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Characteristic Volatiles from Young and Aged Fruiting Bodies of Wild *Polyporus sulfureus* (Bull.:Fr.) Fr.

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The volatile compounds of fresh fruiting bodies of wild *Polyporus sulfureus* (Bull.:Fr.) Fr. growing on oak trees were isolated by continuous liquid−liquid extraction (CLLE) and investigated by high-resolution gas chromatography−mass spectrometry (HRGC-MS) on two GC columns of different polarity (DB-5 and ZB-WAX), and by gas chromatography−olfactometry (GC−O). A total of 40 major volatile compounds from the young samples were identified and semiquantified. Five odorous compounds were determined to be responsible for the characteristic flavor of young *Polyporus sulfureus*: 1-octen-3-one, 1-octen-3-ol, 3-methylbutanoic acid, phenylethanol, and phenylacetic acid. Four volatiles investigated by GC−O and detected by GC-MS were determined as the characteristic odorants of aged species: 2-methylpropanoic acid, butanoic acid, 3-methylbutanoic acid, and phenylacetic acid. The comparative results revealed that the volatile composition of the fruiting bodies even from the same fungal species may greatly vary with its host, location, and age.

KEYWORDS: Polyporus sulfureus; volatile compounds; fruiting bodies; odor; characteristic odorants; GC-MS

INTRODUCTION

Polyporus sulfureus (Bull.:Fr.) Fr. (synonym: *Laetiporus sulfureus* (Bull.: Fr.) Murrill), is a member of the class Aphyllophorales (Polypore) fungi. It is widely distributed in Asia, Europe, and North America. This fungus commonly occurs on hardwoods or conifers. Its fruiting bodies are annual, spongy to leathery, with bright orange color on one side, up to 40 cm wide. It can be harvested as an edible fungus and has long been used in Asian herbal medicine (*1*).

Because fungal metabolites represent a wide diversity of chemical species, the investigation of the secondary metabolism of fungi arouses great scientific interest. Furthermore, along with certain *Ascomycota*, polypores are a major source of biologically active compounds in the fungal kingdom. Due to its enormous ability of high value compounds production or biotransformation, investigation and utilization of *Polyporus sulfureus* (*P. sulfureous*) have never subsided over the past 50 years. In the 1960s, biosynthesis of eburicoic acid brought *P. sulfureus* in focus (*2*). This triterpenoid acid amounts to 30% of the dry weight of *P. sulfureus* (*3*). Both, natural products exhibiting biological activity and specific enzymes catalyzing their biosynthesis in this fungus have been studied in recent years (*2*, *4*−*6*). Fungal biotransformation of low-cost substrates to high value flavor and aroma compounds appears to have great commercial potential (*7*). An important prerequisite of this effort is to know the genuine volatile constituents of a specific natural fungus. Moreover, a detailed investigation of volatile compounds is a basic start to reveal the mechanism of flavor formation in the fruiting bodies of fungi.

Most of the previous investigations on *P. sulfureus* focused on nonvolatile compounds. Seven volatile amines were described by List and Menssen (*8*). Forty-one years later, 26 volatile compounds from a mixed sample of young and old basidiocarps collected on *Salix alba* trees were investigated by GC-MS using a DB-1 column (*9*). However, it was observed that fruiting bodies of wild *P. sulfureus* smelled pleasantly when young, whereas its aged specimens emitted a strong pungent odor. This study was performed to elucidate the significant odor differences between young and aged fruiting bodies by analyzing their volatile composition.

MATERIALS AND METHODS

Materials. *Polyporus sulfureus* was collected from oak trunks in Wisent park, Springe (northwest of Germany). The fresh fruiting bodies of young *P. sulfureus*, about 10 days old, were picked in June 2004, while the aged fruiting bodies of *P. sulfureus* were harvested in October 2003. The fruiting bodies showed a white pore layer.

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 sulfate and sodium chloride were obtained from Roth (Karlsruhe, Germany).

Extraction of Volatiles. Fresh fruiting bodies (250 g) were cut into cubes of about 2 cm × 2 cm × 2 cm size. The samples were mixed

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with 400 mL of methanol immediately, and 1 mL of methyl nonanoate (468 mg/L in pentane/ether (1:1.12)) solution was added as internal standard. The mixture was homogenized by Ultra-Turrax (Jahnke and Kunkel, Germany) and centrifuged at 18800*g* at 5 °C using an RC28S centrifuge (Kendro, Germany) 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 CLLE apparatus, and 250 mL of pentane/ether (1:1.12) was placed into a 500 mL round-bottom 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 sulfate. The pentane/ether extract was concentrated at 42 °C using a Vigreux column to a final volume of about 1 mL for GC analysis. Headspace sampling, in the first third of the chromatogram (about 15 min), gave very similar results, but several techniques applied (SPME, Tenax; data not shown) were not able to accumulate enough of the higher boiling compounds to allow for sound mass spectrometric analyses.

High-Resolution GC-MS. High-resolution GC-MS (HRGC-MS) analysis using a polar phase was conducted on a Fisons GC 8000 equipped with a ZB WAX column (30 m × 0.32 mm i.d. × 0.25 μm film thickness, Phenomenex, USA) connected to a Fisons MD800 mass selective detector. HRGC-MS analysis using a nonpolar phase was conducted on a HP5890 Series II GC equipped with a DB-5 column (30 m × 0.32 mm i.d. × 0.25 μm film thickness, Varian, Germany) connected to a HP quadrupole mass spectrometer 5989A. Both HRGC-MS instruments were operated at 70 eV in the EI mode over the range of 33−300 amu. The linear carrier gas (He) velocity was 38 cm/s. Chemical ionization (CI) was carried out with methane as reactant gas. The oven temperature program was held at 40 °C for 2 min, raised at 5 °C/min to a final temperature of 250 °C, and held constant for 5 min at 250 °C. The injection volume was 1 μL.

GC-Olfactometry. GC−olfactory (GC−O) was performed on a Sato Chrom GC equipped with a DB-WAX column (30 m × 0.32 mm i.d. × 0.25 μm film thickness, SGE, Germany) with a H_2 linear velocity of 52 cm/s. One part was led to the flame ionization detector (FID); the other one, to a heated sniff-port (250 °C). The oven temperature was held at 40 °C for 2 min, raised at 5 °C/min to a final temperature of 250 °C, and held constant for 5 min at 250 °C. A panel of 10 persons was used to note the odor impression induced by eluting compounds; each panelist sniffed for about 15−20 min and then took over from the one recording the sensory statements. Characteristic odor impressions were considered to be valid, if at least 50% of the judges reproducibly signaled a sensory perception. The injection volume was 2 μL.

Identification and Semiquantification. Linear retention indices (RIs) were calculated according to the Kovats method using *n*-alkanes (C_7−C_{28}) as external references (*10*). Mass spectral identification was completed by comparing spectra with commercial mass spectral databases WILEY, NIST, and LIBTX and by comparison with authentic reference standards if available. Experimental results of odor quality and retention indices of volatiles were additionally compared with published data (*11*−*14*). Approximate concentrations of volatile compounds were calculated according to the internal standard method using methyl nonanoate and the HP ChemStation Software (Agilent Technologies, USA).

RESULTS AND DISCUSSION

The volatile compounds identified in the extract of young *P. sulfureus* consisted of 11 acids, 9 esters, 7 alcohols, 5 hydrocarbons, 5 aldehydes, and 3 ketones. **Table 1** summarizes these 40 volatile compounds and their RIs both on a polar column and a nonpolar one. According to the quantification by internal standard, 25 of them were present in concentrations of more than 100 μg/(kg of fruiting bodies). Compared with most other fresh mushrooms, the concentration of volatile compounds from *P. sulfureus* was high. This agreed with the low water content and strong odor of the fresh fruiting bodies. The most abundant compounds detected were 1-octen-3-ol and linoleic acid. 2-Methyl-1-propanol, 3-octanone, and linoleic acid methyl ester belonged to the top abundant group as well.

Fatty Acids and Methyl-Branched Carboxylic Acids. The predominant constituents were carboxylic acids and their esters. Seven linear free fatty acids covering a wide range of chain length were detectable. It is well-known that fatty acids play a critical role in flavor formation, both in microorganisms and in plants, as well as in animal products. Not only do they contribute to the odor by themselves but also act as flavor precursors under enzymatic oxidation, decarboxylation, or esterification reactions (*15*). The profile and changes of fatty acids in specific fungi significantly contribute to the formation and transformation of volatile compounds. The determination of the fatty acid content and the major fatty acids in the basidiospores by GC and GC-MS was recently reported as a new method for a chemotaxonomical index of fungi (*16*). In *P. sulfureus* an odd long-chain fatty acid, pentadecanoic acid, and its methyl ester were detected. Odd long-chain fatty acids have been detected in various fungi such as *Penicillium atrovenetum*, *Rhizoctonia solani*, and *Auricularia polytricha* (*17*−*19*), although they occur rarely in nature. The production of odd-chain fatty acids, including pentadecanoic acid by microorganisms was patented (*20*). Three methyl-branched carboxylic acids and one of their esters, 2-methylpropanoic acid, 3-methylbutanoic acid, 2-methylhexanoic acid, and 2-methylbutanoic acid methyl ester, were traceable. Natural methyl-branched carboxylic acids relevant to the flavor industry cannot be produced by traditional hydrolyzing of vegetable oil or animal fats. An alternative producing procedure is the oxidative conversion of the corresponding alcohols or aldehydes by microorganisms (*21*). Studies on the bacterial production of 3-methylbutanoic acid (*22*, *23*) and 2-methylbutanoic acid (*24*, *25*) were carried out. A branched-chain amino acid aminotransferase gene (ilvE) plays a role in the formation of 3-methylbutanoic acid in *Lactococcus lactis* and *Staphylococcus carnosus* (*26*, *27*). *Saccharomyces cerevisiae* was also demonstrated to catalyze the production of 3-methylbutanoic acid (*28*). Interestingly, a fungal protease EPg222 was found to catalyze the formation of 3-methylbutanoic acid, as well as of 2-methylpropanol and 2-methylbutanoic acid in a dry fermented sausage (*29*). Therefore, searching for an alternative enzymatic access to specific carboxylic acids, including branched-chain fatty acids, *P. sulfureus* may be selected as a candidate strain.

C_8 Compounds and Benzoid Volatiles. The well-known eight carbon atom (C_8) series compounds discovered in fungi, such as 3-octanone, octanal, 1-octen-3-one, 3-octanol, 1-octen-3-ol, 1-octanol, and (*E*)-2-octen-1-ol were all detected in this fungus. Among them, 1-octen-3-ol, also called mushroom alcohol, contributed the strongest impact to both odor intensity and quality. 1-Octen-3-ol is formed de novo by fruiting bodies of higher fungi through enzymatic oxidative degradation of linoleic acid (*30*). Mosandl et al. (*31*) have stated that the mushroom note is attributed to the (*R*)-(−)-enantiomer. Recent studies indicated that 1-octen-3-ol from natural sources showed an enantiomeric excess of the (*R*)-(−)-form when compared to its synthetic counterpart (*32*). Apart from its flavor properties, 1-octen-3-ol acts as an attracting odorant of three wood-living generalist beetles (*33*). As the most important C_8 mushroom flavor, its production by submerged liquid culture of fungi was studied (*34*, *35*).

Among the identified volatiles were four benzoid volatiles with pleasant flavor, benzaldehyde, phenylacetaldehyde, phenylethanol, and phenylacetic acid. As attractive target compounds of fungal biotransformation, they can be accumulated by adding

Table 1. Major Volatile Compounds from Young Fruiting Bodies of Wild *Polyporus sulfureus*[a]

no.	compound	retention indices ZB-WAX	retention indices DB5	approx concn[b] (μg/kg fruiting bodies)
1	2-methylbutanoic acid methyl ester[c]	1002	771	++
2	hexanal[d]	1075	786	+++
3	2-methyl-1-propanol[d]	1085	<700	+++++
4	1-butanol[d]	1140	<700	+
5	5-(1-methylethylidene)-1,3-cyclopentadiene[c]	1170	858	+
6	hexanoic acid methyl ester[d]	1180	911	+
7	3-octanone[d]	1244	970	+++++
8	octanal[d]	1277	986	+
9	1-octen-3-one[d]	1289	962	++
10	4-hydroxy-4-methyl-2-pentanone[c]	1346	809	+++
11	nonanal[d]	1380	1085	+++
12	3-octanol[d]	1389	988	+++
13	1-octen-3-ol[d]	1448	973	+++++
14	acetic acid[d]	1464	<700	++++
15	benzaldehyde[d]	1498	933	+
16	1-octanol[d]	1555	1063	+++
17	2-methylpropanoic acid[c]	1579	793	+++
18	hexadecane[d]	1599	1602	++
19	(*E*)-2-octen-1-ol[c]	1612	1059	+++
20	phenylacetaldehyde[d]	1626	1009	+
21	3-methylbutanoic acid[c]	1682	850	+++
22	heptadecane[d]	1700	1702	++
23	2-methylhexanoic acid[c]	1757	941	+++
24	octadecane[d]	1800	1802	++
25	dodecanoic acid 1-methylethyl ester[c]	1832	1618	++
26	hexanoic acid[d]	1860	990	+++
27	2,2,4-trimethyl-3-carboxyisopropylpentanoic acid isobutyl ester[c]	1866	1581	+++
28	phenylethanol[d]	1892	1088	++
29	eicosane[d]	2000	2002	+
30	pentadecanoic acid methyl ester[c]	2108	1814	+++
31	nonanoic acid[c]	2179	1273	+++
32	hexadecanoic acid methyl ester[d]	2210	1916	+++
33	(*Z*)-9-hexadecenoic acid methyl ester[d]	2249	1890	+
34	(*Z*)-9-octadecenoic acid methyl ester[d]	2436	2087	++++
35	(*Z,Z*)-9,12-octadecadienoic acid methyl ester[d]	2484	2079	+++++
36	phenylacetic acid[d]	2589	1248	++++
37	pentadecanoic acid[c]	>2800	1857	+++
38	hexadecanoic acid[d]	>2800	1958	++++
39	octadecanoic acid[d]	>2800	2157	+++
40	(*Z,Z*)-9,12-octadecadienoic acid[d]	>2800	2126	+++++

[a] The volatile compounds are listed in increasing RIs order on a polar column ZB-WAX. [b] +, 10–50; ++, 50–100; +++, 100–500; ++++, 500–1000; +++++, 1000–5000. [c] The compound was identified by comparing mass spectrum with commercial mass spectral databases and RIs on two different polarity columns with published data. [d] The compound was confirmed by authentic reference standard.

the precursor L-phenylalanine into the culture of several fungi (*36*). The approximate concentration of phenylacetic acid was 500−1000 μg/(kg of fruiting bodies).

Characteristic Odorants of the Young Fruiting Bodies of *Polyporus sulfureus*. The fruiting bodies of young *P. sulfureus*, right after harvesting, smelled pure and fresh and emitted a delicate mushroom aroma. Their volatile extract smelled fresh and shiitake-like, accompanied by a faint flowery scent. To evaluate the flavor profile of *P. sulfureus* fruiting bodies, panels of 10 testers were employed. According to Pollien et al. (*37*), a number of eight to 10 judges is required to create a reliable flavor profile by GC−O. The contribution of a single flavor substance to the overall aroma profile may be assessed by comparing the individual odor threshold with the concentration detected in the fruiting bodies. Five volatile compounds were determined to contribute significantly to the overall flavor of the young fruiting bodies (**Table 2**). Obviously, the components responsible for the shiitake-like flavor of the young basidiocarps were primarily 1-octen-3-ol and 1-octen-3-one. The faint flower fragrance was chiefly attributed to phenylacetic acid and second to phenylethanol. A sweaty odor was emitted by 3-methylbutanoic acid. 3-Methylbutanoic acid is a typical odorant with animal notes. It has also been identified as a key odorant of many foods, such as coffee, cheese, wine, bread, salami, soy sauce, milk chocolate, heated sweet butter, and several fresh fruits (*38*).

Table 2. Characteristic Odorous Compounds from the Young Fruiting Bodies of *Polyporus sulfureus*

compound	GC–O odor description	retention indices DB-WAX (GC–O)	retention indices ZB-WAX (GC-MS)	retention indices DB-5 (GC-MS)
1-octen-3-one	mushroom	1293	1289	962
1-octen-3-ol	shiitake	1447	1448	973
3-methylbutanoic acid	sweaty	1676	1682	850
phenylethanol	fruity	1883	1892	1088
phenylacetic acid	honey and flower	2578	2589	1248

Variation of Volatile Composition of Fruiting Bodies. It is known that fruiting bodies of *P. sulfureus* develop a disagreeable odor with age. The fruiting bodies of aged species harvested in October emitted a pungent odor. To clarify this change at a molecular level, characteristic odorants shaping the flavor were also investigated. A bit different from fresh fruiting bodies, the overall flavor of the extract exhibited a moldy and musklike odor with a faint sweet flavor. Four volatile com-

Table 3. Characteristic Odorous Compounds from the Aged Fruiting Bodies of *Polyporus sulfureus*

compound	GC–O odor description	retention indices DB-WAX (GC–O)	ZB-WAX (GC-MS)	DB-5 (GC-MS)
2-methylpropanoic acid	stinky	1588	1585	790
butanoic acid	fermented soybean	1652	1647	860
3-methylbutanoic acid	sweaty	1678	1685	848
phenylacetic acid	honey and flower	2581	2590	1246

pounds were found to contribute significantly to the character flavor of the aged fruiting bodies of *P. sulfureus* (**Table 3**). Compared to the flavor of young samples, a typical "mushroom flavor" could not be perceived. 1-Octen-3-ol was not the characteristic odorant any longer, although it was detectable in traces by GC-MS. Besides, 1-octen-3-one was not detected in the extracts of aged species. The off-flavor was chiefly attributed to the sharp decrease of the content of 1-octen-3-ol, the remarkable increase of the concentration of 3-methylbutanoic acid and 2-methylpropanoic acid, and the appearance of butanoic acid. Compared to the qualitative results of Rapior et al. (*9*), only five compounds, namely, 1-octen-3-ol, benzaldehyde, phenylethanol, pentadecanoic acid, and hexadecanoic acid, coincided. Nearly 90% of the total volatiles identified, including the aged species, and about 80% of compounds reported by Rapior et al. (*9*) did not tally with each other. The enormous difference is probably be due to the varietal diversity within the *P. sulfureus* species and to the growth conditions. The profile of flavor compounds varies with species and variety, as well as with culture conditions (*39*). The specimens Rapior et al. (*9*) investigated were a combination of young and old basidiocarps collected from *Salix alba*. *P. sulfureus* is easily identified by its bright yellow-orange color, which was obviously the reason for naming the species "*sulfureus*", but there are a number of varieties within the species (*40*). The fungus of the present study belongs to *P. sulfureus* variety *semialbinus* Peck (*41*). The characteristic volatiles identified may serve as a chemotaxonomic guide for *P. sulfureus*, until unambiguous techniques, such as PCR (polymerase chain reaction) or RFLP (restriction fragment length polymorphism) have become more widely accessible. The presented results show that the volatile composition of fruiting bodies even of the same fungal species may vary greatly with host, location, and age.

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