous hardwood species, such as oak trees, in late autumn. Because of its appearance, it is aptly and commonly named as beefsteak or ox-tongue fungus. This fungus is consumed as salad or cooked and was once called the poor man's beefsteak (Jahn, 1990).

As fungal metabolites represent a wide diversity of chemical species (Cole & Schweikert, 2003), the investigation of the secondary metabolism of fungi arouses great scientific interest. Starting from the early 1950s, researchers attempted to harness fungi for natural flavour production because of their enormous biochemical potential. Most of the studies on the secondary metabolites of *F. hepatica*, however, have focussed on acetylenic compounds, several of which show antibacterial activities (Barley et al., 1987; Bianco Coletto, 1981; Farrell, Keeping, Pellatt, Martin, & Thaller, 1973; Jones, Lowe, & Shannon, 1966; Schwope, Givan, & Minto, 2003; Tsuge et al., 1999). Furthermore, polysaccharides, D-arabitol, amino acids, chlorogenic acid, ergosterol, and hydrocarbons of this fungus were examined (Casalicchio, Bernicchia, Govi, & Giovanni, 1975; Casalicchio, Paoletti, Bernicchia, & Govi, 1975; Frerejacque, 1939; Nano, Binello, Bianco, Ugazio, & Burdino, 2002; Paris, Durand, & Bonnet, 1960). Nothing has become known about the volatile secondary metabolites of this fungus so far.

In this study, major volatile compounds from the fruiting bodies of wild *F. hepatica* were identified, and

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the characteristic compounds shaping the flavour of this fungus were determined by GC-olfactometry.

2. Materials and methods

2.1. Materials

Ripe fruiting bodies of wild *F. hepatica* were harvested on the oak trees in October 2003 from Wisent Park, Springe.

2.2. Chemicals

Solvents were provided by BASF (Ludwigshafen, Germany) and Baker (Deventer, Netherlands). All solvents were distilled before use. High purity water was prepared with an E pure water purification system (Barnstead, Dubuque, IA). Sodium sulphate and sodium chloride were obtained from Carl Roth GmbH & Co. (Karlsruhe, Germany).

2.3. Methods

2.3.1. Preparation of extract

Fruiting bodies (250 g) were cut into cubes of about $2 \times 2 \times 2$ cm size. The samples were mixed with 400 ml methanol immediately, and 1 ml methyl nonanoate (42.7 mg l^{-1} in pentane/ether (1:1.12)) solution was added as internal standard. The mixture was homogenised by an Ultra-Turrax (Jahnke and Kunkel, Germany) macerator and centrifuged at 3300*g* at 5 °C for 20 min. The solvent layer was recovered as crude extract for continuous liquid liquid extraction (CLLE).

Saturated sodium chloride solution was added into the crude extract to a final volume of 1 l. This mixture was transferred to a CLLE-apparatus and 250 ml pentane/ether (1:1.12) were placed in a 500-ml round flask connected to the CLLE-apparatus. Following an extraction process of 24 h, the pentane/ether fraction was washed with high purity water and dried over anhydrous sodium sulphate.

The pentane/ether extract was concentrated at 42 °C using a Vigreux-column to a final volume of about 1 ml for GC-analysis.

2.3.2. High resolution GC–MS (high resolution gas chromatography–mass spectrometry)

High resolution gas chromatography–mass spectrometry (HRGC–MS) analysis, using a polar phase, was conducted on a Fisons GC8000 apparatus equipped with a (polyethylene glycol) ZB-WAX (30 m × 0.32 mm i.d. × 0.25 μm film thickness, Phenomenex, USA) column (He flow rate 38 $cm^3 s^{-1}$) and connected to a Fisons MD800 mass selective detector.

HRGC–MS analysis, using a non-polar phase, was conducted on a HP5890 Series II GC equipped with a DB-5 (30 m × 0.32 mm i.d. × 0.25 μm film thickness, Varian, Germany) column (He flow rate 38 $cm^3 s^{-1}$) and connected to a HP quadrupole mass spectrometer 5989A.

Both of the instruments of HRGC–MS were operated at 70 eV in the EI mode over the range 33–300 amu. Chemical ionisation (CI) was carried out with methane as reactant gas. The oven temperature programme was held at 40 °C for 2 min, raised to 250 °C at 5 °C min^{-1}, and held at 250 °C for 10 min. The injection volume was 1 μl.

2.3.3. GC-atomic emission detector

GC-atomic emission detector (GC-AED) analysis was carried out on a HP6890 series GC-system equipped with an Optima-5-MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness, J & W Scientific, USA) column (He flow rate 38 $cm^3 s^{-1}$) and connected to a G2350A atomic emission detector (Hewlett–Packard company, USA). The oven temperature programme was held at 50 °C for 5 min, raised to 280 °C at 10 °C min^{-1}, and held at 280 °C for 5 min. The injection volume was 2 μl.

2.3.4. GC-olfactometry

GC-olfactometry (GC-O) was performed on a Sato Chrom GC equipped with a (polyethylene glycol) DB-WAX column (30 m × 0.32 mm i.d. × 0.25 μm film thickness, SGE GmbH, Germany) with a H_2 flow rate of 52 $cm^3 s^{-1}$. One part was led to the FID, the other one to a heated sniff-port (250 °C). The oven temperature was held at 40 °C for 2 min, raised to 230 °C at 5 °C min^{-1}, and held at 230 °C for 15 min. Ten panellists noted the descriptions induced by compounds when they eluted from the sniffing port. Characteristic odour impressions were considered to be valid when at least 50% of the judges reproducibly signalled a sensory perception. The injection volume was 2 μl.

2.3.5. Identification and semi-quantification

Retention indices (RIs) were calculated according to the Kovats method, using *n*-alkanes as external references (Kondjoyan & Berdagué, 1996). Mass spectral identification was completed by comparing spectra with commercial mass spectral databases WILEY, NIST and LIBTX. Experimental results of odour quality and retention indices of volatiles were additionally compared with published data (Adams, 1995; Burdock, 2002; Jennings & Shibamoto, 1980; Kondjoyan & Berdagué, 1996; Rychlik, Schieberle, & Grosch, 1998) and, if available, authentic standards. Approximate concentrations of volatile compounds were calculated according to the internal standard method, using methyl nonanoate and the HP ChemStation Software (Agilent Technologies, USA).

3. Results and discussion

Altogether, 48 volatile compounds were detected in the extract of *F. hepatica*. According to the quantification by internal standard, 36 of them were presented in approximate concentrations of more than 10^{-3} mg 100 g^{-1} fruiting bodies. Table 1 summarises these 48 volatile compounds and their RIs on polar and non-polar columns. The volatile compounds are listed in an increasing RIs order on a polar column.

According to Pollien et al. (1997), a number of 8–10 judges is required to create a reliable flavour profile by GC-O. To evaluate the flavour profile of *F. hepatica* fruiting bodies, panels of 10 testers were employed. A compound was considered to contribute significantly to the overall aroma profile of the fungus if at least

Table 1
Major volatile compounds of fruiting bodies of wild *F. hepatica* (in increasing order of RIs on a ZB-WAX column)

No.	Compounds	RIs		Approximate concentration[a] (mg 100 g^{-1} fruiting bodies)
		ZB-WAX	DB5	
1	Hexanal	1069	n.d.	++
2	2-Methyl-1-propanol	1081	n.d.	++++
3	Sabinene	1097	n.d.	+
4	1-Butanol	1133	n.d.	++++
5	Limonene	1175	1021	+++
6	1,8-Cineole	1179	1019	++
7	Iso amyl alcohol	1194	n.d.	++
8	3-Octanone	1236	n.d.	++
9	Octanal	1270	n.d.	+
10	1-Octen-3-one	1282	961	+
11	(*E*)-2-Heptenal	1297	n.d.	++
12	6-Methyl-5-hepten-2-one	1317	n.d.	+
13	4-Hydroxy-4-methyl-2-pentanone	1339	n.d.	++
14	Ethanedioc acid dimethyl ester	1395	800	+++
15	(*E*)-2-Octenal	1400	n.d.	+
16	1-Octen-3-ol	1439	972	+++++
17	Citronellal	1457	n.d.	+
18	2-Ethyl-1-hexanol	1476	n.d.	++
19	Benzaldehyde	1491	931	++
20	Linalool	1536	1088	++
21	1-Octanol	1543	1069	++
22	2-Methyl-propanoic acid	1579	n.d.	++
23	Benzoic acid methyl ester	1591	1073	+++
24	Phenylacetaldehyde	1616	1011	++++
25	Butanoic acid	1642	863	++
26	Pentanoic acid	1679	n.d.	++++
27	2-Methyl-pentanoic acid	1755	n.d.	++
28	(+)-Cuparene	1785	1488	++++
29	Isopropyl dodecanoate	1821	n.d.	+
30	(*Z*)-2-Methyl-2-butenoic acid	1849	n.d.	++
31	Hexanoic acid	1858	864	+
32	(*E*)-2-Methyl-2-butenoic acid	1862	941	+
33	1-Dodecanol	1956	n.d.	++
34	Cinnamic aldehyde	2007	n.d.	+
35	(*E*)-Nerolidol	2028	1549	++++
36	(*E*)-Methyl cinnamate	2046	1353	++++
37	δ-Deca-2,4-dienolactone	2143	n.d.	++
38	Nonanoic acid	2176	n.d.	+
39	Hexadecanoic acid methyl ester	2198	1910	++++
40	9-Hexadecenoic acid methyl ester	2237	1895	++
41	Decanoic acid	2282	n.d.	+
42	Methyl stearate	2405	2111	++
43	9-Octadecenoic acid methyl ester	2424	2082	+++
44	9,12-Octadecadienoic acid methyl ester	2472	2075	++++++
45	Bisabolol oxide B	2525	1749	++
46	Phenylacetic acid	2613	1257	++
47	Hexadecanoic acid	>2800	1965	++++

n.d., not detectable.
[a] +, $(1–10)\times10^{-4}$; ++, $(1–5)\times10^{-3}$; +++, $(5–10)\times10^{-3}$; ++++, $(1–5)\times10^{-2}$; +++++, $(5–10)\times10^{-2}$; ++++++, $(1–5)\times10^{-1}$.

50% of the panel ascertained the characteristic odour impression imparted by the respective substance. The contribution of a single flavour substance to overall aroma profile may be assessed by comparing the individual odour threshold to the concentration detected in the fruiting bodies. The most abundant volatile detected was 9,12-octadecadienoic acid methyl ester.

To evaluate the presence of sulfurous, chlorine and nitrogen compounds in the sample, GC equipped with an atomic emission detector (GC-AED) was employed. No sulfur-, chlorine- or nitrogen-bearing volatile compounds were detected by GC-AED.

More than 80% of the identified volatiles contained 4–14 carbon atoms. Among them, C8 and C10 compounds were predominant. These results are in good agreement with typical mushroom flavours (Buchbauer, Jirovetz, Wasicky, & Nikiforov, 1993). If the volatiles are classified according to their most likely origin, the compounds originate from lipid oxidation and degradation, such as aliphatic alcohols, aldehydes and ketones from C4 to C10 are the majority group of compound. Most of the compounds containing more than 15 carbon atoms were long chain free fatty acids or their methyl esters.

Eleven volatiles, summarised in Table 2, were found to contribute significantly to the characteristic flavour of *F. hepatica*. Four of them, namely 1-octen-3-one, 1-octen-3-ol, phenylacetaldehyde and phenylacetic acid belong to the C8 group.

The overall flavour of the final extract was dominated by sweet and wild flowery impressions. GC-O investigations revealed three main groups of odorous compounds: rather unpleasant odours, shiitake-like, fruity and flowery. The impression 'sweet' was mainly attributed to the aromatic compounds phenylacetaldehyde and phenylacetic acid, and to the heterocyclus bisabolol oxide B. Furthermore, (*E*)-2-methyl-2-butenoic (tiglic) acid and (*E*)-methyl cinnamate also slightly contributed to the 'sweet' impression. A strong fermented soybean-like odour was imparted by butanoic acid. (*Z*)-9-Hexadecenoic acid methyl ester exhibited a flavour reminiscent of old leather. An unknown volatile emitted a mouldy odour. Though butanoic acid and the unknown compound were present in trace concentrations only, they imparted strong stimuli due to their low threshold values. Several shiitake-like flavours were sniffed in varying intensities during the GC-O investigations with 1-octen-3-ol giving the strongest impact. Besides further C8 compounds, 1-octen-3-ol is the well-known typical flavour compound formed in fruiting bodies of higher fungi by enzymatic oxidative degradation of linoleic acid. 1-Octen-3-ol is also the most important C8 mushroom aromatic compound (Zawirska-Wojtasiak, 2004). Mosandl, Heusinger, and Gessner (1986) indicated that a fruity mushroom-like flavour is attributed to (*R*)-(−)-1-octen-3-ol rather than to (*S*)-(+)-1-octen-3-ol. The flowery and fruity sensations are primarily assigned to phenylacetaldehyde, (*E*)-methyl cinnamate, linalool, bisabolol oxide B and (*E*)-2-methyl-2-butenoic acid.

Among the identified 10 characteristic odorous compounds, C8 derivatives (1-octen-3-one and 1-octen-3-ol), phenylethyl derivatives (phenylacetaldehyde and phenylacetic acid) and monoterpenoid compounds (linalool) have been well studied and reported from fungi (Borgkarlson, Englund, & Unelius, 1994; Breheret, Talou, Rapior, & Bessiereb, 1997; Venkateshwarlu, Chandravadana, & Tewari, 1999).

Methyl cinnamate was detected in *Lentinus lepideus* Fr. (Birkinshaw & Findlay, 1940) as early as 64-years ago. Later it was also found in *Inocybe corydalina* and *Inocybe pyrodora* (Schmitt, 1978). The RIs suggested (*E*)-methyl cinnamate rather than (*Z*)-methyl cinnamate. Viňa (2003) reported that both isomers of methyl cinnamate occurred simultaneously in 12 varieties of aromatic herb *Ocimum* spp. The structure of bisabolol oxide B was derived from the characteristic ions at *m*/*z* 143, 161, 179. The missing molecular ion (*m*/*z* 238) was confirmed by chemical ionisation, yielding an inten-

Table 2
Characteristic odorous compounds from the fruiting bodies of wild *F. hepatica*

Identity	GC-O odor description	Retention indices		
		GC-O (DB-WAX)	GC–MS (ZB-WAX)	GC–MS (DB-5)
1-Octen-3-one	Shiitake	1279	1282	961
1-Octen-3-ol	Shiitake	1435	1439	972
Linalool	Flowery	1528	1536	1088
Phenylacetaldehyde	Fruity & sweet	1614	1616	1011
Butanoic acid	Fermented soybean	1647	1642	863
Unknown	Mouldy	1799	–[a]	–[a]
(*E*)-2-Methyl-2-butenoic acid	Lovage	1867	1862	941
(*E*)-Methyl cinnamate	Fruity	2047	2046	1353
(*Z*)-9-Hexadecenoic acid methyl ester	Old leather	2236	2237	1895
Bisabolol oxide B	Honey & flowery	2511	2525	1749
Phenylacetic acid	Sweet & honey	2601	2613	1257

[a] Trace concentration, index value could not be determined.

sive MH^+-ion at *m/z* 239. Bisabolol oxide B has never previously been reported as a native fungal flavour. However, it resulted from the biotransformation of (−)-α-bisabolol by *Glomerella cingulata* and by *Aspergillus niger* (Miyazawa, Nankai, & Kameoka, 1995). (*E*)-2-Methyl-2-butenoic acid is accessible via regioselective biocatalytic hydrolysis of 2-methyl-2-butenenitrile (Hann et al., 2004). It has been identified as a spicy volatile in fruits and flowers (Idstein, Bauer, & Schreier, 1985; Kollmannsberger, Lorenz, Weinreich, & Nitz, 1998; Morales & Duque, 2002; Ngassoum, Jirovetz, & Buchbauer, 2001), and it exhibits biological activity as a beetle defence substance. To the best of our knowledge, this is the first report on (*E*)-2-methyl-2-butenoic acid from a fungal source. The unequivocal identification of the (*E*)-form, rather than the (*Z*)-form (angelic acid), was performed by comparison with authentic standard substances. From plants, both isomers were isolated (Burger, Nell, Spies, Le Roux, & Bigalke, 1999; Cataneda et al., 1996; Idstein et al., 1985; Kollmannsberger et al., 1998; Morales & Duque, 2002; Ngassoum et al., 2001; Raman & Santhanagopalan, 1979). 2-Methyl-2-butenoic acid and butanoic acid were also found to be the main characteristic odour components from the fresh and dried fruit shell of *Tetrapleura tetraptera* (Thonn.) (Ngassoum et al., 2001).

4. Conclusions

Forty eight volatile compounds, from the fruiting bodies of wild *F. hepatica*, were identified and approximately quantified. Most of them were C4–C14 compounds, and no sulfur-, chlorine- or nitrogen-bearing volatiles were detected. The overall flavour of the volatile extract was sweet and resembled wild flowers. Eleven volatile compounds were determined to be characteristic odorous compounds of this fungus: 1-octen-3-one, 1-octen-3-ol, linalool, phenylacetaldehyde, butanoic acid, (*E*)-2-methyl-2-butenoic acid, (*E*)-methyl cinnamate, (*Z*)-9-hexadecenoic acid methyl ester, bisabolol oxide B, phenylacetic acid, and an uncertain mouldy compound. (*E*)-2-Methyl-2-butenoic acid and bisabolol oxide B have never previously been identified as native volatile secondary metabolites of fungi.

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