Volatile Compounds Generated by Basidiomycetes

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For my dear mother Gāo, Jú-Xiāng

ZUSAMMENFASSUNG

Höhere Pilze aus der Klasse der Basidiomyceten (Ständerpilze) verfügen über ein bemerkenswertes biochemisches Potential zur Synthese von hochwertigen flüchtigen Aromastoffen. Die instrumentell-analytische Untersuchung dieser Inhaltsstoffe insbesondere bei eßbaren Vertretern ist sowohl von grundlegender wissenschaftlicher Bedeutung als auch von potentiellem industriellen Interesse.

Frische Fruchtkörper der Wildpilze *Polyporus sulfureus*, *Lentinus lepideus* und *Fistulina hepatica* wurden im Pilzlehrpfad des Wisentgeheges Springe geerntet. Die flüchtigen Inhaltsstoffe wurden aus den zerkleinerten Fruchtkörpern durch kontinuierliche Flüssig-Flüssig-Extraktion isoliert und mit der Kapillar-Gaschromatographie-Massenspekrometrie-Kopplung (GC-MS) auf zwei unterschiedlich polaren Säulen (DB-5 und ZB-WAX) untersucht. Heteroatomhaltige Komponenten wurden mit der Gaschromatographie-Atomemissionsdetektor-Kopplung (GC-AED) gesucht, und Aromabeiträge mit der Gaschromatographie-Olfaktometrie (GC-O) abgeschätzt.

Aus den jungen frischen Fruchtkörpern von *P. sulfureus*, gewachsen auf Eichenholz, sind 40 flüchtige Hauptkomponenten identifiziert und semi-quantifiziert worden; ebenso 43 Hauptkomponenten aus *L. lepideus*, gewachsen auf altem Kastanienholz und 48 Hauptkomponenten aus *F. hepatica*, gewachsen auf Eichenholz.

Fünf Komponenten waren für den charakteristischen Geruch von jungen Fruchtkörpern von *P. sulfureus* verantwortlich: 1-Octen-3-on, 1-Octen-3-ol, 3-Methylbutansäure, 2-Phenylethanol und Phenylethansäure. Mit zunehmendem Alter der Fruchtkörper verschob sich das Spektrum zugunsten der nun besonders prägenden Aromastoffe 2-Methylpropansäure, Butansäure, 3-Methylbutansäure und Phenylethansäure. Somit sind die sensorischen Veränderungen auf die chemischen Ursachen zurückgeführt worden. Der Vergleich mit Literaturangaben ergab, dass die Aromazusammensetzung selbst in der gleichen Spezies von Wirtsholz, Standort und der Seneszenz abhängig sein kann.

Von den 19 erstmals beschriebenen Sesquiterpenen von *L. lepideus* haben sich 11 einer abschließenden Identifizierung entzogen. Die fünf intensivsten Geruchskomponenten der jungen Fruchtkörper waren n-Nonanal, (2*E*)-Nonenal, Germacene D, 2-Vinylmalonsäuremethylpropylester und Nonansäure. Das schwefelhaltige Sesquiterpen „Mintsulfide“ wurde zum ersten Mal als Pilzmetabolit nachgewiesen.

Das Aromaprofil der Fruchtkörper von *F. hepatica* war gekennzeichnet durch das häufige Auftreten von Monoterpenen. Zu den 11 sensorisch prägendsten Komponenten gehörten 1-Octen-3-on, 1-Octen-3-ol, Linalool, Phenylacetaldehyd, Butansäure, (2*E*)-Methyl-2-butensäure, (*E*)-Zimtsäuremethylester, (9*Z*)-Hexadecensäuremethylester, Bisabololoxid B, Phenylethansäure sowie eine nicht identifizierte Verbindung mit muffigem Geruch. (2*E*)-Methyl-2-butensäure und Bisabololoxid B wurden zum ersten Mal als Pilzmetabolite beschrieben.

Das Potential von *F. hepatica zur* Aromabildung wurde zusätzlich für Zellkulturen unter sterilen Laborbedingungen untersucht. Submerskulturen in Standardnährlösung bildeten 39 flüchtige Hauptkomponenten, während aus Oberflächenkulturen, gewachsen auf Eichenholzpulver, 53 Komponenten identifiziert und semi-quantifiziert wurden. Insgesamt bildeten die Oberflächenkulturen im Vergleich ein größeres Spektrum von Aromastoffen in viel höheren Konzentrationen, so auch für die Schlüsselkomponente 1-Octen-3-ol. Die Bildung der besonders interessanten Terpenoide hängt offenbar von den Kultivierungsbedingungen ab. Die enzymatischen Besonderheiten von *F. hepatica* beim selektiven Ligninabbau könnten zur Erzeugung von nichtphenolischen, methoxybenzenoiden Aromastoffen aus Ligninabfallströmen genutzt werden.

Schlagwörter: Basidiomycete, flüchtig, charakteristisch, Aromastoff, *F. hepatica*, *P. sulfureus*, *L. lepideus*, Kultur

SUMMARY

Basidiomycetes are capable of producing a wide diversity of volatile flavors with high value. The investigation of these compounds of edible mushroom fungi are of both theoretical and commercial significance.

The wild mushrooms *Polyporus sulfureus*, *Lentinus lepideus*, and *Fistulina hepatica* were harvested from trees in Wisent Park, Springe, northwest of Germany. The volatile constituents of their fresh fruiting bodies 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), gas chromatography-atomic emission detector (GC-AED), and by gas chromatography–olfactometry (GC–O).

Forty major volatile compounds from the young fresh fruiting bodies of *P. sulfureus* growing on oak tree, 43 major volatile compounds from the young fresh fruiting bodies of *L. lepideus* developing on downed old chestnut tree, and 48 major volatile compounds from the fresh fruiting bodies of *F. hepatica* habitating on oak tree, were identified and semiquantified, respectively.

Five odorous compounds were determined to be responsible for the characteristic flavor of the young fruiting bodies of *P. sulfureus*: 1-octen-3-one, 1-octen-3-ol, 3-methylbutanoic acid, phenylethanol, and phenylacetic acid. Four volatiles were determined as the characteristic odorants of the aged fruiting bodies of *P. sulfureus*: 2-methylpropanoic acid, butanoic acid, 3-methylbutanoic acid, and phenylacetic acid. The manifest odor differences between young and aged fruiting bodies of *P. sulfureus* were thus elucidated. This investigation also revealed that the volatile composition of the fruiting bodies even from the same fungal species may greatly vary with its host, location and age.

Investigation on *L. lepideus* showed 19 new sesquiterpenoids including 11 unknown sesquiterpenoids were metabolites of this species. Five odorous compounds, were determined to be responsible for the characteristic flavor of the young fruiting bodies of *L. lepideus*: nonanal, (*E*)-2-nonenal, germacrene-D, 2-vinyl malonic acid methyl propyl ester, and nonanoic acid. The sulfur-containing sesquiterpenoid mintsulfide was identified for the first time in the fungal kingdom and a main odorant 2-vinyl malonic acid methyl propyl ester was proposed.

Investigation of the fruiting bodies of *F. hepatica* showed its wealth of monoterpenes. 11 odorous compounds significantly contributed to the overall flavor of the fruiting bodies of *F. hepatica*: 1-octen-3-one, 1-octen-3-ol, linalool, phenylacetaldehyde, butanoic acid, an unidentified volatile compound with mouldy odor, (*E*)-2-methyl-2-butenoic acid, (*E*)-methyl cinnamate, (*Z*)-9-hexadecenoic acid methyl ester, bisabolol oxide B and phenylacetic acid. (*E*)-2-methyl-2-butenoic acid and bisabolol oxide B were the first time to be reported as metabolites of fungi.

The biochemical potential of *F. hepatica* to produce flavors was further evaluated by cultivation in sterile laboratory environments. A total of 39 volatile compounds generated by the submerged cultured *F. hepatica* in standard nutrition solution (SNS), and 53 volatile compounds by the surface grown *F. hepatica* on oak wood powder, were identified and semiquantified, respectively. In general, the surface cultures contained a wider diversity of volatile classes and produced much higher concentration of key flavors such as 1-octen-3-ol. The results suggested that *F. hepatica* could produce a variety of terpenoids depending on medium. The enzymatic effects of *F. hepatica* on selective breakdown of lignin might be exploited to create natural non-phenolic methoxybenzenoid flavors from lignin wastes.

Key words: Basidiomycete, volatile, characteristic, flavor, *F. hepatica*, *P. sulfureus*, *L. lepideus*, culture

PRELIMINARY REMARKS

This dissertation represents the work performed at the Center of Applied Chemistry, Institute of Food Chemistry, University of Hannover (**Zentrum Angewandte Chemie, Institut für Lebensmittelchemie, Universität Hannover**), directed by Prof. Ralf Günter Berger, from October 2003 to October 2005. The research was supported by German Academic Exchange Service (**Deutscher Akademischer Austauschdienst (DAAD)**) in cooperation with China Scholarship Council (CSC). Parts of the work have already been published in international peer reviewed journals, submitted for publication or presented at conferences.

Full papers:

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2. Wu, S. M.; Krings, U.; Zorn, H.; Berger, R. G. Volatile compounds from the fruiting bodies of beefsteak fungus *Fistulina hepatica* (Schaeffer: Fr.) Fr.. *Food Chem.* **2005**, *92*, 221-226.
3. Wu, S. M.; Krings, U.; Zorn, H.; Berger, R. G. Volatile compounds of the wild mushroom *Lentinus lepideus* grown on chestnut trees. *Adv. Food Sci.* **2005**, in press.
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Poster presentations:

5. Wu, S.; Zorn, H.; Krings, U.; Berger, R. G. Flüchtige Sekundärmetabolite aus Fruchtkörpern von *Fistulina hepatica*. *Lebensmittelchemie* **2005**, *59*, 11.

Chapters 2 to 5 of this dissertation represent the full papers as published (*1-3*) or submitted (*4*).

ABBREVIATIONS

AED	atomic emission detector
CBS	Centraalbureau voor Schimmelcultures
CEC	the Council of the European Communities
CFR	the Code of Federal Regulations in the US
CI	chemical ionization
CLLE	continuous liquid–liquid extraction
CSC	China scholarship council
DAAD	Deutscher Akademischer Austauschdienst
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EI	electron impact ionization
FID	flame ionization detector
FPRL	forest product research laboratory (Princes Risbotough, UK)
GC-O	gas chromatography–olfactometry
HRGC–MS	high resolution gas chromatography–mass spectrometry
ID	identity
IFB	Institut für Forstbotanik (Berlin, Germany)
IR	infrared
IS	internal standard
LC	liquid chromatography
M^+	molecular ion
MH^+	protonated molecular ion (quasi molecular ion)
NMR	nuclear magnetic resonance
OWP	oak wood powder
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RIs	retention indices
rpm	rounds per minute
SNS	standard nutrition solution
SPME	solid phase micro-extraction
v/v	volume/volume
w/w	weight/weight

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1. INTRODUCTION

1.1. Mushrooms as sources of valuable secondary metabolites

Owing to the fungal metabolic versatility, ecological diversity, complex life cycles, and essential role in nature, fungi have attracted the attention of chemists, biochemists, biologists, geneticists, ecologists, and naturalists in myriad ways (Tkacz, & Lange, 2004). The use of fungi for the production of commercially important products has a long tradition, but it has increased rapidly over the past half century (Papagianni, 2004).

Commonly, mushrooms are described as macrofungi with distinctive fruiting bodies, which are large enough to be seen with human naked eyes and can be picked by hand. Based on this definition, more than 12,000 species have been considered as mushrooms. At least 2,000 of them are considered as edible (Chang, 1999). In terms of taxonomy, they belong to the class of basidiomycetes or to the ascomycetes (Mizuno, 1995). Modern molecular biology techniques have revealed that single mushroom species showing identical morphology may comprise many varieties in nature (Hawksworth, 2004). For example, *Polyporus sulfureus* species, has a number of varieties (Banik, Burdsall, & Volk, 1998). According to RFLP (restriction fragment length polymorphism) analyses of mtDNA (mitochondrial DNA), a single fallen tree accommodated at least four distinct parental strains of *Lentinula edodes* (Fukuda, & Mori, 2003). In view of this, and considering unknown wild species remaining unidentified, the number of mushroom strains seems to be immense.

Since ancient times, mushrooms have been part of normal human diets. *Lentinus edodes*, commonly called shiitake, was firstly cultivated in China early between 1000 AD and 1100 AD (Sánchez, 2004). The production of macrofungi for alimentary use is so far the only profitable way of utilizing lignocelluloses wastes. In recent times, the amounts of consumed mushrooms, involving a larger number of species, have greatly increased (Mattila, Könkö, Eurola, Pihlava, Astola, Vahteristo, et al., 2001). Mushrooms have been realized as a rich source of protein and secondary metabolites with novel structures and interesting biological and pharmacological activities (Turner, & Aldridge, 1983). In food sciences, the overall harmonizing effect of a diet balanced with edible mushrooms, highly praised by the Chinese as early as 100 AD (Chang, 1996), is not a myth, but is continually supported by modern scientific investigations (Abraham, 2001; Itonori, Aoki, & Sugita, 2004; Wasser, 2002;

Wojtas, Bieñkowski, Tateyama, Sagami, Chojnacki, Danikiewicz, & Swiezewska, 2004; Zjawiony, 2004). For centuries, mostly in East Asia, more than 200 wild mushroom species have been collected and used for various traditional medical purposes, while being devoid of undesirable side-effects (Sagakami, Aohi, Simpson, & Tanuma, 1991; Sánchez, 2004). Many bioactive molecules with pharmaceutical activities, including volatile compounds, have been recently identified in various edible mushroom species (Cohen, Persky, & Hadar, 2002; Jin, Jung, Shin, Kim, Jeon, & Choi, 2003; Keller, Maillard, Keller, & Hostettmann, 2002; Tsukamoto, Macabalang, Nakatani, Obara, Nakahata, & Ohta, 2003; Yaoita, Matsuki, Iijima, Nakano, Kakuda, & Machida, 2001).

Apart from edibility and medicinal purposes, many edible mushrooms with a characteristic taste and aroma are highly appreciated not only by gourmet chefs but also by flavor and fragrance researchers. Presumably, it was their pleasant aroma that prompted the notorious Roman emperor Nero to name mushrooms "*cibus deorum*", food of the gods (Berger, & Zorn, 2004). The flavor compounds of edible mushrooms are an important factor to evaluate their quality as food materials and even medicine stuffs.

1.2. Significance of producing natural flavors by mushroom fungi

The EU (CEC, 1991) and USA (CFR, 1993) legislations have clarified that "natural" flavor substances can only be prepared either by physical processes (extraction from natural sources), or by enzymatic or microbial processes, which involve precursors isolated from nature (Berger, Krings, & Zorn, 2002; Serra, Fuganti, & Brenna, 2005). The preference of consumers for "natural products" strongly drives the market to develop natural flavors. A flavor sold as natural product is often significantly more expensive than an identical one prepared by chemical synthesis. For example, the price of natural vanillin is 80 to 266 times of that of synthetic vanillin (Walton, Mayer, Narbad, 2003). Furthermore, chiral flavor compounds usually occur in nature as single enantiomers, which are not easily accessible by the less selective classical synthetic approaches (Brenna, Fuganti, & Serra, 2003). Meanwhile, the increasing sensitivity of the ecological systems supports the choice of environmentally friendly processes. As a result, academic research and industrial exploitation of the biosynthetic capabilities of many microorganisms have been stimulated in recent times.

As early as 1923, a first academic discussion on the capabilities of bacteria to form flavors was published (Omelianski, 1923). The importance of flavors originating from

microorganisms was not academically realized until 30 years later, and industrial research in the biotechnological production of natural flavors was not initiated until the early 1980s (Gatfield, 1999). Since the mid-1980s, a considerable number of monographs, multi-authored works, and conference proceedings have focused on the biotechnological production of flavors and aromas (Schrader, & Berger, 2001). The perspectives of generating flavors by fungi have been well outlined by several updated reviews (Agrawal, 2004; Berger, & Zorn, 2004; Vandamme, 2003).

Among all fungal classes, basidiomycetes are probably the most promising candidates to generate desirable flavors. Starting from the early 1950s, researchers attempted to harness mushroom fungi for natural flavor production because of their enormous biochemical potential (Sugihara, & Humfeld, 1954). The class of basidiomycetes shows a complicated sexual cycle, pseudo-tissue formation, and the distinct ability to degrade native cellulose or lignin aerobically. The biotechnological potential of mushrooms is probably far from exploited. Volatile flavors from all chemical classes were found in basidiomycete fruiting bodies and cell cultures (Berger, Krings, & Zorn, 2002). Studies on flavors of mushroom fungi are not only of theoretical but also of commercial significance. Based on the updated researches, five main aspects may be summarized:

- Although flavors formed *de novo* in fruiting bodies of mushrooms suffer from low yield, a detailed investigation of volatile compounds from specific mushroom is a critical and basic start to reveal the mechanism of flavor formation in its fruiting bodies. The full-scale investigations may open up new avenues for discovering related enzymes and genes responsible for the flavor development. This will accelerate the process of engineering the biosynthesis of natural flavors.

- New strains of interest may be isolated from the wild fruiting bodies (Trinci, 1995). Improved understanding of fungal metabolic activity in natural ecological settings may facilitate the searching and screening for attractive flavors. To optimize the selection of isolates from different substrates and habitats will increase the chance to find novel odorous metabolites (Wildman, 1995). The efficiency of biotransformations of low-cost compounds to high value volatiles may be improved by a careful selection of the strain (Kaspera, Krings, Pescheck, Sell, Schrader, & Berger, 2005).

- The increasing amounts of ligninocellulosic wastes from agriculture, such as rice and wheat straw, corn cobs, cotton stalks, and cereal hulls, as well as from woodland, animal husbandry, and manufacturing industries, are often landfilled or burned at great cost to the environment (Rinker, 2002; Anoliefo, Isikhuemhen, & Okosolo, 1999). Many natural flavors, being building blocks of the lignin macromolecule, can be selectively released by mushroom fungi.

- The elucidation of flavor profiles contributes to the discovery of novel secondary metabolites from fungal kingdom, and may serve as a new method for a chemotaxonomical index of fungi (Brondz, Hoiland, & Ekeberg, 2004; Monaghan, Polishook, Pecore, Bills, Nallin-Omstead, & Streicher, 1995). Novel natural components of impact odor will attract interest from aroma-related industries, especially the perfume, fragrance and cosmetic companies, which are in a constant search for new and unusual volatile compounds and scents.

- The investigation of volatile secondary metabolites of wild mushroom fungi may be beneficial for the monitoring of environment changes, as the living organisms evolve to meet environmental challenges (Hawksworth, 2004).

1.3. Brief review on the investigated wild mushroom fruiting bodies and noticeable volatiles

The mushrooms cultivated in the highest amounts worldwide are *Agaricus bisporus* (button mushroom), followed by *Lentinula edodes* (shiitake) and *Pleurotus ostreatus* (oyster mushroom) (Sánchez, 2004). Their popularity is not only based on their nutritional value, but also on their unique flavor and aromatic properties (Cohen, Persky, & Hadar, 2002; Stoop, & Mooibroek, 1999). The secondary metabolisms of these industrially produced species are much better studied than those of wild species. As a result, a breakthrough in industrial production of natural volatiles from mushrooms was the formation of natural 1-octen-3-ol using enzymes from *Agaricus bisporus* (Morawicki, Beelman, Peterson, & Ziegler, 2005). This testifies that fundamental efforts on understanding the volatiles and their formation in the mushroom fungi may also be of commercial interest. This also inspires industrial and academic research to screen more wild species for the production of high value flavor

compounds. An extensive investigation on the volatile secondary metabolites of wild fruiting bodies is an indispensable part of the whole process.

The cultivation of mushroom fruiting bodies represents an economically important biotechnological industry, that has markedly expanded all over the world in the past few decades. Nevertheless, the number of commercially cultivated mushroom species currently amounts to only about 35, and around 20 of them are produced on an industrial scale (Sánchez, 2004). The major problems up to now are that only a few species can be induced to fruit in culture (Cohen, Persky, & Hadar, 2002; Kües, & Liu, 2000). Furthermore, the search for secondary metabolites from wild filamentous fungi has been a consistent source of chemical innovation in screening programs for natural products (Monaghan, & Tkacz, 1990). Consequently, most of the investigated materials were the fruiting bodies picked in the wild.

70 years prior to this study, a paper on odorous volatiles from the fruiting bodies of mushroom was published (Aye, 1933). Considerable progress was not made until the birth of gas chromatography (GC). Searching the SciFinder database and on-line Web of Science until September 2005, a survey showed that more than 220 species of wild basidiomycete mushroom fruiting bodies have been examined for volatile compounds. Around 70% of them were investigated comparatively detailed in the past decade. Especially the French team of Rapior and Breheret has rendered outstanding services to the investigation of volatiles from fungal fruiting bodies. About 120 wild mushroom species have been screened by them since 1994. The well-known mushroom aroma arises from a series eight-carbon aliphatic oxygenated compounds (briefly called C8 compounds), especially 1-octen-3-ol (Mau, Chyau, Li, & Tseng, 1997). After 1-octen-3-ol was firstly isolated from *Armillaria matsutake* (Murahashi, 1938), more and more volatiles were realized to be distinct and typical flavors of fruiting bodies of different mushrooms. A milestone example was the isolation of lenthionine from the fruiting bodies of *Lentinus edodes* (Morita, & Kobayashi, 1966). To avoid stale and redundant review, the reports published prior to 1994, are not restated. Here, except for the common C8 compounds, emphasis is given to noticeable volatiles formed *de novo* by fruiting bodies, together with their respective original wild mushroom species described in selected publications (**Table 1**).

As seen in **Table 1**, more and more compounds of other chemical classes were found to be responsible for the characteristic flavor of fungi. They may be divided into four main groups: terpenoids, sulfur-containing compounds, aromatic compounds, and miscellaneous classes.

The former three classes were most frequently highlighted in many literatures, although esters, lactones, methyl-branched short-chain fatty acids, etc., were also described.

Table 1. Investigated Fruiting Bodies of Wild Mushroom Basidiomycetes and Noticeable Volatile Compounds Since 1994

Mushroom species	Noticeable compounds	References
Agaricus blazei	Benzaldehyde, benzoic acid	Stijve, et al., 2002
Agaricus bisporus	(*E*)-Linalool oxide	Venkateshwarlu, et al., 1999
Agaricus esettei	Benzaldehyde, phenylethanol	Rapior, et al., 2002
Amanita ovoidea	Piperitol, α-fenchene, α-thujene, sabinene hydrate	Breheret, et al., 1997
Auricularia polytricha	Dihydro-5-pentyl-2-(3H)-furanone acid	Lee, et al., 1995
Boletus erythropus	Piperitone	Breheret, et al., 1997
Calocybe indica	p-Anisaldehyde	Venkateshwarlu, et al., 1999
Clitocybe odora	p-Anisaldehyde	Rapior, et al., 2002
Cortinarius herculeus	Geosmin	Breheret, et al., 1999
Cystoderma amianthinum	Geosmin	Breheret, et al., 1999
Cystoderma carcharias	Geosmin, fenchol, fenchone, camphene hydrate	Breheret, et al., 1997 & 1999
Fomes fomentarius	β-Phellandrene, β-mycrene	Faldt, et al., 1999
Fomitopsis pinicola	β-Barbatene, (*E*)-β-farnesene	Faldt, et al., 1999; Rösecke, et al., 2000
Gomphidius glutinosus	Camphene, α-thujene	Breheret, et al., 1997
Gyrophragmium dunalii	Benzaldehyde, phenylethanol	Rapior, et al., 2000
Gloeophyllum odoratum	Drimenol, (*R*)-(–)-Linalool	Kahlos, et al., 1994; Rösecke, et al., 2000
Lactarius rufus	Rufuslactone	Luo, et al. , 2005
Lactarius atlanticus	Altanticones	Clericuzio, et al., 2002
Lentinellus cochleatus	p-Anisaldehyde	Rapior, et al., 2002
Lentinus edodes	Sulfur- and nitrogen-containing volatiles	Cho, et al., 2003; Eri, et al. 2004;
Lepista nuda	(*Z*)-Linalool oxide	Breheret, et al., 1997
Marasmius alliaceus	2,4,5,7-Tetrathiaoctane, 2,3,5-trithiahexane	Rapior, et al., 1997
Phallus impudicus	Dimethyl sulfide, trimethy sulfide, (*E*)-ocimene	Borgkarl, et al., 1994
Piptoporus betulinus	(+)-α-Barbatene, Isobazzanene	Rösecke, et al., 2000
Pleurotus eryngii	Benzaldehyde	Mau, et al., 1998
Pleutrotus ssp	Sotolone	Lizarrage-Guerra, et al., 1997
Termitomyces shimperi	Phenylethanol	Nyegue, et al., 2003
Trametes suaveolens	Methyl anisate	Rösecke, et al., 2000
Tricholoma caligatum	α-Thujene	Breheret, et al., 1997
Tricholoma matsutake	Methional	Cho, et al. 2005
Tricholoma sulfureum	Indole, 3-formylindole, linalool	Rapior, et al., 1998
Ustilago maydis	(*E*, *E*)-Deca-2,4-dienal, vanillin	Lizarrage-Guerra, et al., 1997
Volvariella volvacea	Octa-1,5-dien-3-ol	Mau, et al., 1997

Many terpenoids and sulfur-containing compounds are highly valued as flavors and fragrances so that a special fragrance chemistry on terpenoids and sulfur-containing odorants has recently formed (Candela, Fellous, Joulain, & Faure, 2002; Goeke, 2002). Sulfur-containing compounds are appreciated, as they often show very low odor thresholds. They may change the overall olfactory impressions of fragrant mixtures, even if present only in trace amounts. Biochemistry of sulfur flavors in *Lentinula edodes* is thought to be similar to that of well-studied plants of the genus *Allium*, such as Chinese chive, garlic, and onion. Why the mushroom produces such large quantities of exotic sulfur species remains a mystery (Sneeden, Harris, Pickering, Prince, Johnson, Li, Block, & George, 2004). Little has become known on the biosynthetic pathways of terpenoids in the fruiting bodies of mushrooms.

1.4. Analysis of volatiles from the fruiting bodies of mushroom fungi

1.4.1. Sampling of volatile constituents from the fruiting bodies of mushroom fungi

Sample preparation is one of the most critical aspects of the analysis of complex matrices for trace components, and can also be the most time consuming (Poole, & Wilson, 2000). Volatile compounds are most often isolated by taking advantage of their volatility and nonpolar nature. In a typical analysis for volatiles in mushrooms, the fruiting bodies should be first homogenized. As reviewed in **1.3.**, volatile compounds in fruiting bodies of mushroom fungi comprise constituents belonging to different chemical classes and are present in dramatically different concentrations. Similarly, a large number of isolation strategies which have been adopted to fruits, vegetables, and tree leaves, may be employed to the mushroom fruiting bodies. 11 main sample preparation methods for isolating volatile constituents have been available so far (Chaintreau, 2001). However, no exclusive method for collection of volatiles from a complex system can be simultaneously described as optimal (Petersen, & Poll, 2000; Wilkes, Conte, Kim, Holcomb, Sutherland, & Miller, 2000). To date, no single technique can meet all the demands. The ideal method of choice should be cheap, rapid, simple, environmentally friendly, highly sensible and reproducible. Many comparative studies revealed that methods for isolation of volatiles depended on the type of matrices, the compounds of interest, and the required sensitivity (Cavalli, Fernandez, Lizzani-Cuvelier, & Loiseau, 2003; Lee, Kim, & Lee, 2003; Shen, Sha, Deng, Fu, Chen, & Zhang, 2005; Wanakhachornkrai, & Lertsiri, 2003). New attempts focus on the combination of two or even three methods, the development of new materials, and the introduction of new technologies.

Prior to GC analysis, at least seven main sample preparation methods have been employed for isolating volatile constituents from fruiting bodies of mushrooms (**Table 2**).

Table 2. Methods of Sample Preparation for the Analysis of Volatiles from Fruiting Bodies of Mushroom Fungi Prior to GC Analysis

Methods	Fundamental principles	Reference examples
Continuous liquid–liquid extraction	Partition	Wu, et al., 2005
Direct solvent extraction	Partition	Rapior, et al., 2000
Dynamic headspace	Volatility	Kabbaj, et al., 2002
Simultaneous distillation–solvent extraction	Partition & volatility	Cho, et al., 2003
Solid phase microextraction	Partition	Zeppa, et al., 2004
Steam distillation	Volatility	Eri, et al., 2004
Stir bar sorptive extraction	Partition	Eri, et al., 2004

Figure 1. Apparatus of continuous liquid–liquid extraction

Solid-phase microextraction is becoming increasingly popular in the field of flavor and fragrance analysis (Jelen, Kaminski, & Wasowicz, 2000). Nevertheless, it still demands improvements because the surface area of its adsorbent is sometimes too small to adsorb sufficient amounts of volatiles from faint or complex samples (Ishikawa, Ito, Ishizaki, Kurobayashi, & Fujita, 2004). Meanwhile, other sampling methods of volatiles, including new attempts such as liquid-phase microextraction (Jiang, Basheer, Zhang, & Lee, 2005), are currently used in parallel. In contrast, continuous liquid–liquid extraction (CLLE) (**Figure 1**) is still regarded as a versatile, reliable and robust sample preparation technique (Apps, & Tock, 2005). CLLE dates back at least to 30 years (Stage, & Gemmeker, 1963). The advantages of CLLE can be summarized as follows:

- In contrast to the solvent-free sampling methods such as head space, the extract can be concentrated to allow the detection of trace volatiles (sub µg/L). Moreover, a small volume of a single concentrated extract (for example, 1 mL) supplies enough material for repeated analysis by GC and GC-MS with different columns, sensitivities, or detectors, as well as for GC-olfactometry, aroma dilution analysis, and for micropreparative-GC.

- As a limited volume of solvent is continuously recycled, solvent purity is less critical than with the larger volumes used in batch extractions (Elss, Preston, Hertzig, Heckel, Richling, & Schreier, 2005).

- Compared to other systems, the glassware is simple and the procedure is straightforward (Etievant, 1996). It requires no gas chromatographic inlet hardware beyond a split-splitless injector.

- The extraction process is gentle and can be run as long as necessary with little attention. It is especially valuable in exploratory studies on flavors, where the composition of samples in terms of viscosity, suspended solids and concentration of volatiles is variable and unpredictable (Apps, & Tock, 2005).

1.4.2. Analysis of collected volatile constituents

Usually, the volatile compounds emitted by the fruiting bodies of basidiomycetes are analyzed by coupled gas chromatography–mass spectrometry (GC–MS) and GC-olfactometry

(Berger, & Zorn, 2004). An identification of a flavoring substance must pass scrutiny of the latest available analytical techniques. In practice, this means that any particular substance must be identified by at least two methods, e.g., by comparison of chromatographic and spectrometric data (which may include GC, MS, IR, and NMR) with those of an authentic sample (The American Chemical Society, 2005).

In this study, GC retention indices on columns of different polarity and mass spectrometric information given by MS with electron impact ionization (EI) were used. To ensure the integrity of the results, it is indispensable to check each peak with the hits given by the computerized matching of an unknown spectrum with databases. The retention indices on polar and apolar columns were calculated and compared to published data. The retention indices of some of the volatiles from the fruiting bodies of *F. hepatica* on DB5 column, which were not indicated in the earlier report (Wu, Krings, Zorn, & Berger, 2005), were re-examined and revised due to better-understanding of the computerized matching system (**Table 11**). The sensory properties of flavors, determined by GC-O, were additionally compared with those reported from literature. If necessary, an accurate mass determination by high resolution MS with chemical ionization (CI) was performed to confirm the identification of the compound. Additionally, GC-atomic emission detector (GC-AED) analyses were performed to detect sulfur-, chloride-, and nitrogen-containing volatiles. For those compounds that could not be identified, the molecular ions and the eight most intense ions were listed.

The volatile fraction of fruiting bodies of a mushroom consists of many compounds, of which only a small number significantly contributes to the flavor. GC-O has been used widely in the isolation and characterization of odorants from complex natural products for more than 30 years (Srinivasan, 2005). Those compounds, which impressed the panelists intensively, are addressed as characteristic or key odorants (Belitz, Grosch, & Schieberle, 2004).

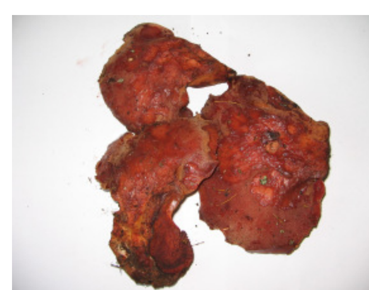
1.5. Choice of wild fruiting bodies

Generally, the composition of volatiles is affected by differences in strain, substrate, fruiting conditions, developmental stage, and the age of the fresh mushroom sample. Nevertheless, selecting the fruiting bodies of *Fistulina heaptica*, *Lentinus lepideus*, and *Polyporus sulfureus* (**Figure 2**) for the investigation was not arbitrary. First of all, the fruiting bodies of the three fungi have been consumed as foods or medicinal stuffs. *F. hepatica* was once called “poor man’s beefsteak” (Jahn, 1990). *P. sulfureus*, commonly named “Chicken-of-The-Woods”, has

long been used in herbal medicine in China (Zjawiony, 2004). *L. lepideus* has been commercially consumed in East Asia and utilized to produce a latest natural immune enhancing medicine (Jin, Jung, Shin, Kim, Jeon, & Choi, 2003). Secondly, *P. sulfureus* arose our interest due to the unrevealed great differences of flavor between young and aged fruiting bodies. *L. lepideus*, a well-known sesquiterpene-producing fungus (Rösecke, Pietsch, & König, 2000), developed its fruiting bodies on chestnut trees, which was a newly-found host.

Polyporus sulfureus

Lentinus lepideus



Fistulina hepatica

Figure 2. Fresh fruiting bodies of the wild mushrooms (Wisent Park, Springe, Germany)

1.6. Purposes of this study

Market-driven industrial research was concentrated on screening microorganisms and enzymes and applying the knowledge obtained for the development of suitable production processes (Gatfield, 1999). Natural flavors can be produced by two biotechnological processes, biotransformation (or bioconversion) and *de novo* synthesis. In general, compared to the selective biotransformation using precursors or concentrated enzymes, the *de novo* synthesis using the whole metabolic spectrum of mushroom fungi growing on basic cultivation media or natural hosts, produces a much wider range of aromas and suffers from much lower productivities or poor concentrations of the target compounds. Therefore, most of the studies on *de novo* synthesis of mushroom fungi were on a laboratory scale and thus lacked economic profitability (Bluemke, & Schrader, 2001; Cohen, Persky, & Hadar, 2002). However, as described above, attempts of this category have helped and will continue to aid the study of the enzyme systems involved, of the reaction pathways during the synthesis (Agrawal, 2004), and of relationships among metabolites-, strains-, medium-, and culture conditions. Meanwhile, novel odorants of interest or new fungal secondary metabolites may be discovered. Promising new technologies and materials, for example, the dynamic removal of the formed volatiles from the fermentation broth by *in situ* product removal, may help to

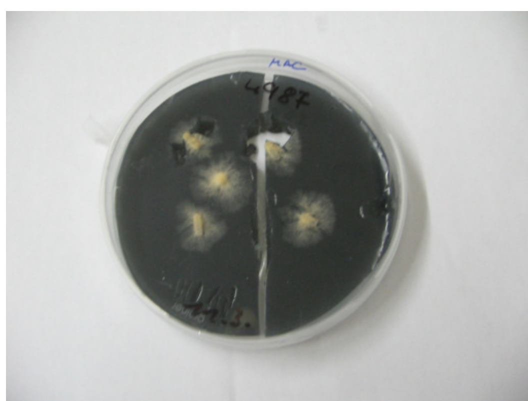
overcome the limitations of *de novo* synthesis. Mainly for these reasons, comprehensive investigations on volatile compounds formed *de novo* both by fruiting bodies of the three fungi and by cultures of *F. hepatica* were performed.

- Volatile constituents from the fresh fruiting bodies of *F. hepatica* were investigated, and characteristic odorants of this fungus were determined.

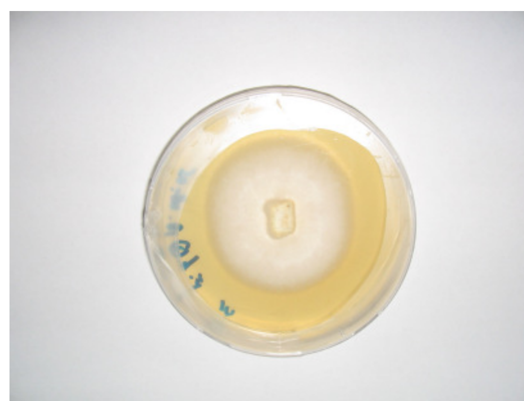
- The composition of volatiles from young and aged fruiting bodies of *P. sulfureus* were analyzed, and characteristic odorants of them were determined. Significant odor differences between the young and aged fruiting bodies of this fungus were elucidated.

- Volatile compounds from the young fruiting bodies of wild *Lentinus lepideus* grown on chestnut wood were comprehensively examined and compared with previously reported volatiles generated by this species. Characteristic odorants of the fruiting bodies of this fungus grown on chestnut wood were determined.

- *F. hepatica* (DSMZ 4987) (**Figure 3**) was grown submerged and in surface cultures. Volatile compounds generated by both cultures were investigated and compared.



On charcoal-agar medium



On SNS-agar medium

Figure 3. Mycelia morphology of *F. hepatica* (DSMZ 4987) on agar medium

2. CHARACTERISTIC VOLATILES FROM YOUNG AND AGED FRUITING BODIES OF WILD *POLYPORUS SULFUREUS* (BULL.:FR:)FR.

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3. VOLATILE COMPOUNDS OF THE WILD MUSHROOM *LENTINUS LEPIDEUS* GROWN ON CHESTNUT TREES (in press: *Adv. Food Sci.* copyright [2005] with permission from Advances in Food Sciences)

3.1. Abstract

Young fresh fruiting bodies of wild *Lentinus lepideus* were harvested from downed old chestnut trees. The volatile compounds were isolated by continuous liquid–liquid extraction (CLLE) and investigated by high resolution gas chromatography–mass spectrometry (HRGC–MS) on two columns of different polarity (DB-5 and ZB-WAX), gas chromatography-atomic emission detector (GC-AED), high resolusion-mass spectrometry (HR–MS), and by gas chromatography-olfactometry (GC-O). A total of 43 major volatile compounds were identified and semi-quantified. 19 sesquiterpenoids including 11 unknowns were newly found in this fungus. A sulfur-containing sesquiterpenoid mintsulfide was identified for the first time in the fungal kingdom, and a main odorant, 2-vinyl malonic acid methyl propyl mixed ester, was proposed. Five odorous compounds were responsible for the characteristic flavor of the young fruiting bodies: nonanal, (*E*)-2-nonenal, germacrene-D, 2-vinyl malonic acid methyl ester propyl ester, and nonanoic acid. The results showed that this wild variety of *Lentinus lepideus* formed a wide diversity of natural sesquiterpenoids, but lacked, in contrast to previous studies, cinnamic acid derivatives and 1-octen-3-ol.

Keywords: *Lentinus lepideus*; volatile compounds; chestnut; characteristic; sesquiterpenoids.

3.2. Introduction

The commercial importance of volatile secondary metabolites has prompted recent interest in understanding their formation and in engineering their biosynthesis (Dudareva, & Negre, 2005). The biotransformation of sustainable low-cost substrates to high value natural flavor compounds by higher fungi and isolated enzymes has been highlighted by several reviews

(Agrawal, 2004; Berger, & Zorn, 2004; Serra, Fuganti, & Brenna, 2005). A comprehensive investigation of the genuine volatile constituents of a specific wild fungus is not only a basic start to reveal the pathways of flavor formation, but also an important prerequisite for industrial development. The production of R-(–)-1-octen-3-ol by *Agaricus bisporus* has set an example of technical applicability (Liu, Zhou, Zeng, & Ouyang, 2004; Morawicki, Beelman, Peterson, & Ziegler, 2005).

Lentinus lepideus is a creosote tolerant, brown rot basidiomycete. It is not only consumed mainly in east Asia as an edible mushroom, but also has been generally confirmed as an industrially important microorganism (Kim, Kang, Jin, Kim, Shim, & Choi, 2000). It has been utilized in paper manufactures (Ueno, Higaki, Onishi, & Watanabe, 1990), industrial waste treatment (Collett, 1992; Sakaguchi, Nakajima, Okada, & Iseda, 1990; Samson, Langlois, Lei, Piche, & Chenevert, 1998; Uesono, Deguchi, Nishida, Takahara, & Katayama, 1992), and recent production of a natural immune-enhancing medicine (Jin, Jung, Shin, Kim, Jeon, & Choi, 2003). The specific volatile compounds of *L. lepideus* have attracted scientific interest over the last sixty years. Early in 1940, two volatile compounds, methyl 4-methoxycinnamate and methyl cinnamate, from the fruiting bodies of this fungus growing on Scots pine sapwood were identified. The mycelium of this strain was found to generate methyl 4-methoxycinnamate on malt-agar medium as well (Birkinshaw, & Findlay, 1940). A number of papers concentrated on the biochemistry of methyl 4-methoxycinnamate formation (Eberhardt, 1956; Nord, & Vitucci, 1947; Shimazono, Schubert, & Nord, 1958; Shimazono, 1959). Since the arising of capillary gas chromatography, many volatile metabolites from this fungus, cultured on different artificial media, were reported (Abraham, Hanssen, & Mohringer, 1988; Collet, 1992; Ohta, Shimada, Hattori, Higuchi, & Takahashi, 1990; Shimada, Ohta, Kurosaka, Hattori, Higuchi, & Takahashi, 1989; Sprecher, 1981; Sprecher, & Hanssen, 1982; Taubert, 2000; Towers, Singh, Van Heerden, Zuiches, & Lewis, 1998; Wat, & Towers, 1975). Among them, cinnamic acid derivatives and sesquiterpenoids arose most general concern. However, nothing has become known about the biodegradation of chestnut lignocellulosic wastes by *L. lepideus* to flavor compounds. The fruiting bodies of *L. lepideus* are commonly found on dead pine tree in spring and show a scent reminiscent of pine resin, whereas the wild *L. lepideus* investigated in this paper was found on downed old chestnut trees. The study aimed at a comprehensive investigation of the volatile metabolites and characteristic odorants from the young fruiting bodies of wild *L. lepideus* grwon on old chestnut woods, and at comparing the results with those already documented for this fungus.

3.3. Materials and methods

3.3.1. Materials

The fresh young fruiting bodies of wild *L. lepideus* were picked from downed old chestnut trees in “Wisent Park” (Springe, Germany) on October 31, 2003.

3.3.2. Chemicals

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

3.3.3. 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 with 400 mL of methanol immediately, and 1 mL of methyl nonanoate (427 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 18,800 g at 5 °C using an RC28S centrifuge (Kendro Laboratory Products GmbH, Germany) for 20 min. The supernatant fluid was recovered as a crude extract for continuous liquid–liquid extraction (CLLE): Saturated sodium chloride solution was added to the crude extract to a final volume of 1 L. This mixture was transferred to the 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.

3.3.4. High-Resolution GC–MS and High-Resolution EIMS

High-resolution GC–MS (HRGC–MS) analysis using a polar phase was conducted on a Fisons GC 8000 equipped with a (polyethylene glycol) ZB-WAX (30 m × 0.32 mm i.d. × 0.25 µm film thickness, Phenomenex, USA) column 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 (30 m × 0.32 mm i.d. × 0.25 µm film thickness, Varian, Germany)

column 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. 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.

HR-EIMS data were collected on a GC–MS instrument at 70 eV consisting of an Agilent GC 6890N coupled to an AMD M 40 - $QuAS^3AR$ (AMD Intectra, Germany) double focusing sector field mass spectrometer in the positive ion mode and the same chromatographic conditions as for GC-FID analysis. Accurate masses were measured using PFTBA as the calibration gas. Chemical ionization (CI) was carried out using methane as reactant gas.

3.3.5. 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 × 0.32 mm i.d. × 0.25 μm film thickness, J&W Scientific, USA) column (He flow rate 38 cm/s) and connected to a G2350A atomic emmision detector (Hewlett-Packard, USA). The oven temperature program was held at 50 °C for 5 min, raised to 280 °C at 10 °C/min, and held at 280 °C for 5 min. The injection volume was 2 μL.

3.3.6. 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 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 retention times and sensory statements. Characteristic odor impressions were considered to be valid, if at least 50% of the judges reproducibly signaled an intensively sensory perception. The injection volume was 2 μL.

3.3.7. Identification and semiquantification

Linear retention indices (RIs) were calculated according to the Kovats method using *n*-alkanes (C_7-C_{28}) as external references (Kondjoyan & Berdagué, 1996). 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 (Adams, 1995; Burdock, 2002; Jennings, & Shibamoto, 1980; Kondjoyan, & Berdagué, 1996; Rychlik, Schieberle, & Grosch, 1998). Approximate concentrations of volatile compounds were calculated according to the internal standard method using methyl nonanoate and the HP ChemStation Software (Agilent Technologies, USA).

3.4. Results and discussion

In order to achieve exhaustive recovery of the genuine volatile fraction of the fruiting bodies and to allow over a dozen of injections of the same extract containing solvent for GC-O, GC-AED, GC-FID, and GC-MS, CLLE was chosen as the separation technique. In total, 43 volatile compounds were identified in the extract from the young fruiting bodies of *L. lepideus*. They comprised 7 acids, 12 esters, 10 hydrocarbons, 4 alcohols, 4 aldehydes, 2 ketones, and 4 others. In order of elution on a ZB-WAX column, they are listed in **Table 6**.

3.4.1. 2-Vinyl malonic acid methyl propyl ester

The most abundant compounds were hexadecanoic acid and 2-vinyl malonic acid methyl ester propyl ester. Their respective approximate concentration attained one to five mg/kg fruiting bodies. 2-Vinyl malonic acid methyl ester propyl ester (RI 1772 on ZB-WAX) was the dominating odorant. The mass spectral data for this compound are listed in **Table 7**. A library search using commercial MS databases (Wiley, NIST) yielded one reasonable suggestion, dimethyl-(2-methylallyl)-malonate, only. Despite of the wide compliance of the 70 eV mass spectra this structure was excluded. An intensive molecular at m/z 186 and an intensive fragment ion at m/z 155 were missing in the EI mode as well as the ion at m/z 139.

Table 6. Major Volatile Compounds from the Young Fruiting Bodies of Wild *Lentinus lepideus* [a]

No.	Compounds	Retention indices		Approximate concentration [b] (μg/kg fruiting bodies)
		ZB-WAX	DB5	
1	Decane [c]	1000	999	++++
2	3-Methyl-2-pentanone[d]	1005	752	+++
3	2-Methylbutanoic acid methyl ester[d]	1009	771	++++
4	3-Methylbutanoic acid methyl ester[d]	1019	766	+++
5	Acetic acid butyl ester[d]	1075	812	++
6	2-Methyl-1-propanol[c]	1087	<700	+
7	1,3-Dimethyl-5-methoxypyrazole[d]	1103	901	+
8	3-Acetyl-2,5-dimethyl furan[d]	1118	905	++
9	Heptanal[d]	1176	896	+
10	Hexanoic acid methyl ester[c]	1186	936	+
11	Limonene[c]	1190	1021	+
12	3-Methyl-1-butanol[d]	1199	731	++
13	2-Pentylfuran[d]	1224	991	+
14	3-Methyl-3-buten-1-ol[d]	1242	726	+
15	Octanal[d]	1278	984	+
16	Nonanal[c]	1380	1086	++
17	(*E*)-2-Nonenal[d]	1514	1139	++
18	(*E*)-Caryophyllene[d]	1564	1411	++
19	β-Elemene[d]	1576	1386	+
20	2-Undecanone[d]	1587	1277	+
21	Hexadecane[c]	1598	1599	++
22	Benzoic acid methyl ester[d]	1603	1073	+
23	Valencene[c]	1672	1364	++
24	Bicyclogermacrene[d]	1676	1486	++
25	Germacrene-D[d]	1683	1472	++
26	β-Himachalene[d]	1705	1457	+
27	β-Bisabolene[d]	1711	1498	+++
28	2-Vinyl malonic acid methyl propyl ester[f]	1772	1202	++++++
29	Dodecanoic acid 1-methylethyl ester[d]	1821	1614	++
30	2-Methyl-propanoic acid 1-(1,1-dimethylehtyl)-2-methyl-1,3-propanediyl ester[d]	1857	1578	++
31	Hexanoic acid[d]	1861	864	++
32	1-Dodecanol[d]	1956	1466	++++
33	Tetradecanoic acid 1-methylethyl ester[d]	2024	1812	++
34	Mintsulfide[d, e]	2105	1726	+
35	Nonanoic acid[d]	2170	1272	++
36	Hexadecanoic acid methyl ester[c]	2198	1906	+++
37	(*Z*)-9-Octadecenoic acid methyl ester[c]	2433	2081	+++
38	(*Z, Z*)-9,12-Octadecadienoic acid methyl ester[c]	2472	2073	++++
39	Dodecanoic acid[c]	2496	1568	++
40	Tetradecanoic acid[c]	2715	1771	++
41	(*Z*)-9-Octadecenoic acid[c]	>2800	1885	++
42	Hexadecanoic acid[c]	>2800	1976	++++++
43	Octadecanoic acid[c]	>2800	2181	+++

[a] The volatile compounds are listed in increasing RIs order on a polar column ZB-WAX.
[b] +: 1-10; ++: 10-50; +++: 50-100; ++++: 100-500; +++++: 500-1,000; ++++++: 1,000-5,000.
[c] The compound was confirmed by comparing with those mass spectra and RIs of authentic reference database of this institute.
[d] The compound was identified by comparing mass spectrum with commercial mass spectral databases and RIs on two different polarity columns with published data.
[e] Mintsulfide was specially detected by GC-AED using an Optima-5-MS column.
[f] The compound was confirmed with HREI-MS.

The CI mass spectrum, however, showed a protonated molecular ion (MH^+) at m/z 187. The overall fragmentation pattern was in good agreement with a branched malonic acid ester

structure. The double peaks at m/z 155/154 and 127/126 were attributed to the α-cleavage of the alcoholic moieties, methanol and propanol respectively, of the two ester groups with and without hydrogen rearrangement from the side chain of branched malonic acid. This hydrogen rearrangement was confirmed with a commercial available diethyl ester of ethylidene malonic acid. The symmetric diester exhibited only one double peak at m/z 141/140 resulting from the cleavage of ethanol. Combining all spectral data the structure of 2-vinyl malonic acid methyl propyl ester is proposed.

Table 7. Accurate Mass Detection of Fragment Ions of Proposed 2-Vinyl malonic acid methyl propyl ester

m/z / [%]	Determined mass [u]	Elemental composition	Calculated mass [u] (± mu)
187 [MH^+, CI]	187.0935	$C_9H_{15}O_4$	187.0970 (+ 0.0035)
186 [trace]	n.d.[a]	n.d.[a]	
155 [40]	155.0686	$C_8H_{11}O_3$	155.0708 (+0.0022)
154 [15]	154.0632	$C_8H_{10}O_3$	154.0630 (-0.0002)
139 [7]	139.0403	$C_7H_7O_3$	139.0395 (-0.0008)
127 [100]	127.0750	$C_7H_{11}O_2$	127.0759 (+0.0009)
126 [25]	126.0738	$C_7H_{10}O_2$	126.0681 (-0.0057)
111 [15]	111.0488	$C_6H_7O_2$	111.0446 (-0.0042)
95 [57]	95.0428	C_6H_7O	95.0497 (+0.0069)

[a] not determined.

3.4.2. Distinctive individuality of volatile secondary metabolite of this wild species

The presence of sulfurous and chlorine volatile compounds in the sample was selectively and sensitively evaluated using a GC equipped with an atomic emission detector (GC-AED). No chlorine-containing volatile compounds were detected. One sulfur-containing compound, mintsulfide, was detected by GC-AED and further comfirmed by GC-MS. The predominant chemical classes within the volatile constituents were sesquiterpenoids, fatty acids and their esters. Carboxylic acids and their esters were frequently found in the volatile metabolites from edible basidiomycetes. However, a rare branched carboxylic acid esters, 2-methyl-propanoic acid 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester, showed the specificity of volatile composition of this fungus. Although this ester was not previously reported as a secodary metabolite in the fungal world, it was detected in green tea flower (Baik, Bock, Han, Cho, Bang, & Kim, 1996), propolis (Greenaway, May, Scaysbrook, & Whatley, 1991), black bream and rainbow trout (Guillén, & Errecalde, 2002).

The abundantyl occuring sesquiterpenoids were the most distinctive feature of this wild fungus. Altogether, 19 sesquiterpenoids were detected in the young fruiting bodies. Markedly different from previously reported varieties of this species, another eight sesquiterpenoids were identified: (*E*)-caryophyllene, β-elemene, valencene, bicyclogermacrene, germacrene-D, β-himachalene, β-bisabolene, and mintsulfide. In terms of functionality, they comprised seven sesquiterpene hydrocarbons, and one sesquiterpene sulfide. The sulfur-bearing mintsulfide has never been reported as a native secondary metabolite in the fungal kingdom before. As a minor constituents, mintsulfide has been found in essential oils of different higher plants (Goeke, 2002; Maurer, & Hauser, 1983; Morteza-Semnani, & Saeedi, 2005).

Table 8. Unknown Sesquiterpenoids from the Young Fruiting Bodies of Wild *Lentinus lepideus*

No.	Retention indices		Characteristic mass spectral ions (EI) [a]	Approximate concentrations (µg/kg fruiting bodies)[b]
	ZB-WAX	DB5		
1	1446	1365	M^+, 204 (20.38); 162 (24.30), 161 (100.00), 147 (15.52), 134 (16.41), 133 (13.28), 121 (22.97), 119 (23.52), 105 (19.73)	+++
2	1468	1303	M^+, 204 (26.30); 147 (52.48), 133 (77.41), 121 (82.56), 119 (78.05), 107 (79.42), 105 (100.00), 93 (57.05), 91 (66.29)	+++
3	1497	1391	M^+, 204 (30.23); 162 (21.96), 161 (100.00), 147 (66.03), 133 (23.12), 121 (19.25), 119 (36.53), 105 (30.20), 91 (21.70)	++
4	1502	1383	M^+, 204 (4.63); 136 (100.00), 135 (29.73), 121 (91.15), 107 (31.31), 105 (15.17), 93 (33.56), 91 (19.71), 79 (16.81)	+
5	1550	1408	M^+, 204 (16.60); 161 (70.21), 84 (40.35), 69 (100.00), 56 (84.99), 70 (60.14), 55 (94.80), 43 (39.75), 41 (68.20)	++
6	1622	1343	M^+, 204 (27.94); 121 (80.67), 120 (40.17), 119 (100.00), 105 (62.99), 93 (65.36), 91 (88.71), 81 (47.15), 79 (45.28)	++
7	1646	1458	M^+, 204 (9.20); 147 (20.35), 121 (56.63), 119 (100.00), 107 (22.46), 105 (42.17), 93 (60.97), 91 (24.66), 79 (30.54)	++
8	1742	1519	M^+, 204 (11.01); 133 (48.24), 121 (97.67), 119 (100.00), 105 (62.01), 93 (98.13), 92 (41.51), 91 (65.33), 79 (45.95)	+++
9	1882	1475	M^+, 222 (0.61); 107 (25.73), 95 (31.44), 93 (24.71), 91 (24.31), 84 (100.00), 81 (30.49), 69 (30.48), 43 (45.67)	++++++
10	2065	1611	M^+, 220 (35.64); 136 (48.83), 135 (62.62), 121 (37.28), 109 (100.00), 108 (39.83), 107 (42.40), 93 (46.58), 81 (41.18)	++
11	2159	1496	M^+, 222 (0.75); 119 (73.02), 93 (63.63), 83 (44.39), 69 (100.00), 57 (39.28), 55 (55.10), 43 (40.81), 41 (43.82)	++

[a] Characteristic mass spectral ions consisted of molecular ion (M^+) and the other eight ions with the first highest relative abundance given by GC-MS on a ZB-WAX column.

[b] +: 1-10; ++: 10-50; +++: 50-100; ++++: 100-500; +++++: 500-1,000; ++++++: 1,000-5,000.

Additionally, 11 unkown sesquiterpenoids were detected in the fruiting bodies of this fungus (**Table 8**). Temporarily, their identities remained uncertain because no references mass spectra are available in the databases. Besides, their respective retention indices could not be matched with those of the published data. So far, more sesquiterpenoids have been reported from plants than from microorganisms, fungi, marine invertebrates, and insects. The present data show that diversity of sesquiterpenoids of fungi is not necessaarily inferior to higher plants (Fraga, 2001; Schrader, & Berger, 2001). However, gerneration of sesquiterpenoids by *L. lepideus* was highly affected by strain specificity and culture conditions. Another six strains of *L. lepideus* CBS 450.79 (Taubert, 2000), FPRL 7B, CBS I, CBS II, IFB 27a, and IFB 27b (Sprecher, & Hanssen, 1982) were studied for their capability to produce desirable volatiles. Out of them, only FPRL 7B showed positive results to generate sesquiterpenoids *de novo*. Twenty one sesquiterpenoids were previously detected in the liquid culture of strain FPRL 7B (**Table 9**).

Table 9. Previous Identified and Semi-identified Sesquiterpenoids from *Lentinus lepideus* FPRL 7B[a]

Sesquiterpenoid group	Compound	References
Sesquiterpene hydrocarbons	α –copaene; α –elemene; (*E*)-β-farnesene; γ-muurolene; α-muurrolene; δ-cadinene; cadina-1,4-diene; calacorene; $C_{15}H_{22}$[b]; $C_{15}H_{24}$[b]	Hanssen, 1982
Sesquiterpene alcohols	δ-cadinol ; (+)-T-cadinol; (-)-α-cadinol; (-)-T-muurdol; cubenol; epicubenol, (E)-farnesol; drimenol	Hanssen, 1985
Sesquiterpene ethers	Lentideusether; isolentideusether; 10-hydroxylentideusether	Abraham, & Hanssen, 1988

[a] FPRL 7B was obtained from Forest Products Research Laboratory, Princes Risborough, UK.

[b] The compound was partly identified.

In contrast to previous studies on *L. lepideus*, no cinnamic acid derivatives were detected. Methyl 4-methoxycinnamate was frequently reported as a characteristic secondary metabolite of *L. lepideus* species (Birkinshaw, & Findlay, 1940; Nord, & Vitucci, 1947; Eberhardt, 1956; Ohta & Shimadam, 1991; Shimazono, Schubert, & Nord, 1958; Taubert, 2000; Wat, & Towers, 1975). Phenylalaine ammonia-lyase, hydroxylase and three O-methyltransferases were proposed to contribute to the formation of cinnamic acid derivatives (Ohta & Shimadam, 1991; Power, Towers, & Neish, 1965; Wat, & Towers, 1975).

3.4.3. Characteristic odorants of the fruiting bodies

The charcteristic eight carbon atom (C8) compound 1-octen-3-ol, commonly found in high concentration in many edible mushrooms, was not detected. Only one C8 aldehyde, octanal, was determined as a minor constituent. GC-O investigation revealed that no mushroom-like flavor was perceivable. Another variety of *L. lepideus*, strain CBS 450.79, was recently investigated for its volatiles in submerged culture (Taubert, 2000). Among the 22 volatiles identified, no terpenoids were detected. Only two compounds, the general fusel oil constituent 2-methyl-1-propanol and 3-methyl-1-butanol, coincided with the results in this study. Furthermore, five cinnamic acid derivatives and 1-octen-3-ol were typical characteristic compounds produced by strain CBS 450.79.

Fruiting bodies of *L. lepideus* gathered on pine trees were characterized as aromatic and reminiscent of pine resin. In contrast, the fresh young fruiting bodies studied here were collected on downed old chestnut trees and emitted a very faint pure and fresh aroma. To create a reliable flavor profile by GC-O, ten trained testers were employed. Five volatile compounds were determined to contribute significantly to the overall flavor of the young fruiting bodies (**Table 10**).

Table 10. Characteristic Odorous Compounds from the Young Fruiting Bodies of Wild *Lentinus lepideus*

Characteristic odorants	GC-O Odor Description	Retention Indices		
		DB-WAX (GC-O)	ZB-WAX (GC-MS)	DB-5 (GC-MS)
Nonanal	Slightly rotted citrus	1375	1380	1086
(*E*)-2-Nonenal	Stale and moldy	1512	1514	1139
Germacrene-D	Butter	1683	1675	1472
2-Vinyl malonic acid methyl propyl ester	Walnut	1787	1772	1202
Nonanoic acid	Waxy and cheese	2210	2198	1906

Obviously, the compound responsible for the stale flavor of the young basidiocarps was primarily (*E*)-2-nonenal. During GC-O, germacrene-D and 2-vinyl malonic acid methyl propyl ester reminded the panelists of the pleasant flavor of some fatty foods, such as butter and walnut. However, their contribution to the overall flavor of the extract was somewhat weakened by the three nine-carbon atom (C9) compounds nonanal, nonanoic acid and (*E*)-2-nonenal. (*E*)-2-nonenal is an oxidation product from lipids either by auto-oxidation or by

lipoxygenase catalyzed degradation (De Buck, De Rouck, Aerts, & Bonte, 1998). Due to its very low odor threshold, (*E*)-2-nonenal is considered as an impact flavor constituent in foods, drinks and fragrances. One unfavorable aspect of (*E*)-2-nonenal is the papery off-flavor of stale beer. As a key quality parameter, its concentration should be less than 0.05 μg/L in fresh beer.

Altogeter, the results may assist in evaluating the biochemical potential of this wild strain and in developing advanced techniques of cultivation of fruiting bodies on chestnut wood wastes.

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4. VOLATILE COMPOUNDS FROM THE FRUITING BODIES OF BEEFSTEAK FUNGUS *FISTULINA HEPATICA* (SCHAEFFER: FR.) FR.

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4.1. 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. Eleven odorous compounds significantly contributed to the overall flavour of *Fistulina 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

4.2. 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 *Fistulina hepatica*, however, focused on acetylenic compounds, several of which show antibacterial activities (Barley, Graf, Higham, Cathryn, Jarrah, & Jones, 1987; Bianco Coletto, 1981; Farrell, Keeping, Pellatt, Martin, & Thaller, 1973; Jones, Lowe & Shannon, 1966; Schwope, Givan & Minto, 2003; Tsuge, N., Mori, T., Hamano, T., Tanaka, H., Shin-Ya, K., & Seto, H., 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 the characteristic compounds shaping the flavour of this fungus were determined by GC-olfactometry.

4.3. Materials and methods

4.3.1. Materials

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

4.3.2. Chemicals

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

4.3.3. Methods

4.3.3.1. Preparation of extract

Fruiting bodies (250 g) were cut into cubes of about 2 × 2 × 2 cm size. The samples were mixed with 400 mL of methanol immediately, and 1 mL of methyl nonanoate (42.7 mg/L in pentane/ether (1:1.12)) solution was added as internal standard. The mixture was homogenised by Ultra-Turrax (Jahnke and Kunkel, Germany) and centrifuged at 3,300 g at 5 °C for 20 min. The solvent layer was recovered as crude extract for continuous liquid–liquid extraction (CLLE).

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 of pentane/ether (1:1.12) were placed into 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.

4.3.3.2. High resolution GC–MS (HRGC–MS)

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/s) 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/s) 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, and held at 250 °C for 10 min. The injection volume was 1 µL.

4.3.3.3. GC-atomic emission detector (GC-AED)

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/s) 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, and held at 280 °C for 5 min. The injection volume was 2 μL.

4.3.3.4. GC–olfactometry (GC–O)

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/s. 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, 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.

4.3.3.5. 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 et al., 1996; Rychlik, Schieberle & Grosch, 1998) and, if available, authentic standards. For non-identified compounds, the characteristic mass spectral ions were given. Approximate concentrations of volatile compounds were calculated according to the internal standard method, using methyl nonanoate and the HP ChemStation Software (Agilent Technologies, USA).

4.4. Results and discussion

4.4.1. Volatile compositions of the fruiting bodies

Table 11. Major Volatile Compounds from Fresh Fruiting Bodies of Wild *F. hepatica* [a]

No.	Compound	RIs		Approximate concentration[b] (μg/kg fruiting bodies)
		ZB-WAX	DB5	
1	Hexanal[c]	1069	786	++
2	2-Methyl-1-propanol[c]	1081	<700	++++
3	Sabinene[d]	1097	968	+
4	1-Butanol[c]	1133	<700	++++
5	Limonene[c]	1175	1021	+++
6	1,8-Cineole[d]	1179	1019	++
7	3-Methyl-1-butanol[d]	1194	731	++
8	3-Octanone[d]	1236	970	++
9	Octanal[c]	1270	984	+
10	1-Octen-3-one[c]	1282	961	+
11	(*E*)-2-Heptenal[d]	1297	955	++
12	6-Methyl-5-hepten-2-one[d]	1317	988	+
13	4-Hydroxy-4-methyl-2-pentanone[d]	1339	809	++
14	Ethanedioc acid dimethyl ester[e]	1395	800	+++
15	(*E*)-2-Octenal[d]	1400	1058	+
16	1-Octen-3-ol[c]	1439	972	+++++
17	Citronellal[d]	1457	1152	+
18	2-Ethyl-1-hexanol[c]	1476	1026	++
19	Benzaldehyde[c]	1491	931	++
20	Linalool[d]	1536	1088	++
21	1-Octanol[c]	1543	1069	++
22	2-Methyl-propanoic acid[d]	1579	793	++
23	Benzoic acid methyl ester[d]	1591	1073	+++
24	Phenylacetaldehyde[d]	1616	1011	++++
25	Butanoic acid[c]	1642	863	++
26	Pentanoic acid[c]	1679	906	++++
27	2-Methyl-pentanoic acid[d]	1755	846	++
28	(+)-Cuparene[d]	1785	1488	++++
29	Dodecanoic acid 1-methylethyl ester[d]	1821	1614	+
30	(*Z*)-2-Methyl-2-butenoic acid[d]	1849	898	++
31	Hexanoic acid[c]	1858	864	+
32	(*E*)-2-Methyl-2-butenoic acid[c]	1862	941	+
33	1-Dodecanol[c]	1956	1466	++
34	Cinnamic aldehyde[d]	2007	1250	+
35	(*E*)-Nerolidol[d]	2028	1549	++++
36	(*E*)-Methyl cinnamate[d]	2046	1353	++++
37	δ-Deca-2,4-dienolactone[d]	2143	1465	++
38	Nonanoic acid[c]	2176	1272	+
39	Hexadecanoic acid methyl ester[c]	2198	1910	++++
40	(*Z*)-9-Hexadecenoic acid methyl ester[c]	2237	1895	++
41	Decanoic acid[c]	2282	1376	+
42	Octadecanoic acid methyl ester[c]	2405	2111	++
43	(*Z*)-9-Octadecenoic acid methyl ester[c]	2424	2082	+++
44	(*Z, Z*)-9,12-Octadecadienoic acid methyl ester[c]	2472	2075	++++++
45	Bisabolol oxide B[d]	2525	1749	++
46	Phenylacetic acid[d]	2613	1257	++
47	Hexadecanoic acid[c]	>2800	1965	++++
48	(*Z, Z*)-9,12-Octadecadienoic acid [d]	>2800	2128	+++++

[a] The volatile compounds are listed in increasing RIs order on a polar column ZB-WAX.
[b] +: 1-10; ++: 10-50; +++: 50-100; ++++: 100-500; +++++: 500-1,000; ++++++: 1,000-5,000.
[c] The compound was confirmed by comparing mass spectra and RIs with those of database using authentic standard references established by this institute..
[d] The compound was identified by comparing mass spectrum with commercial mass spectral databases and RIs on two different polarity columns with published data.
[e] The compound was indicated by comparing mass spectrum with commercial mass spectral databases.

Altogether, 48 volatile compounds were detected in the extract of *Fistulina hepatica*. According to the quantification by internal standard, 36 of them were presented in approximate concentrations of more than 10 µg/ (kg fruiting bodies). **Table 11** 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 ten testers were employed. A compound was considered to contribute significantly to the overall aroma profile of the fungus, if at least 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 sulphurous, chlorine and nitrogen compounds in the sample, GC equipped with an atomic emission detector (GC-AED) was employed. No sulphur-, chlorine- and 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.

4.4.2. Characteristic flavour compounds of the fruiting bodies

Eleven volatiles, summarised in **Table 12**, 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.

Table 12. 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.

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, & Bessiere, 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 and Murillo (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 intensive MH^+-ion at m/z 239. Bisabolol oxide B has never been reported as a native fungal flavour before. 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 (*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, Gomez, Mata, Lotina-Hennsen, Anaya, & Bye, 1996; Idstein, et al., 1985; Kollmannsberger, et al., 1998; Morales, et al., 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).

4.5. Conclusion

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- and 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.

4.6. Acknowledgements

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5. VOLATILES FROM SUBMERGED AND SURFACE CULTURED BEEF-STEAK FUNGUS *FISTULINA HEPATICA*

(submitted)

5.1. Abstract

Cultures of *Fistulina hepatica* DSMZ 4987 were grown submerged in standard nutrition solution (SNS) or on oak wood powder (OWP), repectively. The harvested cells were disintegrated, their volatile constituents isolated by continuous liquid–liquid extraction (CLLE), investigated by gas chromatography and by high resolution gas chromatography–mass spectrometry (HRGC–MS), and compared. Altogether, 39 volatile compounds were generated by the submerged liquid cultures, while 53 volatile compounds by the surface cultures, most of them in much higher concentrations than by the submerged cultures. The surface cultures contained aldehydes, methoxybenzenoids, and hydrocarbons, which were absent from the submerged cultures, and emitted a much stronger mushroom aroma. Accordingly, the concentration of 1-octen-3-ol in the surface cultures was more than 100 times higher than in the submerged liquid cultures. 20 compounds, mainly long chain fatty acids and their methyl esters, and short chain aliphatic alcohols, were common to both systems.

Keywords: *Fistulina hepatica*; volatiles; cultures; oak wood; non-phenolic methoxybenzenoids; terpenoids

5.2. Introduction

Since the early 1950s it was attempted to produce natural flavours by fungi, because of their enormous biochemical potential (Sugihara, & Humfeld, 1954). Driven by both, an increasing consumer preferences for natural aromas, and by improved biotechnological means research on fungal biotransformations experiences a revival (Berger, & Zorn, 2004). Particular

attention is paid to basidiomycetes because of their complex metabolism and the food status of the edible representatives.

The trivial name of *F. hepatica* is explained by the shape, size and colour of the fruiting bodies which resemble a roasted piece of beef. Unlike most other brown rot fungi, *F. hepatica* is not hazardous to trees and forests, but rather cherished by the wood industry, as it produces attractive reddish brown staining on the surface of oak wood with little effects on the mechanical properties of wood (Schwarze, Baum, & Fink, 2000). Furthermore, the fungus was attested to generate new antibacterial substances (Schwope, Givan, & Minto, 2003). Recently, it was reported that the fruiting bodies of a wild *F. hepatica* contained a wide diversity of volatile secondary metabolites, such as (*E*)-2-methyl-2-butenoic acid and a wealth of odorous terpenoids (Wu, Krings, Zorn, & Berger, 2005).

To extend the earlier studies and to prepare for a biotechnological process using *F. hepatica* cells, the *F. hepatica* strain DSMZ 4987 was grown in submerged culture and, in parallel, on oak wood powder (OWP). Aim of the present study was to investigate the major volatile compounds generated under two different conditions of growth and to compare with those reported for the fruiting bodies.

5.3. Experimental

5.3.1. Strain and media

The examined strain of *F. hepatica* (DSMZ 4987) cultured on charcoal-agar medium (30 g/L malt extract; 3 g/L medical charcoal; 20 g/L agar) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). It was inoculated on standard nutrition solution (SNS)-agar and maintained at 4 °C.

The SNS was prepared on the basis of Sprecher medium (Sprecher, 1959): 30 g/L D-(+)-glucose monohydrate, 4.5 g/L L-asparagine monohydrate, 1.5 g/L potassium dihydrogen phosphate, 0.5 g/L magnesium sulfate, 3.0 g/L yeast extract granulated, and 1 ml/L trace element solution. The composition of the trace element solution was 80 mg/L iron (III) chloride hexahydrate, 90 mg/L zinc sulfate heptahydrate, 27 mg/L manganese sulfate monohydrate, 5 mg/L copper sulfate pentahydrate, and 400 mg/L Titriplex III. For preparing

SNS-agar medium, agar (15 g/L) was added to the SNS. The pH of the SNS was adjusted to 6.0 using sodium hydroxide prior to sterilisation. The medium was autoclaved at 120 °C for 20 min.

German oak (*Quercus robur* L.) wood stripes were provided by a timber workshop in Hannover, Germany. They were rinsed with bidistilled water and air-dried at room temperature. The dried stripes were ground using a commercial blender and sieved through a 1 mm sieve. The powder was autocaved in Erlenmeyer flasks at 120 °C for 40 min. The bottom of each 1000 mL of Erlenmeyer flask was evenly covered with 6 g of OWP.

5.3.2. Culture conditions

5.3.2.1. Submerged liquid cultures

To prepare pre-cultures, 10 mm × 10 mm agar plugs from the leading mycelia edge were inoculated into a 300 mL Erlenmeyer flask containing 100 mL of the SNS, and homogenised using an Ultra Turrax (Janke & Kunkel, Staufen, Germany). After cultivation in the dark for 14 days at 24 °C on a rotary shaker (Infors, Multitron, Switzerland) with 150 rpm, the pre-cultures were homogenised. 20 mL of homogenised pre-cultures were transferred into a 500 mL Erlenmeyer flask containing 250 mL of fresh SNS for preparation of the main cultures. These were cultivated in the dark for another 21 days at 24 °C and 150 rpm to make up for a total cultivation period of 35 days.

5.3.2.2. Surface cultures

6 g of autoclaved OWP in each 1000 mL Erlenmeyer flask were inoculated with 20 mL of pre-cultures. The flasks were statically incubated at 25 °C and ambient light for 35 days.

5.3.3. Isolation of volatiles

5.3.3.1. Isolation of volatiles from sterilized SNS medium

750 mL of the sterilized SNS medium were mixed with 400 mL of methanol, and 1 mL of methyl nonanoate (46.8 mg/L in pentane/ether (v/v, 1:1.12)) solution was added as internal standard. 850 mL of a saturated sodium chloride solution were added and the mixture submitted to continuous liquid–liquid extraction (CLLE): using pentane/ether (v/v, 1:1.12) as an extraction solvent. After 24 h the pentane/ether phase was washed with water, dried over

anhydrous sodium sulfate, and concentrated at 42 °C using a Vigreux-column to a final volume of about 1 mL for GC analysis.

5.3.3.2. Isolation of volatiles from OWP

5.3.3.2.1. Extraction of volatiles from OWP

25 g of autoclaved OWP were soaked in 500 mL of pentane/ether (v/v, 1: 1.12) in darkness for 72 h at 4 °C. The mixture was filtered, and 1 mL of methyl nonanoate (468 mg/L in pentane/ether (v/v, 1:1.12)) solution was added as internal standard. The organic fraction was dried over anhydrous sodium sulfate and concentrated at 42 °C using a Vigreux-column to a final volume of about 1 mL. The GC-FID chromatogram showed that further purification of the extract was necessary.

5.3.3.2.2. Silica gel fractionation of the volatile extract

20 g of silica gel 60 (0.040-0.063 mm, Merk, Darmstadt, Germany) were dried at 150 °C for 24 h and then homogenised with 4.5 % (w/w) of bidistilled water for 12 h before use. The size of the silica gel-column was 15 mm × 200 mm. The column was washed with 20 mL of pentane before elution and its temperature was maintained at 10-15 °C during the elution using a Julabo UC cooling system (Julabo Labortechnik, Seelbach, Germany). The volatiles were eluated with pentane, pentane/ether (v/v, 1:1.12), ether and finally with methanol at an elution speed of 75 mL/h. The volume of each solvent was 150 mL and the collection container was kept at 0 °C. All of the eluates were dried over anhydrous sodium sulfate and concentrated at 42 °C using a Vigreux-column to about 1 mL.

Thus, the volatiles listed below were confirmed as either enzymatic breakdown products of OWP, or as *de novo* formed products of *F. hepatica*.

5.3.3.3. Isolation of volatiles from cultures

Three flasks of the main cultures (750 mL) were filtered at 4 °C and the supernatant was kept at 0 °C. The separated wet mycelia were disrupted as follow: The mycelia were mixed with 70% (v/v) of methanol, and the mixture was ground at 5 °C for 30 min using a KDL-A Dyno-Mill (Willy A. Bachofen, Basel, Switzerland). The diameter of glass beads (Carl Roth, Karlsruhe, Germany) was 0.25-0.50 mm. According to a previous study on disruption of filamentous fungi (Taubert, Krings, & Berger, 2000), the amounts of mycelia (w), 70% methanol (v) and glass beads (v) were 5-35 g, 50 mL and 80 mL, respectively. The cell

homogenates were centrifuged using an RT-7Plus centrifuge (Kendro Laboratory Products, Langenselbold, Germany) at 3,300 g at 0 °C. The centrifugated supernatant was combined with the filtered supernatant of the first step. The combined solutions were mixed with up to 400 mL of methanol, and 1 mL of methyl nonanoate (46.8 mg/L in pentane/ether (v/v, 1:1.12)) solution was added as internal standard. Then, the mixture was subjected to CLLE.

Surface cultures were disrupted as described above for the wet mycelia and the centrifuged supernatant was submitted to CLLE.

5.3.4. Chemicals

Solvents were provided by BASF (Ludwigshafen, Germany) and Baker (Deventer, the Netherlands). All solvents were distilled before use. High purity water was prepared with an E pure water purification system (Barnstead, Dubuque, Iowa, USA). Sodium sulfate and sodium chloride were obtained from Carl Roth (Karlsruhe, Germany). Magnesium sulfate and iron (III) chloride hexahydrate were supplied by RdH Laborchemikalien (Seelze, Germany). Zinc sulfate heptahydrate and copper sulfate pentahydrate were from Fluka (Buchs, Switzerland). The other reagents were received from Merck (Darmstadt, Germany).

5.3.5. Analysis of volatiles

5.3.5.1. High resolution GC–FID

High resolution GC–FID (HRGC–FID) using a polar phase was performed on a Trace GC equipped with a (polyethylene glycol) DB-WAX column (30 m × 0.32 mm i.d. × 0.25 µm film thickness, SGE, Germany). HRGC-FID using an apolar phase was carried out on a Fisons GC8000 equipped with a DB-5 column (30 m × 0.32 mm i.d. × 0.25 µm film thickness, Varian, Germany). The linear velocity of H_2 was 52 cm/s. 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. The injection volume was 1 µL cool on column.

5.3.5.2. 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 (polyethylene glycol) ZB-WAX (30 m × 0.32 mm i.d. × 0.25 µm film thickness, Phenomenex, USA) column connected to a Fisons MD800 mass selective detector. HRGC–MS analysis using an apolar phase was conducted on an HP5890 Series II GC equipped with a DB-5 (30 m × 0.32 mm i.d. × 0.25 µm film thickness, Varian, Germany)

column connected to an 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. Helium was used as the carrier gas at a linear velocity of 38 cm/s. Chemical ionization (CI) was carried out with methane as reactant gas. The oven temperature program was the same as described above. The injection volume was 1 µL cool on column.

5.3.5.3. Identification and semiquantification

Linear retention indices (RIs) were calculated according to the Kovats method using *n*-alkanes (C_7-C_{28}) as external references (Kondjoyan & Berdagué, 1996). 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. Odor qualities and retention indices were additionally compared with published data (Adams, 1995; Burdock, 2002; Jennings, & Shibamoto, 1980; Kondjoyan, & Berdagué, 1996; Rychlik, Schieberle, & Grosch, 1998). Approximate concentrations of volatile compounds were calculated according to the internal standard method using methyl nonanoate and the HP ChemStation Software (Agilent Technologies, USA).

5.4. Results and discussion

The initially dispersed oak wood powder was completely overgrown after 35 days of cultivation. The colour of surface cultured mycelia turned from old-cotton into yellow ochre. The same visual change was also observed during the storage of mycelia on SNS-agar plates. In contrast, the submerged mycelial pellets remained cotton-white to the end. After 35 days of growth, the whole surface cultures weighed 35.26 g. The wet mycelial pellets isolated from the 750 ml of submerged liquid cultures weighed 45.87 g.

5.4.1. Volatile compounds generated by *F. hepatica* in SNS medium

In total, 39 volatile compounds generated by submerged cultures of *F. hepatica* were identified. They comprised 16 alcohols, 12 esters, 8 acids, 2 ketones, and a lactone (**Table 13**). The most abundant compounds were linoleic acid and its methyl ester. Their approximate concentration attained 500-1,000 µg/L. The predominant chemical classes within the volatile constituents were short chain aliphatic alcohols, long chain fatty acids and their esters. Although four C8 “mushroom-flavour” compounds were detectable, they were present at no more than 10 µg/L, which was much lower than their concentration level in the fruiting bodies

of a wild *F. hepatica* (Wu, Krings, Zorn, & Berger, 2005). Comparable concentrations of the key mushroom flavour compound 1-octen-3-ol have been reported for submerged SNS-cultures of the basidiomycete strains *Kuehneromyces mutabilis*, *Pleurotus sapidus*, *Nigroporus durus*, and *Polyporus umbellatus* (Abraham, & Berger, 1994).

Table 13. Major Volatile Compounds from Submerged Liquid Culture *F. hepatica* in SNS Medium [a]

No	Compounds	RIs		Approximate concentration[b] (μg/L)
		ZB-WAX	DB5	
1	2-Pentanone[c]	983	<700	++++
2	Butanoic acid methyl ester[c]	992	710	+++
3	2-Butanol[c]	1022	<700	+
4	1-Propanol[c]	1030	<700	++++
5	Acetic acid butyl ester[c]	1070	812	+
6	2-Hexanone[d]	1074	790	+
7	Pentanoic acid methyl ester[c]	1081	823	+
8	2-Methyl-2-propanol[c]	1102	914	+
9	2-Pentanol[d]	1117	706	+
10	1-Pentanol[d]	1255	760	+
11	1-Hexanol[d]	1351	864	+
12	4-Methyl-pentanoic acid methyl ester[c]	1436	1085	+
13	1-Octen-3-ol[d, f]	1453	974	+
14	1-Heptanol[d]	1462	971	+
15	1-Octanol[d, f]	1556	1070	+
16	4-Terpineol [c]	1589	1175	+
17	(*E*)-2-Octen-1-ol[c]	1611	1059	+
18	L-(-)Menthol [c]	1631	1160	+
19	Nonanol[c]	1665	1172	+
20	α-Terpineol [d]	1687	1186	+
21	2-Hydroxy-benzoic acid methyl ester[c]	1747	1190	+
22	1-Decanol[d]	1767	1275	+
23	Hexanoic acid[d]	1866	990	+
24	1-Dodecanol[d, f]	1961	1466	+
25	γ-Nonalactone[d]	2005	1358	+
26	Tetradecanoic acid methyethyl ester[c]	2023	1812	+
27	Octanoic acid[d]	2070	1182	+
28	Pentadecaoic acid methyl ester[c]	2108	1820	+
29	Nonanoic acid[d, f]	2180	1272	+
30	Hexadecanoic acid methyl ester[d, f]	2202	1915	+++
31	(*Z*)-9-Hexadecenoic acid methyl ester[d, f]	2237	1890	+
32	Octadecanoic acid methyl ester[d, f]	2409	2112	++
33	(*Z*)-9-Octadecenoic acid methyl ester[d, f]	2426	2085	+++
34	(*Z, Z*)-9,12-Octadecadienoic acid methyl ester[d, f]	2476	2076	+++++
35	Tetradecanoic acid[d]	2715	1770	++
36	Pentadecanoic acid[c]	>2800	1857	++
37	Hexadecanoic acid[d, f]	>2800	1958	++++
38	Octadecanoic acid[d]	>2800	2158	+++
39	(*Z, Z*)-9,12-Octadecadienoic acid[d]	>2800	2130	+++++

[a] The volatile compounds are listed in increasing RIs order on a polar column ZB-WAX.
[b] +: 1-10; ++: 10-50; +++: 50-100; ++++: 100-500; +++++: 500-1,000.
[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 comparing mass spectra and RIs with those of database using authentic standard reference established by this institute.
[f] The compound was detected in the fruiting bodies of wild *F. hepatica* as well.

5.4.2. Volatile compounds generated by surface cultures of *F. hepatica* on OWP

F. hepatica typically grows on oak trees. To find out more about the effects of natural substrates on the metabolism of volatiles and to seek for a sustainable outlet of oak wood wastes, *F. hepatica* was cultured on sterilised OWP. Altogether, 53 volatile compounds were identified as products of *F. hepatica* grown on oak wood (**Table 14**).

Among the compounds identified were 20 alcohols, 14 esters, 7 hydrocarbons, 4 fatty acids, 4 methoxybenzenoids, 2 ketones, and 2 aldehydes. The most abundant compounds were 2-methyl-1-propanol, hexadecanoic acid, linoleic acid and its methyl ester. Their approximate concentration were 1,000-5,000 µg/kg. The key flavour 1-octen-3-ol, 1,2,3,4-tetramethoxybenzene, propiovanillone, and the diterpene biformene were present in appreciable amounts. Fourteen volatiles found in the surface cultures also occurred in the fruiting bodies of a wild *F. hepatica*. Most of them were either lipid- or lignin-derived volatiles. Surprisingly, no other characteristic odorants of the fruiting bodies of wild *F. hepatica* but 1-octen-3-ol was formed by the surface cultures. The most abudant compounds linoleic acid, its methyl ester, and its breakdown product 1-octen-3-ol were created in similar proportions in both the surface cultures and in the wild fruiting bodies. This suggests that the activities of lipoxygenase and hydroperoxide lyase, which catalyse the degradation of linoleic acid to 1-octen-3-ol, were similar on OWP and in the fruiting bodies on live oak trees.

Interestingly, no remains of the strong pungent off-flavor of OWP were detectable after cultivation. This was mainly due to the complete disappearance of the characteristic odorants of OWP butanoic acid, 2-methylpropanoic acid and acetic acid. They may, like the medium and longer-chain aliphatic carboxylic acids of OWP, have served as an immediate carbon source for fungal growth.

Table 14. Volatile Compounds Created by Surface Cultured *F. hepatica* on Oak Wood Powders [a]

No.	Compounds	Retention indices		Approximate concentration [b] (μg/kg cultures)
		ZB-WAX	DB5	
1	Butanoic acid methyl ester [c, f]	981	710	++++
2	Decane [c]	998	1000	++++
3	2-Methyl-butanoic acid methyl ester [c]	1008	771	+++
4	(-)-α-Pinene [c]	1012	927	++++
5	3-Methyl-butanoic acid methyl ester [c]	1019	766	++
6	2-Butanol [c, f]	1030	<700	++
7	1-Propanol [c, e]	1043	<700	++++
8	2-Hexanone [d]	1077	788	+
9	Pentanoic acid methyl ester [c]	1085	823	+
10	2-Methyl-1-propanol [c, e]	1094	<700	++++++
11	Undecane [c, w]	1098	1100	+++++
12	1-Butanol [c, w]	1147	<700	++
13	Hexanoic acid methyl ester [c]	1184	915	+
14	1-Pentanol [c, f]	1252	760	+
15	Octanal [c]	1282	1004	++
16	1-Hexanol [c, e, f]	1353	862	++
17	2-Nonanone [d]	1379	1093	+
18	Tetradecane [c]	1398	1400	++
19	Ethanedioic acid dimethyl ester [d]	1409	826	++
20	(*Z*)-Linalool oxide [d]	1431	1068	+
21	1-Octen-3-ol [c, f]	1452	976	+++++
22	Pentadecane [c]	1500	1500	++
23	Benzaldehyde [c]	1506	933	+
24	1-Octanol [c, f]	1557	1076	++
25	(-)-Isopulegol [d]	1564	1145	+
26	D-Fenchyl alcohol [d]	1574	1110	+
27	4-Terpineol [c, f]	1588	1175	++
28	Hexadecane [c]	1600	1599	++
29	(*E*)-2-Octen-1-ol [c, f]	1613	1060	++
30	L-(-)-Menthol [c, f]	1631	1160	++
31	1-Nonanol [c, f]	1658	1172	+
32	α-Terpineol [c, e, f]	1687	1193	++
33	1-Decanol [c, f]	1760	1275	++
34	p-Cymen-8-ol [d]	1843	1420	++
35	1,2,3-Trimethoxy-benzene [d]	1955	1309	+
36	Tetradecanoic acid methyl ester [c]	1998	1706	++
37	(*E*)-Nerolidol [c]	2033	1562	++
38	Pentadecanoic acid methyl ester [d, f]	2102	1816	+++
39	Tetradecanol [c]	2169	1665	++
40	Hexadecanoic acid methyl ester [c, f]	2205	1916	+++++
41	1,2,3-Trimethoxy-5-(2-propenyl)-benzene [d]	2221	1550	++
42	Hexadecanoic acid 1-methylethyl ester [d]	2232	1981	++
43	Heptadecanoic acid methyl ester [d]	2309	2028	++
44	1,2,3,4-Tetramethoxybenzene [d]	2321	1533	+++++
45	Octadecanoic acid methyl ester [c, f]	2415	2117	+++
46	(*Z*)-9-Octadecenoic acid methyl ester [c, f]	2434	2086	++++
47	(*Z, Z*)-9,12-Octadecadienoic acid methyl ester [c, e, f]	2480	2079	++++++
48	Biformene [d]	2642	2048	++++
49	Propiovanillone [d]	2693	1582	++++
50	Pentadecanoic acid [d, f]	>2800	1857	+++
51	Hexadecanoic acid [c, e, f]	>2800	1971	++++++
52	Octadecanoic acid [c, e, f]	>2800	2162	++++
53	(*Z, Z*)-9,12-Octadecadienoic acid [c, e, f]	>2800	2131	++++++

[a] The volatile compounds are listed in increasing RIs order on a polar column ZB-WAX.

[b] +: 1-10; ++: 10-50; +++: 50-100; ++++: 100-500; +++++: 500-1,000; ++++++: 1,000-5,000.

[c] The compound was confirmed by comparing mass spectra and RIs with those of database using authentic standard references established by this institute.

[d] The compound was identified by comparing mass spectrum with commercial mass spectral databases and RIs on two different polarity columns with published data.

[e] The compound was also detected in the oak wood powders themselves, but its concentration remarkably increased due to fermentation.

[f] The compound can also be generated by the submerged cultured *F. hepatica* in SNS medium.

5.4.3. Comparison of volatile compounds derived from submerged cultures and from surface cultures

The production of volatiles is well known to depend on the culture conditions. Overall similarities and differences of volatile products between the surface cultures and the submerged liquid cultures can be summarized by comparing **Table 13** and **Table 14**.

Generally, the concentrations of the volatiles from the surface cultures were much higher compared to of those of the submerged cultures. A similar phenomenon was also observed with solid-state cultures and submerged cultures of the basidiomycete *Pleurotus ostreatus* (Kabbaj, et al., 2002). While 20 compounds were produced in both culture systems, significant differences became apparent. With the exception of 1-pentanol and 1-nonanol, which were present in similar concentrations, the concentrations of 15 volatiles formed in the surface cultures were five to ten times higher than those of the submerged cultures. Pentadecanoic acid methyl ester, hexadecanoic acid methyl ester, and hexadecanoic acid in the surface cultures were found in 10 to 50 times higher concentrations compared to the submerged cultures. In particular, the concentration of 1-octen-3-ol in the surface cultures exceeded 100 times the level of the submerged cultures. Accordingly, an intensive fresh mushroom aroma was emitted from the surface cultures, while the submerged cultures imparted a faint mushroom flavour only.

The volatiles common to both were mainly lipid-derived. Except for 2-hexanone, 19 compounds represented either alcohols or long chain fatty acids or their methyl esters. An odd long chain fatty acid, pentadecanoic acid, was formed in both cultures, while it was not detected in the fruiting bodies of the wild *F. hepatica*. Remarkably, a wide range of short and medium chain aliphatic acids detected in the liquid cultures and in the fruiting bodies, were absent in the surface cultures. No hydrocarbons, only one benzenoid compound, and only three terpenols were found in the liquid cultures. In contrast, six aromatic compounds and

seven hydrocarbons including two terpene hydrocarbons appeared in the surface cultures. Two aldehydes were identified in the surface cultures, while none was detected in the liquid cultures. Compared to SNS, the most notable changes of volatile classes associated with the presence of OWP referred to methoxybenzenoids and terpenoids.

5.4.4. Methoxybenzenoid volatile compounds

When grown on OWP, *F. hepatica* formed three non-phenolic methoxybenzenoids (1,2,3-trimethoxybenzene, 1,2,3-trimethoxy-5-(2-propenyl)-benzene, 1,2,3,4-tetramethoxybenzene), and a methoxyphenone (1-(4-hydroxy-3-methoxyphenyl)-1-propanone). The two non-methoxybenzenoids benzaldehyde and p-cymen-8-ol, are known as biodegradation results of wood decaying fungi (Millington, Leach, Wyllie, & Claridge, 1998; Rocha, Delgadillo, & Correia, 1996). The presence of methoxybenzenoids is an indicator of the attack of lignin by *F. hepatica*. 1,2,3-Trimethoxybenzene and 1,2,3-trimethoxy-5-(2-propenyl)-benzene (elemicine) have been confirmed as lignin units by pyrolysis GC-MS (Camarero, Bocchini, Galletti, & Martínetz, 1999). 1,2,3,4-Tetramethoxybenzene and 1-(4-hydroxy-3-methoxyphenyl)-1-propanone (propiovanillone) were also described as genuine structural fragments of lignin (Kersten, Kalyanaraman, Hammel, Reinhammar, & Kirk, 1990; Ishizu, Nakano, & Migita, 1962).

1,2,3-Trimethoxybenzene was previously found as one of the key flavour compounds of a fermented tea Puer-tea (Kawakami, 2002). Elemicine, a major volatile in many higher plants (Mansour, Maatooq, Khalil, Marwan, & Sallam, 2004), is a natural insecticide possessing also antioxidant activity (Park, Lee, Shibamoto, & Takeoka, 2003). Three strains of basidiomycetes isolated from Puer-tea can autonomously generate elemicine (Gong, Watanabe, Yagi, Etoh, Sakata, Ina, & Liu, 1993).

Propiovanillone was found in toasted or combusted wood (Cutzach, Chatonnet, Henry, & Dubourdieu, 1997; Fine, Cass, & Simoneit, 2002) and in a smoke flavouring of *Thymus vulgaris* L. (Guillén, & Manzanos, 1999). While wood combustion produces desirable phenolic methoxybenzenoids flavours such as propiovanillone, many undesirable volatile methoxyphenols are simultaneously released (Kjällstrand, Ramnäs, & Petersson, 1998). Contrary to the thermal decomposition, the more selective “enzymatic combustion” (Kirk, & Farrell, 1987) by fungi leads to a different spectrum of volatiles without significant amounts of methoxyphenols.

A wide variety of extracellular fungal enzymes including laccase, manganese peroxidase, lignin peroxidase, phenoloxidase, chloroperoxidase, glyoxal oxidase, aryl alcohol oxidase, and versatile peroxidase are involved in the biodegradation of lignin (Hakala, Lundell, Galkin, Maijala, Kalkkinen, & Hatakka, 2005; Ortiz-Bermúndez, Srebotnik, & Hammel, 2003). However, fungal degradation on lignin seemed to depend on species (Adaskaveg, Gibertson, & Dunlap, 1995; Otjen, Blanchette, Effland, & Leatham, 1987). According to a recent proposal on the mechanism of fungal attack on non-phenolic lignin (Kapich, Steffen, Hofrichter, & Hatakka, 2005), the non-phenolic methoxybenzenoids found here might result from manganese peroxidase-initiated lipid peroxidation.

5.4.5. Terpenoids produced by *F. hepatica*

Earlier investigations on the fruiting bodies indicated that edible basidiomycetes are capable of synthesising a variety of volatile terpenoids (Breheret, Talou, Rapior, & Bessiere, 1997; Rösecke, Pietsch, König, 2000). Nine terpenoids, including monoterpenes, sesquiterpenes and diterpene were present in the surface cultures. Three of them L-(-)-menthol, α-terpineol, and 4-terpineol were also detected in lower amounts in the submerged cultures. α-Pinene and (*Z*)-linalooloxide were metabolites of several basidiomycetes (Abraham, & Berger, 1994; Breheret, Talou, Rapior, & Bessière, 1997; Rösecke, Pietsch, König, 2000). Amounts of D-fenchyl alcohol in cork slabs obviously increased upon the attack of the cork by a saprophytic basidiomycete *Armillaria mellea* (Rocha, Delgadillo, & Correia, 1996). D-fenchyl alcohol was also detected in the fruiting bodies of wild basidiomycete *Cystoderma carcharias* (Breheret, Talou, Rapior, & Bessière, 1997). (*E*)-Nerolidol occurred in the fruiting bodies of the same species (Wu, Krings, Zorn, & Berger, 2005). The diterpene biformene was first isolated and identified from *Dacrydium biforme*, and was later also detected in essential oils of plant leaves (Carman, & Grant, 1961). Fruiting bodies of the basidiomycete *Fomitopsis pinicola* were reported to contain biformene (Rösecke, Pietsch, König, 2000). These results confirm that various terpenoids are formed by basidiomycetes on wooden substrates. The monoterpene alcohol (–)-isopulegol, a constituent of some essential oils (Rajeswara-Rao, Kaul, Syamasundar, & Ramesh, 2003), has been used in the flavour and fragrance industry. It was also found in the liquid cultures of ascomycete *Ceratocystis coerulescens* (Koch, & Sinnwell, 1987). Alternatively, (–)-isopulegol can be obtained from citronellal through biotransformation using the euascomycete *Paecilomyces varioti* (Deodhar, Pipalia, &

Karmarkar, 2002). (–)-Isopulegol is reported here as a product of basidiomycetes for the first time.

Under different culture conditions, altogether17 terpenoids were formed by *F. hepatica*. The species therefore may lend itself to the detailed study of the pathway of formation of volatile terpenoids and related genes and enzymes in basidiomycetes.

5.5. Conclusion

Laboratory cell cultures of basidiomycetes may, under suitable conditions, be developed into multicellular fruiting bodies with stipe, cap and gills indicating a totipotent fungal physiology (Money, 2002). Accordingly, different sets of chemical and physical conditions of cultivation of submerged grown pellets, hydrophobic surface mats (Smits, Wick, Harms, & Keel, 2003), and of intact fruiting bodies (Wu, Krings, Zorn, & Berger, 2005) must result in biochemical differentiation. Best investigated are surface cultures which were shown to produce reactive oxygen species (Cohen, Jensen, Houtman, & Hammel, 2002) and numerous enzymes catalysing the efficient degradation of lignin (Kapich, Steffen, Hofrichter, & Hatakka, 2005) and of other uncommon substrates (Zorn, Bouws, Takenberg, Nimtz, Getzlaff, Breithaupt, & Berger, 2005). Some mechanistic insight has been gained into these processes, and some immediate chemical inducers, such as 2,5-dimethoxybenzyl alcohol (for laccases) (Gonzalez, Terron, Zapico, Tellez, Yaguee, Carbajo, & Gonzalez, 2003) and iron (for general mRNA regulation and iron acquisition) (Assmann, Ottoboni, Ferraz, Rodriguez, & de Mello, 2003) have been identified. Little is known, however, on the role of water activity (Fernandes, Loguercio-Leite, Esposito, & Menezes Reis, 2005) and light (Idnurm, & Heitman, 2005) on cellular differentiation. Candidate genes controlling light responses are now being searched using opsin or phytochrome motives or by insertional mutagenesis. Substrate effects on the composition of the exo-proteome were reported recently (Zorn, Peters, Nimtz, & Berger, 2005).

Not enough causal correlations are available to design optimised cultivation systems for the production of volatile flavours. The data presented show that the biotechnologically favoured submerged variant is not promising for this purpose, but surface cultivation suffers from a number of technical and operational drawbacks, such as substrate transport limitations, down-stream and monitoring problems. Novel cultivation approaches are obviously needed to produce fungal flavours successfully on a larger scale.

5.6. Acknowledgments

I am greatly grateful to DAAD (GermanAcademic Exchange Service) and to CSC (China Scholarship Council) for financial supports.

6. APPENDIX

6.1. Volatile compounds from standard nutrition solution (SNS) medium

6.1.1. Introduction

Standard nutrition solution (SNS) was prepared according to Sprecher (1959) and has been found to be suitable for higher basidiomycetes. The autoclaved SNS medium emits a slight flavor of commercial yeast accompanied by a faint sweet scent. Volatiles from sterilized SNS medium itself are mainly due to presence of yeast extract and chemical reactions during sterilization, such as Maillard reaction and sugar degradation. Despite hundreds of volatiles identified in yeast extracts, the volatile composition of the sterilized SNS medium remained uninvestigated. Above all, volatile compounds from the medium itself cannot be ignored, when volatile secondary metabolites produced by submerged liquid cultures demand comprehensive determination. The results of investigation on volatiles through fermentation in SNS should either eliminate or quantitatively deduct those compounds originating from the chemical blank. For this reason, the present study was undertaken to determine the volatile composition of sterilized SNS.

6.1.2. Materials and methods (seen in 5.2.)

6.1.3. Results and discussion

6.1.3.1. General description of volatile compositions

In order to achieve exhaustive recovery of the genuine volatile fraction, CLLE was chosen for extraction. **Table 15** lists the compounds identified by GC-MS analysis, semiquantitative concentrations, as well as retention indices both on a polar and an apolar column. A total of 28 volatile compounds were identified and quantified in the autoclaved SNS medium. These included 2 pyrazines, 2 sulfur-bearing compounds, 2 alcohols, 4 aldehydes, 5 acids, 6 hydrocarbons, and 7 esters. According to the quantification by internal standard, 25 of them were present at low concentration of no more than 50 μg/L. The most abundant compound detected was 2-methyl-1-propanol.

Table 15. Volatile Compounds Identified in the Sterilized Standard Nutrition Solution [a]

No	Compounds	RIs		Approximate concentration [b] (µg/L)
		ZB-WAX	DB5	
1	Decane [c]	1000	1000	+++
2	2-Methyl-butanoic acid methyl ester [d]	1009	771	++
3	3-Methyl-butanoic acid methyl ester [d]	1019	766	+
4	4-Methyl-decane [d]	1054	1060	+
5	2-Methyl-decane [d]	1057	1067	+
6	Dimethyl disulfide [d, e]	1064	746	++
7	Hexanal [c]	1069	786	+
8	2-Methyl-1-propanol [c]	1087	<700	++++
9	Undecane [c]	1100	1100	+++
10	1-Butanol [c, e]	1139	<700	+
11	Hexanoic acid methyl ester [c]	1182	936	+
12	Dodecane [c]	1200	1199	+
13	Pyrazine [d, e]	1208	712	+
14	Heptanoic acid methyl ester [d]	1279	1013	+
15	2,5-Dimethyl-pyrazine [d, e]	1310	895	+
16	Dimethyl trisulfide [d, e]	1354	976	++
17	2-Methyl-octanoic acid methyl ester [d]	1380	1154	++
18	2-Furancarboxaldehyde [d, e]	1448	830	++
19	Acetic acid [c]	1466	<700	++
20	Benzaldehyde [c, e]	1496	933	++
21	Nonanoic acid ethyl ester [c]	1521	1297	++
22	2-Methyl-propanoic acid [d, e]	1575	793	++
23	Hexadecane [c]	1600	1600	+
24	Phenylacetaldehyde [c, e]	1618	1011	++
25	Butanoic acid [c, e]	1644	860	+++
26	Pentanoic acid [c, e]	1677	906	+++
27	3-Methyl-butanoic acid [c, e]	1689	873	++
28	Dodecanoic acid 1-methylethyl ester [d]	1822	1616	+

[a] The volatile compounds are listed in increasing RIs order on a polar column ZB-WAX.

[b] +: 1-10; ++: 10-50; +++: 50-100; ++++: 100-500.

[c] The compound was confirmed by comparing with those mass spectra and RIs of authentic reference database of the institute.

[d] The compound was identified by comparing its mass spectrum with commercial mass spectral databases and RIs on two different polarity columns with published data.

[e] The compound was previously reported as a volatile component of yeast extract by Ames & Mac Leod (1985).

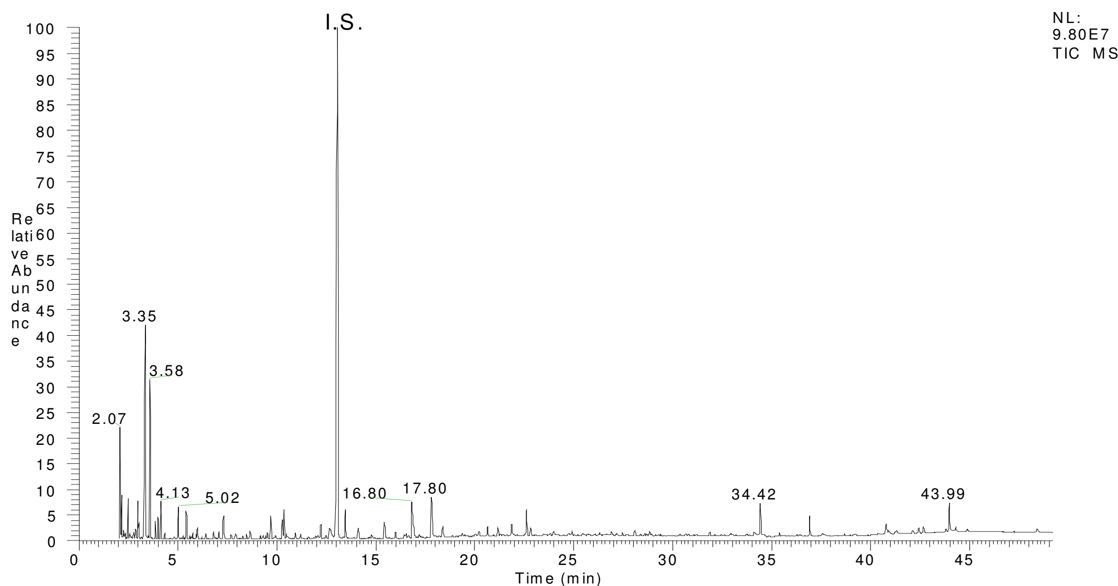

Figure 4. HRGC chromatogram of volatiles from sterilized SNS (ZB-WAX)

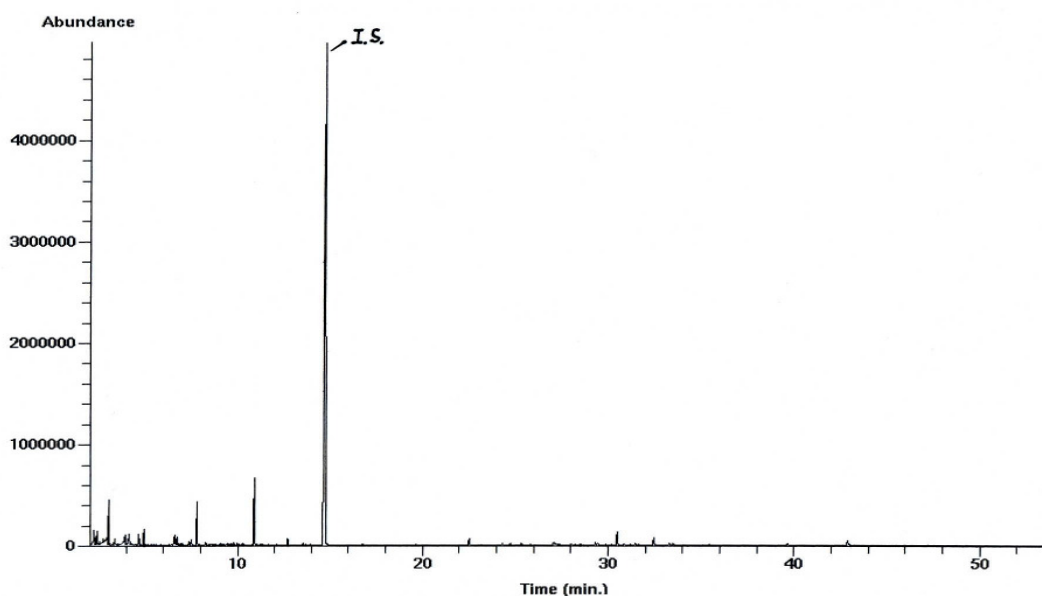

Figure 5. HRGC chromatogram of volatiles from sterilized SNS medium (DB5)

6.1.3.2. Comparison with those previous investigation on yeast extracts

As shown in **Table 15**, 10 volatiles match well with a previous investigation on the volatiles of a yeast extract (Ames, & Leod, 1985). At least 10 compounds in the SNS originate from yeast extract itself. Sulfide compounds and pyrazine volatiles in yeast extract were well described and discussed by Izzo and Ho (1991). The former were most likely produced by the

thermal degradation of methionene while the latter resulted from Strecker degradation and Maillard reaction.

Studies on the aroma extract dilution analysis and published data of odor threshold of volatiles may assess the contribution of each component to the flavor of medium. GC-O investigation revealed that 2-methyl-1-propanol, 2-methyl-propanoic acid, butanoic acid, 3-methyl-butanoic acid, and phenylacetaldehyde resulted in the strongest odor impressions. Therefore, they were responsible for the flavor profile of sterilized SNS medium. Two of them, 3-methyl-butanoic acid and phenylacetaldehyde were also previously determined as the impact odorants of the dry yeast extract through aroma extract dilution analysis (Kotseridis, & Baumes, 2000).

6.2. Volatile compounds from oak wood powders

6.2.1. Introduction

Oak trees are the most popular natural host of many wild mushroom fungi. Numerous studies were carried out on the volatile compositions of oak wood, because oak wood is widely used to maturate various alcoholic beverages (Cadahía, de Simón, & Jalocha, 2003; Pisarnitsky, Klimov, & Brazhnikova, 2004). However, composition of oak wood depends on many factors, including the variety, climate, age, location, etc. For example, in wine industry, effects of oak-related volatile compounds on wine aroma were studied by many researchers (Gómez-Plaza, Pérez-Prieto, Fernández-Fernández, & López-Roca, 2004; Morales, Benitez, & Troncoso, 2004).

The objective of this study was the determination of volatile compounds of sterilized oak wood powders (OWP). The volatile compounds originating from OWP were eliminated or quantitatively deducted from the volatiles of surface cultures of *F. hepatica* using OWP as a substrate.

6.2.2. Materials and methods (as in 5.2.)

6.2.3. Results and discussion

The volatile extract of the OWP analyzed by GC-FID (**Figure 6 & 7**) showed that further purification of the extract was required.

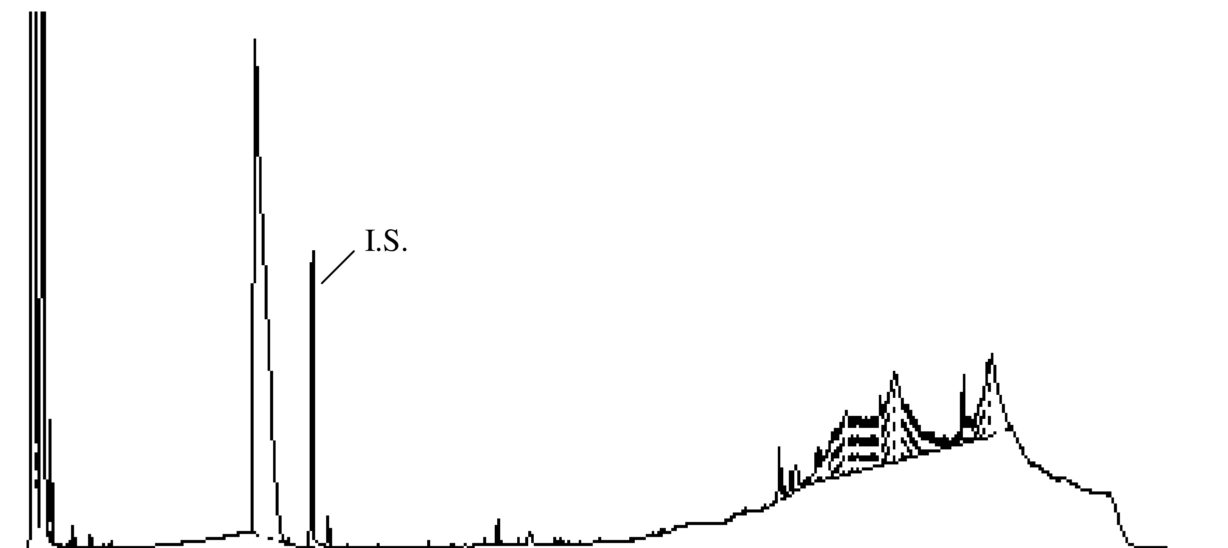

Figure 6. HRGC chromatogram of volatiles from OWP (DB-WAX)

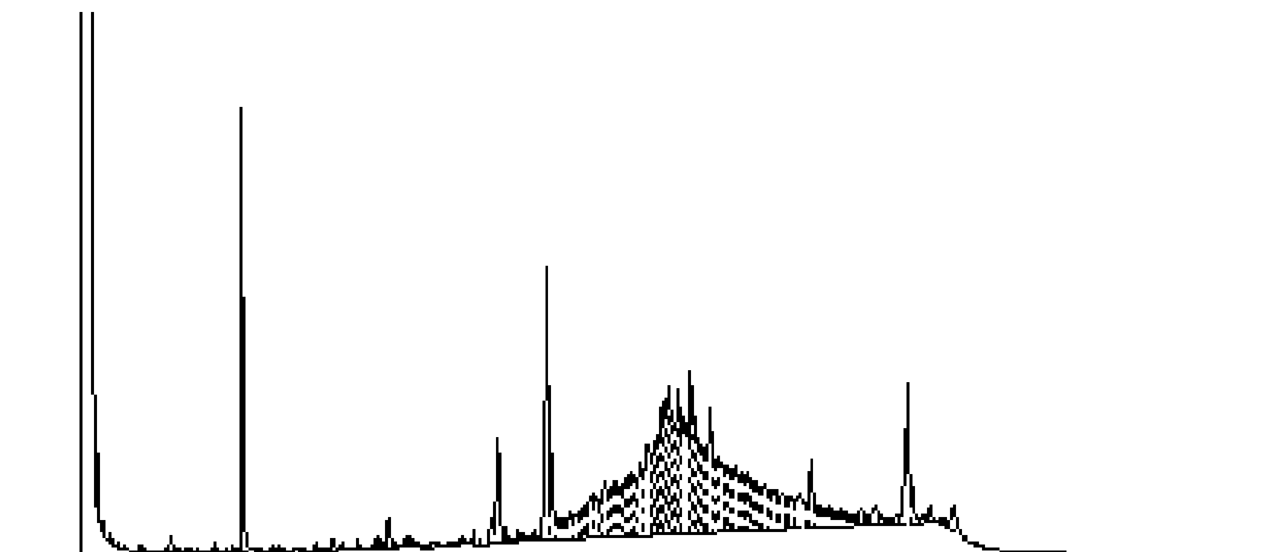

Figure 7. HRGC chromatogram of volatiles from OWP (DB5)

Table 16. Elution Sequence of Compounds on Silica Gel-LC-Column (water content 4.5% (w/w))

Fraction	Volume (mL)	Solvent	Polarity	Eluate
1	150	Pentane	Apolar	Ether, hydrocarbons
2	150	Pentane/Ether (1:1.12)	Moderate polar	Alcohol, aldehyde, ketone, phenol, ester
3	150	Ether	Polar	Acid, primary alcohol, lactone
4	150	Methanol	Highly polar	Acid, lactone

After fractionation with silica gel column using solvents with different polarity (**Table 16**), the volatile compounds in the pentane eluate were branched long chain hydrocarbons. These hydrocarbons were non-natural compounds and originated from lubricating oil, which was commonly used in the timber workshop. The chromatograms of the other three eluates are shown as follow (**Figure 8–10**).

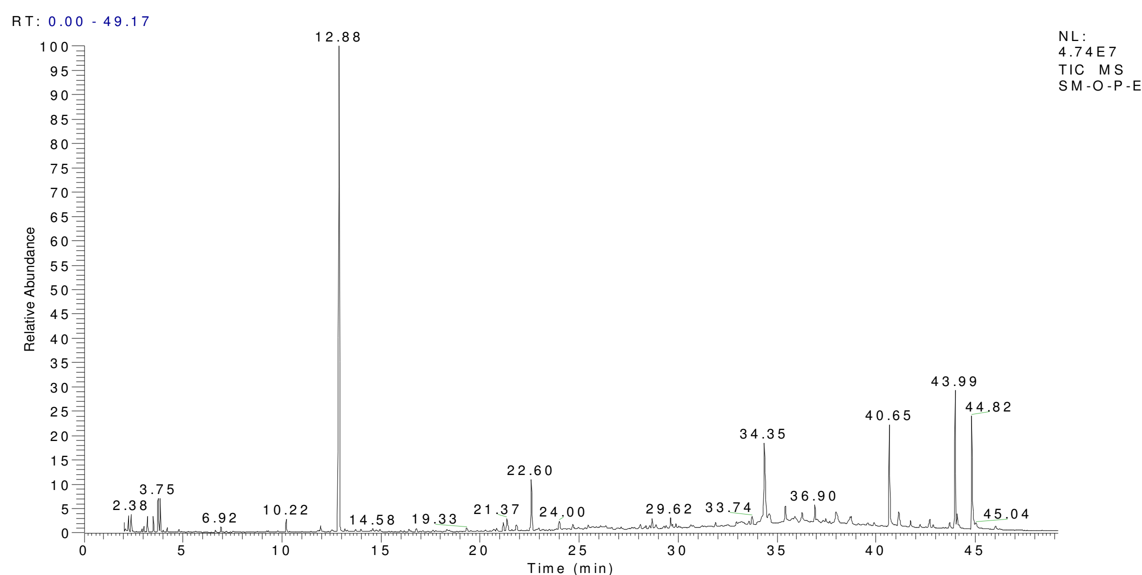

Figure 8. Fractionation of OWP extract: Pentane/Ether fractions

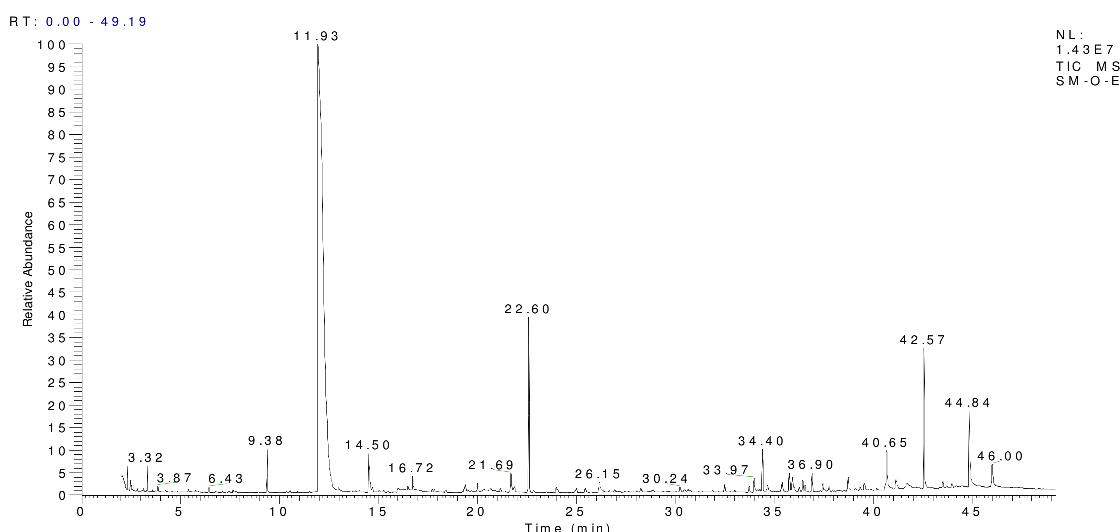

Figure 9. Fractionation of OWP extract: Ether fractions

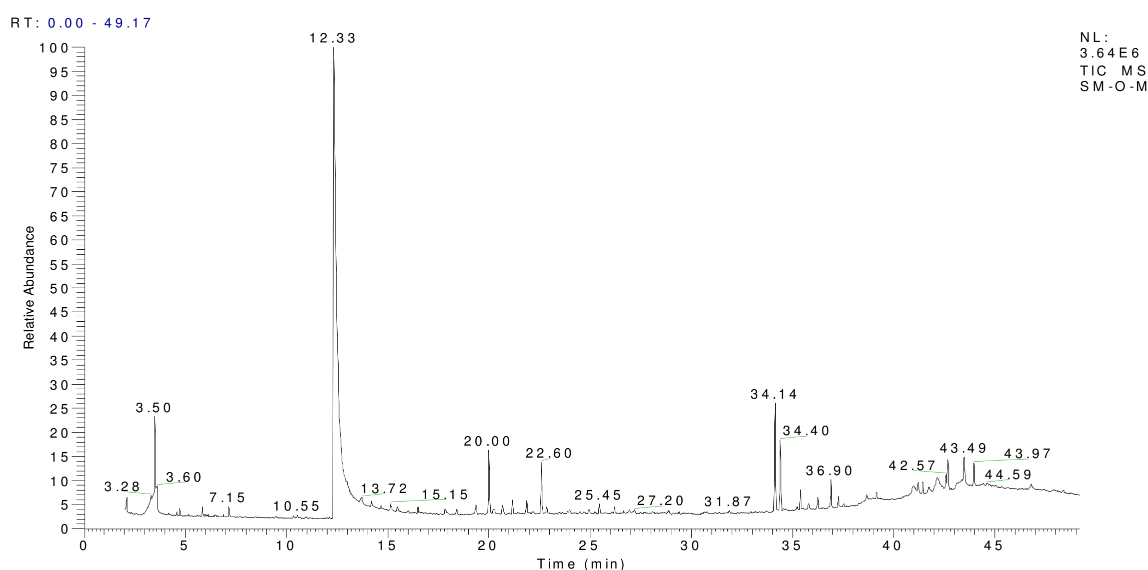

Figure 10. Fractionation of OWP extract: Methanol fractions

Table 17 lists the compounds identified by GC-MS analysis, semiquantitative concentrations, as well as retention indices both on a polar and an apolar column. Altogether, 48 volatile compounds were identified and quatified in the solvent extract of autoclaved OWP. These included 1 aldehyde, 4 hydrocarbons, 5 ketones, 8 alcohols, 11 esters, 14 acids, and 5 others. According to the quantification by internal standard, most of them were present at concentration of more than 100 µg/kg. The most abundant compound detected was acetic acid with more than 10 mg/kg. This is in agreement with previous reports (Balaban, & Uçar, 2004; Fengel, & Wegener, 1984).

Table 17. Major Volatile Compounds from Oak Wood Powder [a]

No.	Compounds	Retention indices		Approximate concentration (µg/kg)
		ZB-WAX	DB5	
1	2-Pentanone [d]	990	<700	+
2	1-Propanol [d]	1030	<700	+
3	Acetic acid butyl ester [d]	1066	812	++
4	Hexanal [c]	1067	786	+++
5	2-Methyl-1-propanol [c]	1081	<700	++++
6	3-Pentanol [d]	1093	763	+++
7	Undecane [c]	1099	1102	+++
8	4-Methyl-3-penten-2-one [d]	1114	778	++++
9	1-Butanol [c]	1132	<700	+++
10	1,1-Diethoxy-hexane [d]	1230	1085	+
11	Acetic acid hexyl ester [d]	1259	1008	+
12	Heptanoic acid methyl ester [d]	1273	1013	+
13	6-Methyl-5-hepten-2-one [c]	1319	988	+
14	4-Hydroxy-4-methyl-2-pentanone [c]	1338	811	+++
15	1-Hexanol [d]	1341	862	+
16	Hexanoic acid 2-propenyl ester [d]	1360	1071	++
17	2-Methyl-octanoic acid methyl ester [d]	1375	1154	+++
18	Acetic acid [c]	1454	<700	+++++++
19	4-Hepten-1-ol [d]	1487	870	+++++
20	Nonanoic acid ethyl ester [c]	1520	1285	+++
21	1,1-Diethoxy-nonane [d]	1522	1377	+
22	Propanoic acid [c]	1543	<700	++++
23	5,5-Diethoxy-2-pentanone [d]	1563	1134	+
24	2-Methyl-propanoic acid [c]	1571	790	+
25	1-Methyl-4-(1-methylethyl)-cyclohexanol [d]	1621	1162	+++
26	Butanoic acid [c]	1631	860	+++
27	Pentanoic acid [c]	1673	906	+
28	α-Terpineol [c]	1676	1177	+++
29	Naphthalene [d]	1698	1160	+++
30	2-Methyl-naphthalene [d]	1809	1270	+++
31	Dodecanoic acid 1-methylethyl ester [d]	1824	1620	+++
32	1-Methyl-naphthalene [d]	1844	1288	+
33	Hexanoic acid [c]	1849	992	+++
34	Tetradecanoic acid 1-methylethyl ester [d]	2026	1818	+++
35	Octanoic acid [c]	2062	1185	+++
36	Triacetin	2077	1350	+++
37	Nonanoic acid [c]	2169	1273	++
38	β-Eudesmol [d]	2192	1633	++++
39	Hexadecanoic acid ethyl ester [d]	2243	1984	+++
40	(*Z*)-9-Hexadecenoic acid ethyl ester [c]	2257	1954	+++
41	Decanoic aicd [c]	2275	1376	++
42	(*Z, Z*)-Octadecadienoic acid methyl ester [c]	2474	2079	+++
43	Dodecanoic acid [c]	2487	1568	+++
44	Tetradecanoic acid [c]	2710	1769	+++
45	Hexadecanoic acid [c]	>2800	1977	+++++
46	3,4,5-Trimethoxy-phenol [d]	>2800	1599	+++++
47	Octadecanoic acid [c]	>2800	2157	+++
48	(*Z, Z*)-9,12-octadecadienoic acid [c]	>2800	2140	++++++

[a] The volatile compounds are listed in increasing RIs order on a polar column ZB-WAX.

[b] +: 10-50; ++: 50-100; +++: 100-500; ++++: 500-1,000; +++++: 1,000-5,000; ++++++: 5,000-10,000; +++++++:10,000-15,000.

[c] The compound was confirmed by comparing with those mass spectra and RIs of authentic reference database of this institute..

[d] The compound was identified by comparing mass spectrum with commercial mass spectral databases and RIs on two different polarity columns with published data.

6.3. Major volatile compounds from the aged fruiting bodies of wild *P. sulfureus*

Table 18. Major Volatile Compounds from Aged Fruiting Bodies of *Polyporus sulfureus* [a]

No.	Compounds	Retention indices		Approximate concentration[b] (µg/kg)
		ZB-WAX	DB5	
1	Butanoic acid methyl ester [d]	992	710	+++++
2	Decane [d]	1000	1001	++++++
3	2-Methylbutanoic acid methyl ester [c]	1003	770	+++++
4	3-Methylbutanoic acid methyl ester [c]	1010	766	++++
5	3,7-Dimethylnonane [c]	1060	1074	+++++
6	Hexanal [d]	1072	786	+++
7	2-Methyl-1-propanol [d]	1083	<700	+++++
8	Undecane [d]	1092	1100	+++++
9	1-Butanol [d]	1136	<700	+++
10	Limonene [d]	1177	1023	+++
11	3-Methyl-1-butanol[d]	1195	732	+++
12	1-Dodecene [c]	1234	1389	+++
13	Octanal [d]	1273	985	++
14	Nonanal [d]	1375	1088	++++
15	Methyl acetoacetate [c]	1396	801	++++
16	1-Octen-3-ol [d]	1440	972	++
17	Benzaldehyde [d]	1494	935	+
18	(*E*)-2-Nonenal [c]	1509	1139	+
19	1-Octanol [d]	1546	1066	++
20	Propanoic acid [d]	1552	<700	+++
21	2-Methylpropanoic acid [c]	1585	790	++++
22	Butanoic acid [d]	1647	860	++++
23	α-Terpineol [d]	1679	1180	+
24	3-Methylbutanoic acid [c]	1685	878	+++++
25	Hexanoic acid [d]	1865	990	+++
26	Phenylethanol [d]	1892	1086	++++
27	1-Dodecanol [c]	1959	1466	+++
28	Tetradecanoic acid 1-methylethyl ester [c]	2027	1818	++++
29	Octanoic acid [d]	2070	1185	++++
30	Pentadecanoic acid methyl ester [c]	2102	1812	+++
31	Nonanoic acid [d]	2182	1273	++++
32	Hexadecanoic acid methyl ester [d]	2202	1913	+++++
33	(*Z*)-9-Hexadecenoic acid methyl ester [d]	2227	1898	++++
34	Octadecanoic acid methyl ester [d]	2409	2110	+++
35	(*Z*)-9-Octadecenoic acid methyl ester [d]	2429	2085	+++++
36	(*Z, Z*)-9, 12-Octadecadienoic acid methyl ester [d]	2475	2077	+++++
37	Dodecanoic acid [d]	2498	1569	+++
38	Phenylacetic acid [d]	2592	1251	+++
39	Hexadecanoic acid [d]	>2800	1962	+++++++
40	Heptadecanoic acid [c]	>2800	1912	++++
41	Octadecanoic acid [d]	>2800	2160	+++++
42	(*Z, Z*)-9, 12-Octadecadienoic acid [d]	>2800	2128	+++++++

[a] The volatile compounds are listed in an increasing RIs order on a polar column ZB-WAX.
[b] +: 10-50; ++: 50-100; +++: 100-500; ++++: 500-1,000; +++++: 1,000-5,000; ++++++: 5,000-10,000; +++++++: 10,000-15,000.
[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 comparing with those mass spectra and RIs of authentic reference database of this institute.

6.4. HRGC chromatograms of volatile extracts described in the main body of this dissertation

6.4.1. HRGC chromatograms of volatile extract from the young fruiting bodies of wild *P. sulfureus*

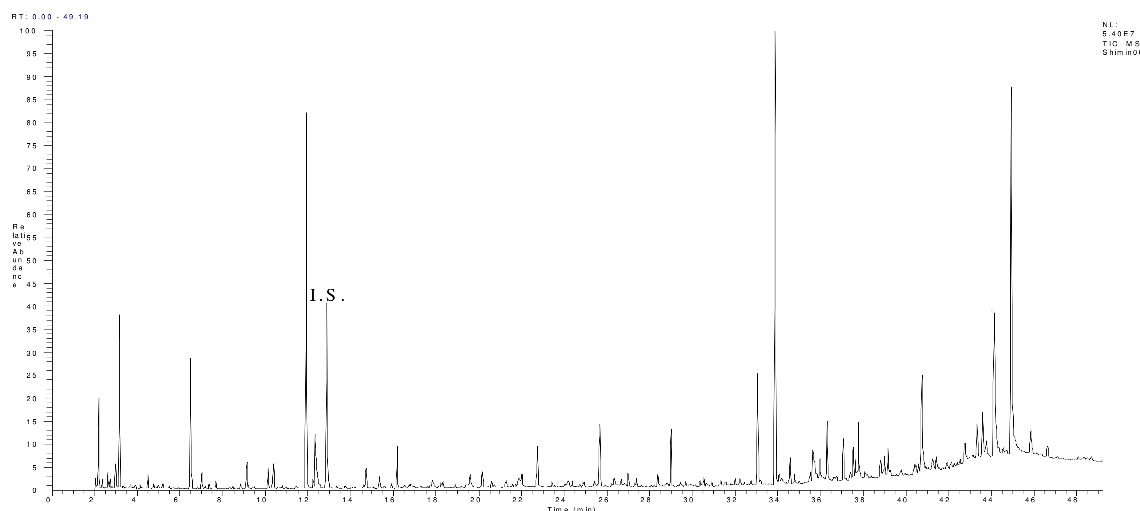

Figure 11. HRGC–MS chromatogram of the volatile extract from the young fruiting bodies of wild *P. sulfureus* (ZB-WAX)

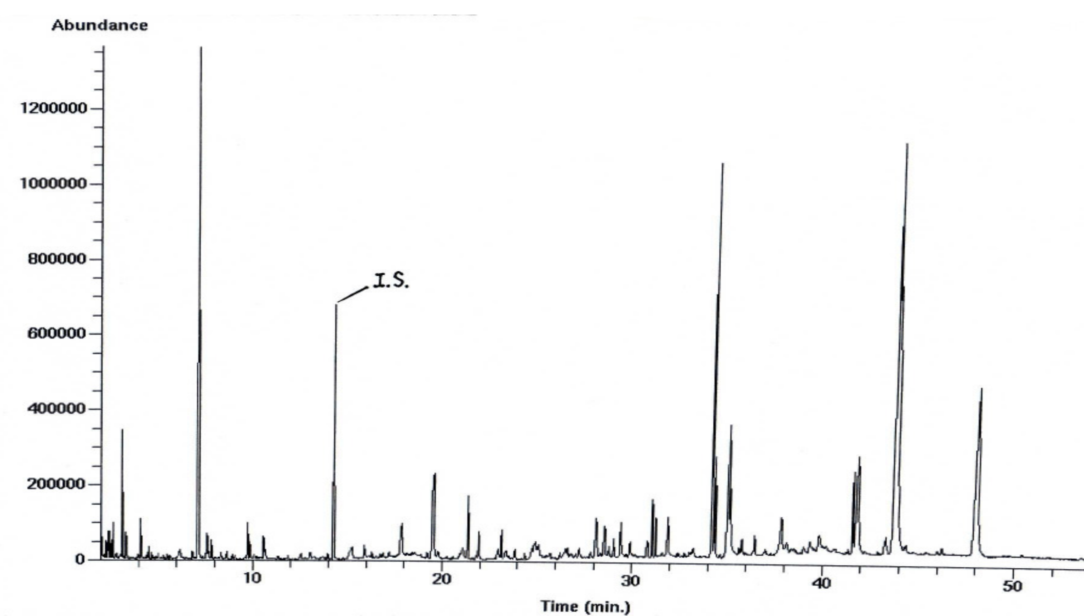

Figure 12. HRGC–MS chromatogram of the volatile extract from the young fruiting bodies of wild *P. sulfureus* (DB5)

6.4.2. HRGC chromatograms of volatile extract from the aged fruiting bodies of wild *P. sulfureus*

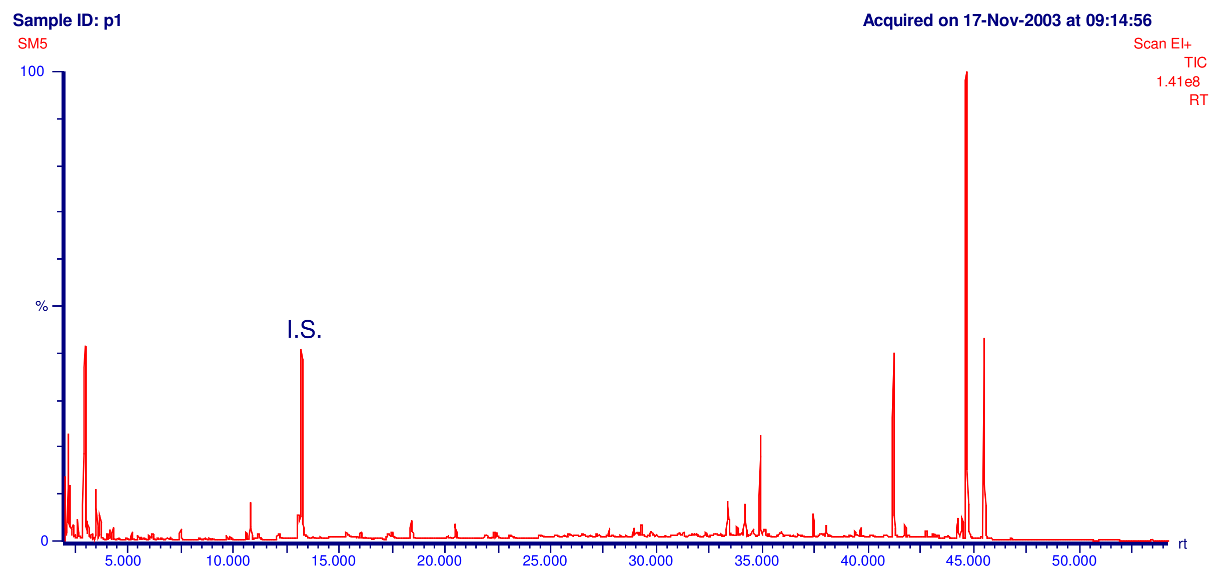

Figure 13. HRGC–MS chromatogram of the volatile extract from the aged fruiting bodies of wild *P. sulfureus* (ZB-WAX)

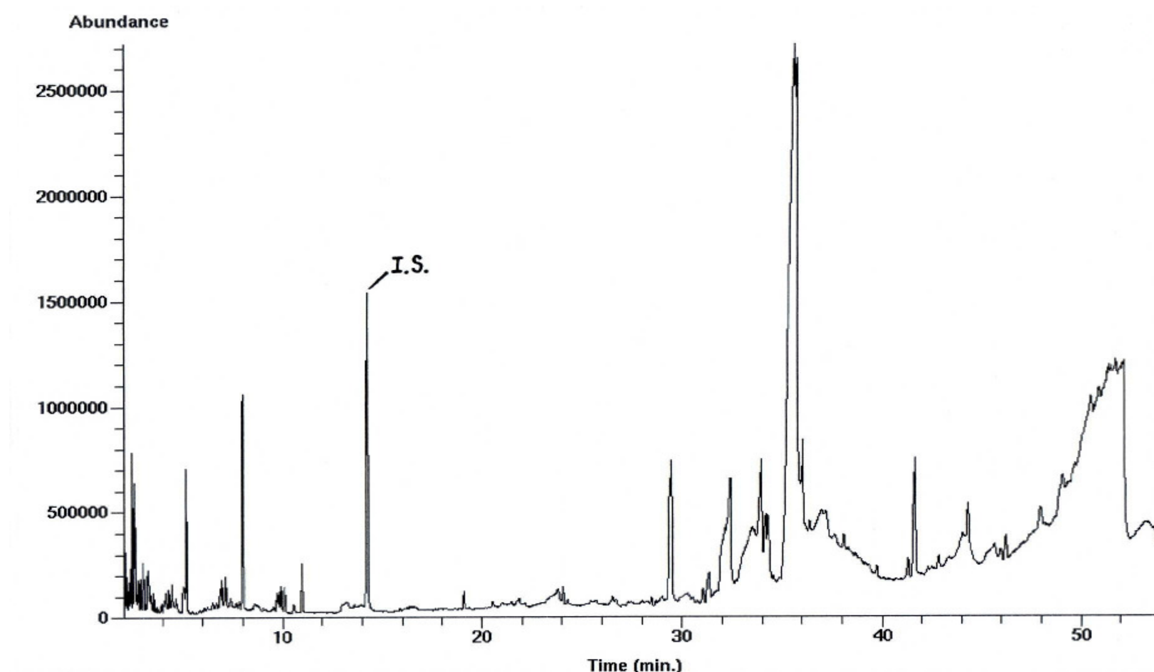

Figure 14. HRGC–MS chromatogram of the volatile extract from the aged fruiting bodies of wild *P. sulfureus* (DB5)

6.4.3. HRGC chromatograms of volatile extract of the young fruiting bodies of wild *L. lepideus*

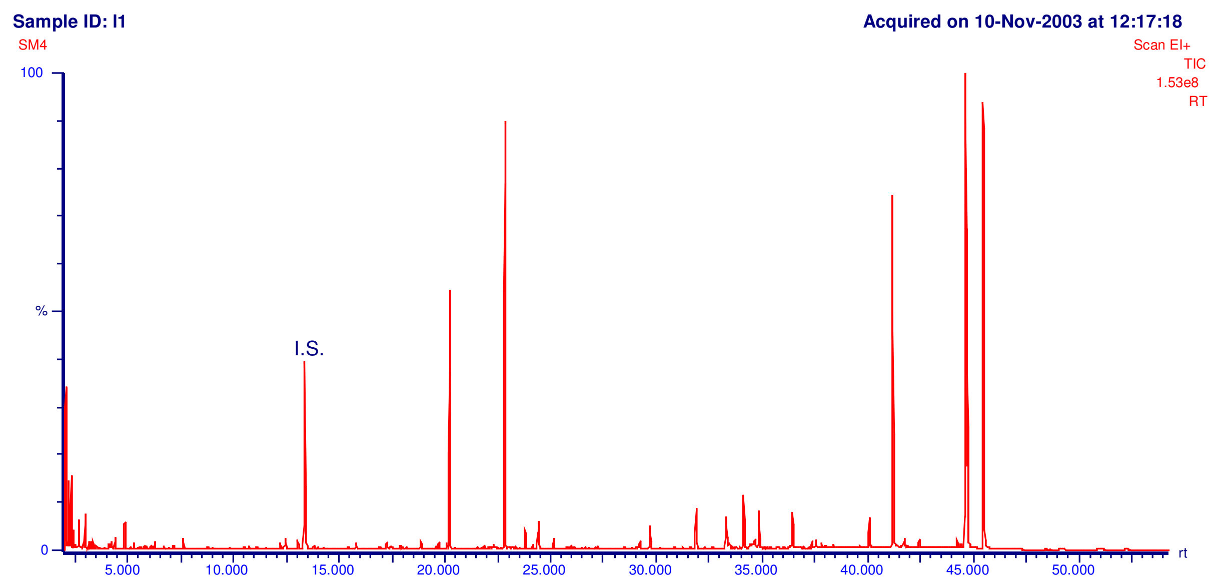

Figure 15. HRGC–MS chromatogram of the volatile extract from the young fruiting bodies of wild *L. lepideus* (ZB-WAX)

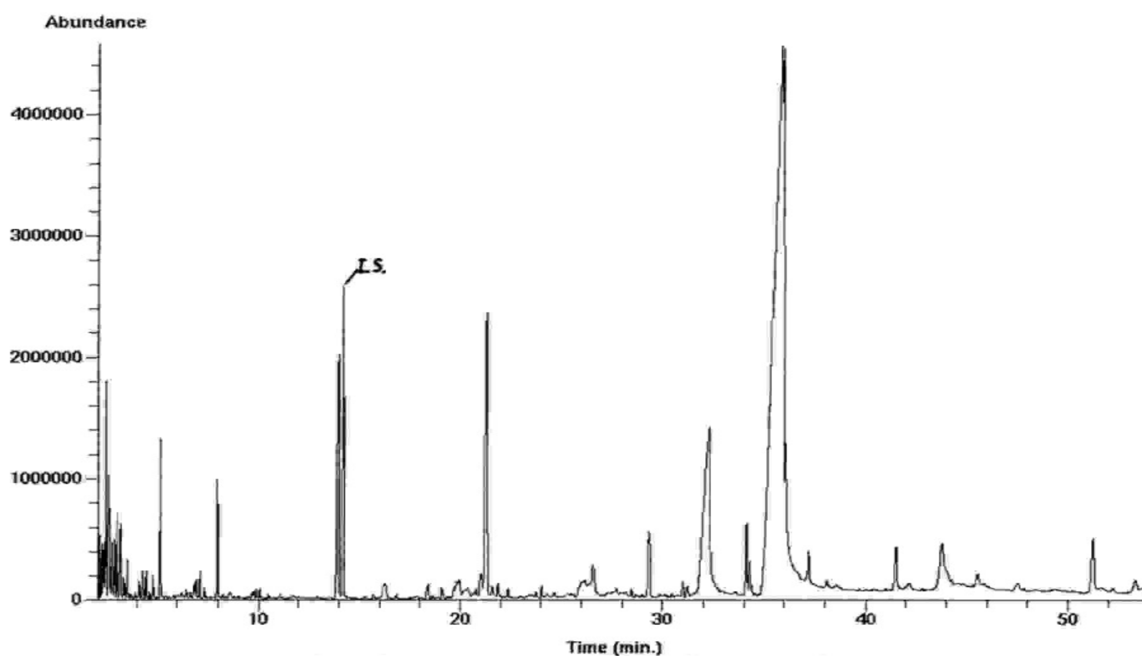

Figure 16. HRGC–MS chromatogram of the volatile extract from the young fruiting bodies of wild *L. lepideus* (DB5)

6.4.4. HRGC chromatograms of volatile extract from the fresh fruiting bodies of wild *F. hepatica*

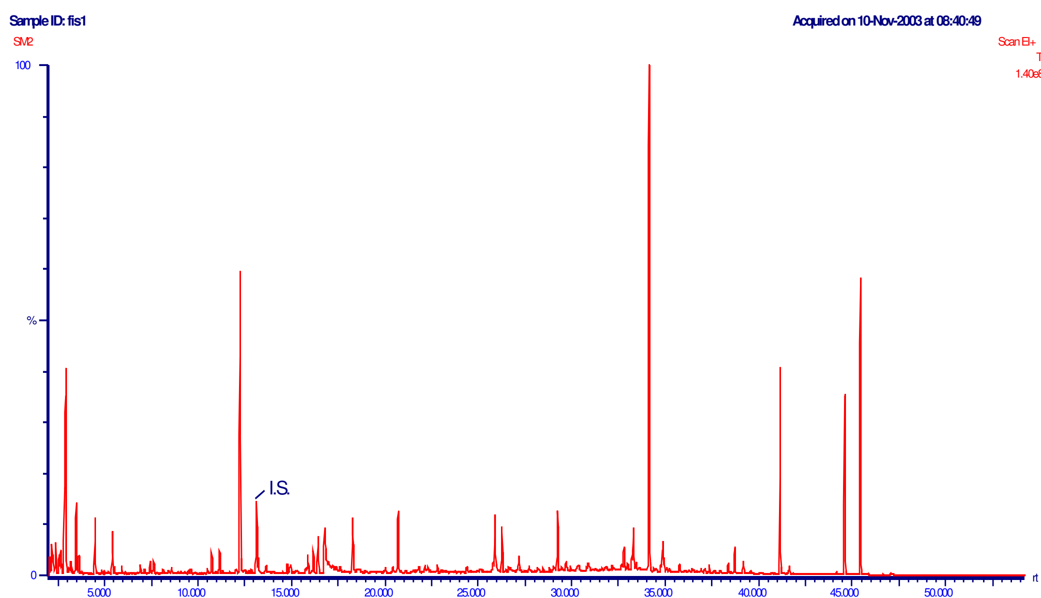

Figure 17. HRGC–MS chromatogram of the volatile extract from the fresh fruiting bodies of wild *F. hepatica* (ZB-WAX)

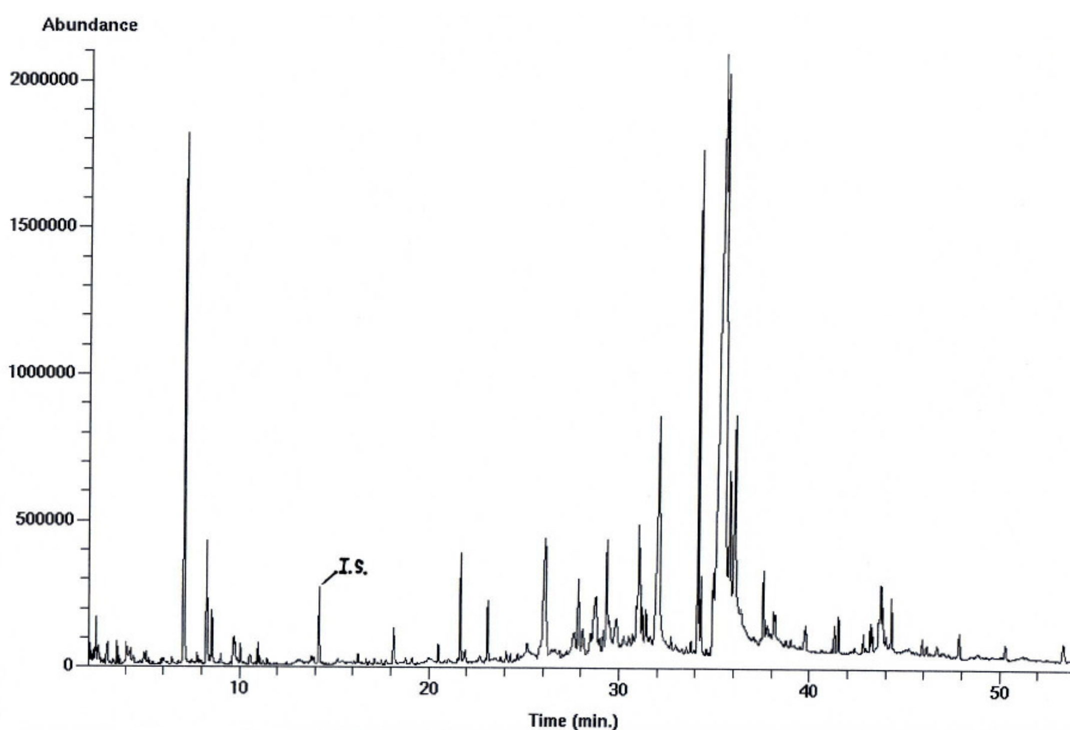

Figure 18. HRGC–MS chromatogram of the volatile extract from the young fruiting bodies of wild *L. lepideus* (DB5)

6.4.5. HRGC chromatograms of volatile extract from liquid cultures of *F. hepatica* growing in SNS medium

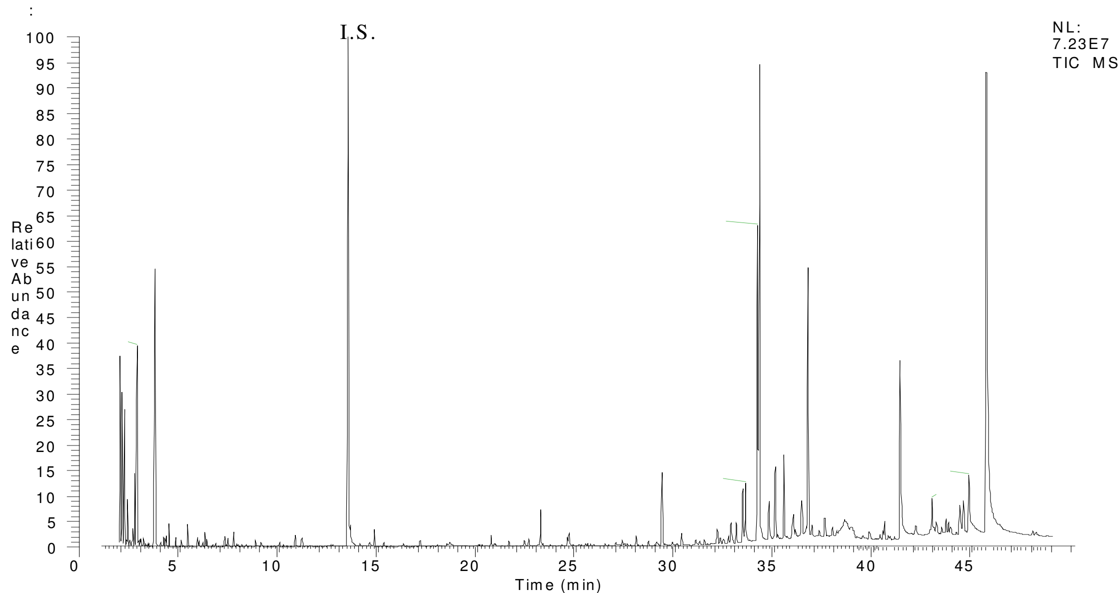

Figure 19. HRGC–MS chromatogram of the volatile extract from the submerged liquid cultures of *F. hepatica* (ZB-WAX)

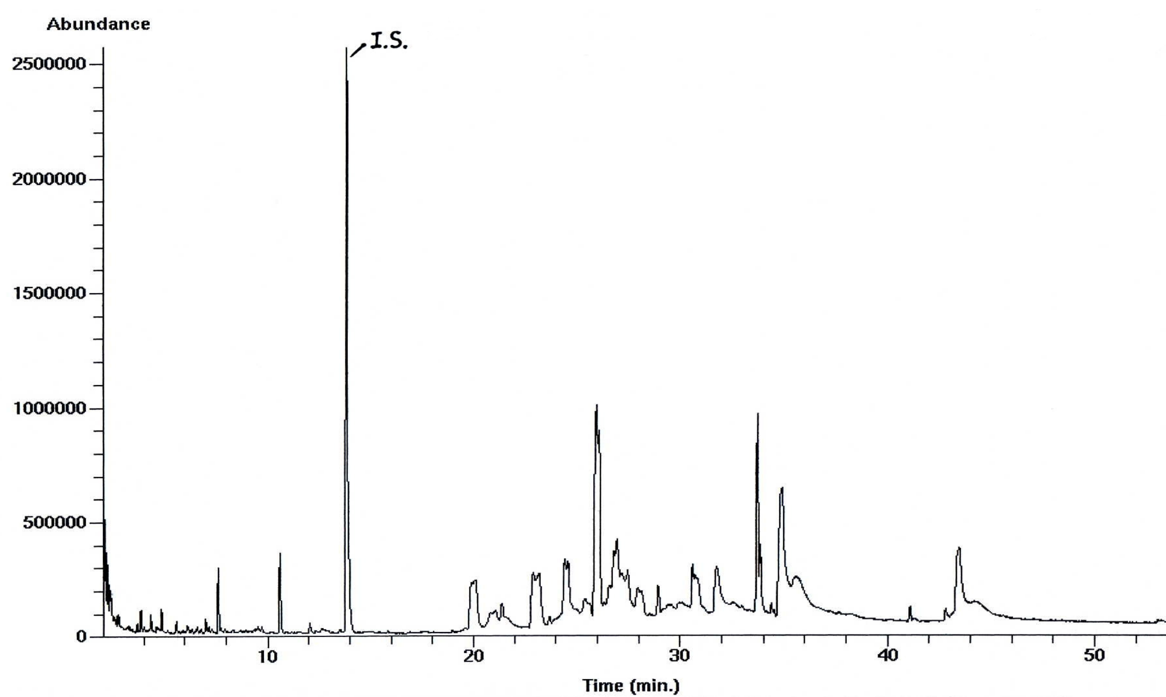

Figure 20. HRGC–MS chromatogram of the volatile extract from the submerged liquid cultures of *F. hepatica* (DB5)

6.4.6. HRGC chromatograms of volatile extract of surface cultures of *F. hepatica* growing on OWP

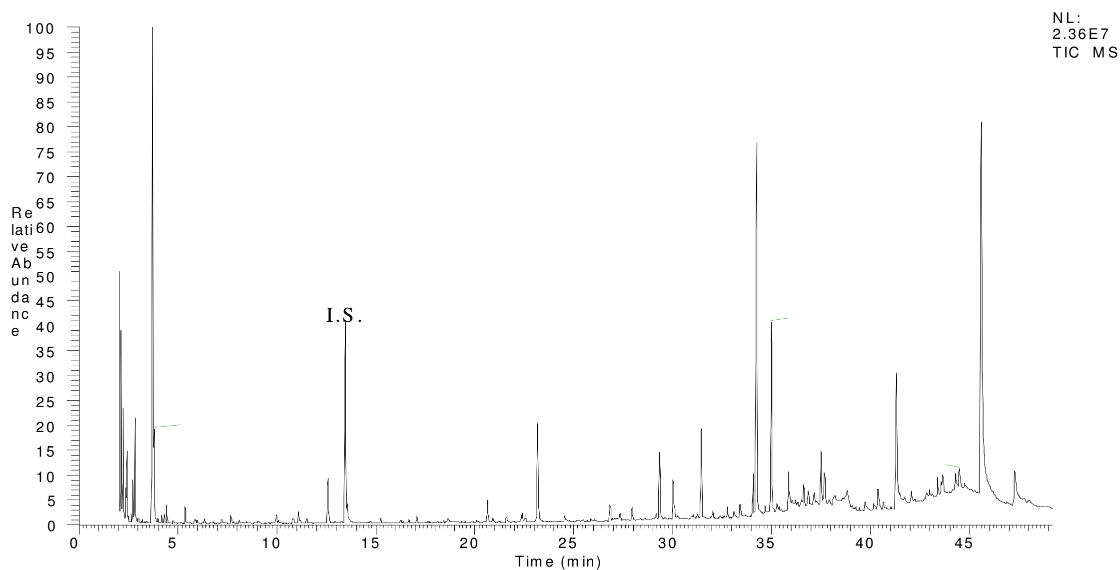

Figure 21. HRGC–MS chromatogram of the volatile extract from surface cultures of *F. hepatica* on OWP (ZB-WAX)

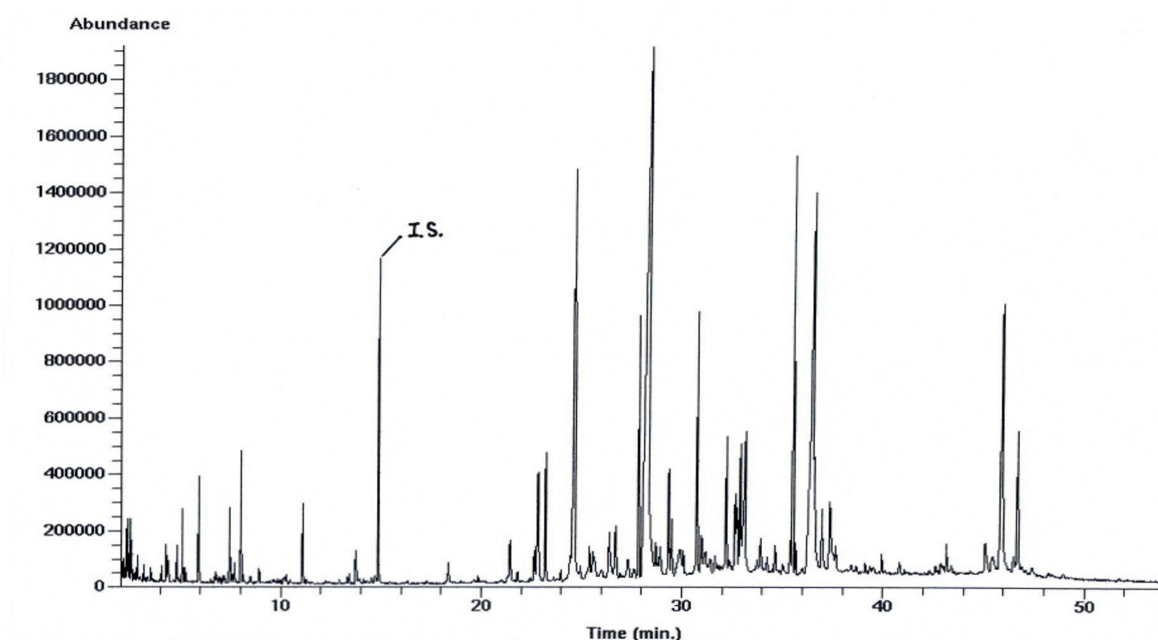

Figure 22. HRGC–MS chromatogram of the volatile extract from surface cultures of *F. hepatica* on OWP (DB5)

6.5. Mass spectra of several volatile compounds

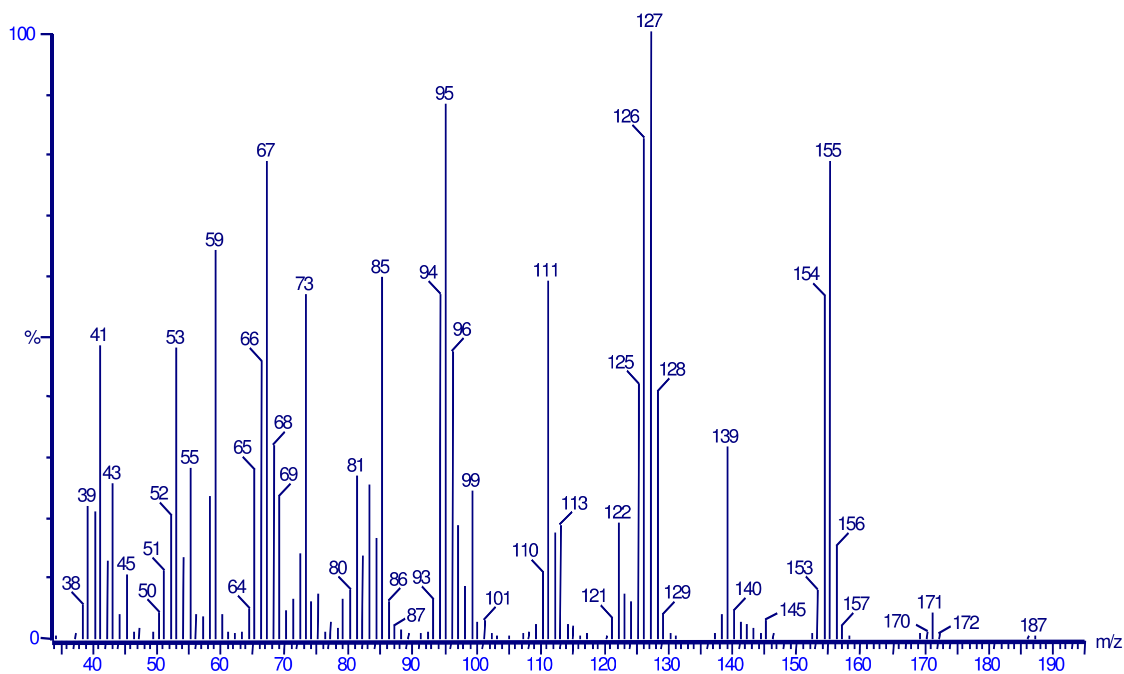

Figure 23. Mass spectrum of proposed 2-vinyl malonic acid methyl propyl ester

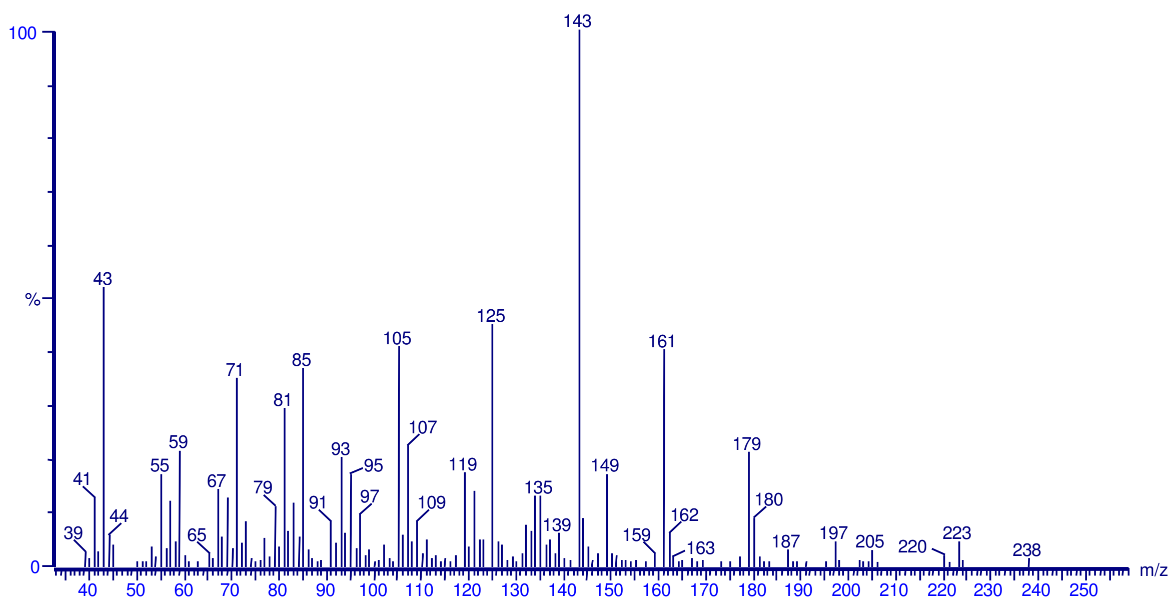

Figure 24. Mass spectrum of bisabolol oxide B

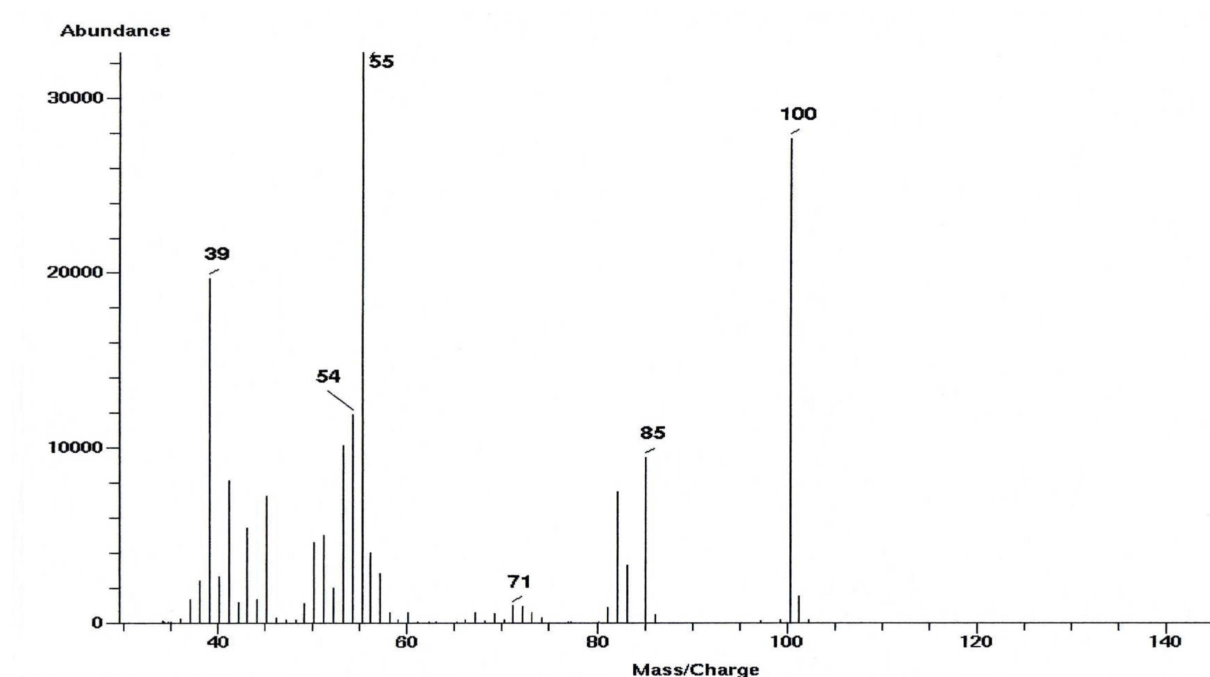

Figure 25. Mass spectrum of (*E*)-2-methyl-2-butenoic acid (tiglic acid)

6.6. Calculation of relative retention indices (RIs)

Retention indices (RIs), also commonly called Kovats indices, were calculated using the following formula based on the formula proposed by Van Den Dool and Kratz (1963).

$$RI(i) = 100 \cdot N + 100 \cdot n \cdot \frac{(\log(t_{R(i)} - t_d) - \log(t_{R(N)} - t_d))}{(\log(t_{R(N+n)} - t_d) - \log(t_{R(N)} - t_d))}$$

Where: RI (i) is the retention index of compound i

N is the number of carbon atoms in alkane N

n is the number difference of carbon atoms between alkane (N+n) and alkane N

$t_{R(i)}$ is the retention time of compound i

$t_{R(N)}$ is the retention time of alkane N

$t_{R(N+n)}$ is the retention time of alkane (N+n)

t_d is the dead time determined by butane

6.7. Proof of contamination of submerged liquid cultures

To be sure that the submerged liquid cultures submitted to extraction were not contaminated during the aerobic cultivation, they were controlled using a light microscope and inoculating on agar plates. One mL homogenized liquid culture was diluted in 100 mL sterilized physiological saline solution, and then 200 µL diluted culture was well-distributed and incubated on SNS-agar plate at 25 °C.

6.8. Enzymatic effects on flavor of the fruiting bodies of *F. hepatica*

In order to enhance enzyme catalyzed flavor generation after cell disruption, according to the literature (Venkateshwarlu, Chandravadana, & Tewari, 1999; Zawirska-Wojtasiak, 2004), the cut pieces of the fruiting bodies were stored for 15 min prior to enzyme inactivation with methanol (**Figure 26**: sample SM1). However, *F. hepatica* showed marginal changes in the flavor profile (**Figure 26**: sample SM2). Based on investigation with GC-MS and GC-O, none of these treatments resulted in any significant changes on volatile quality and quantity.

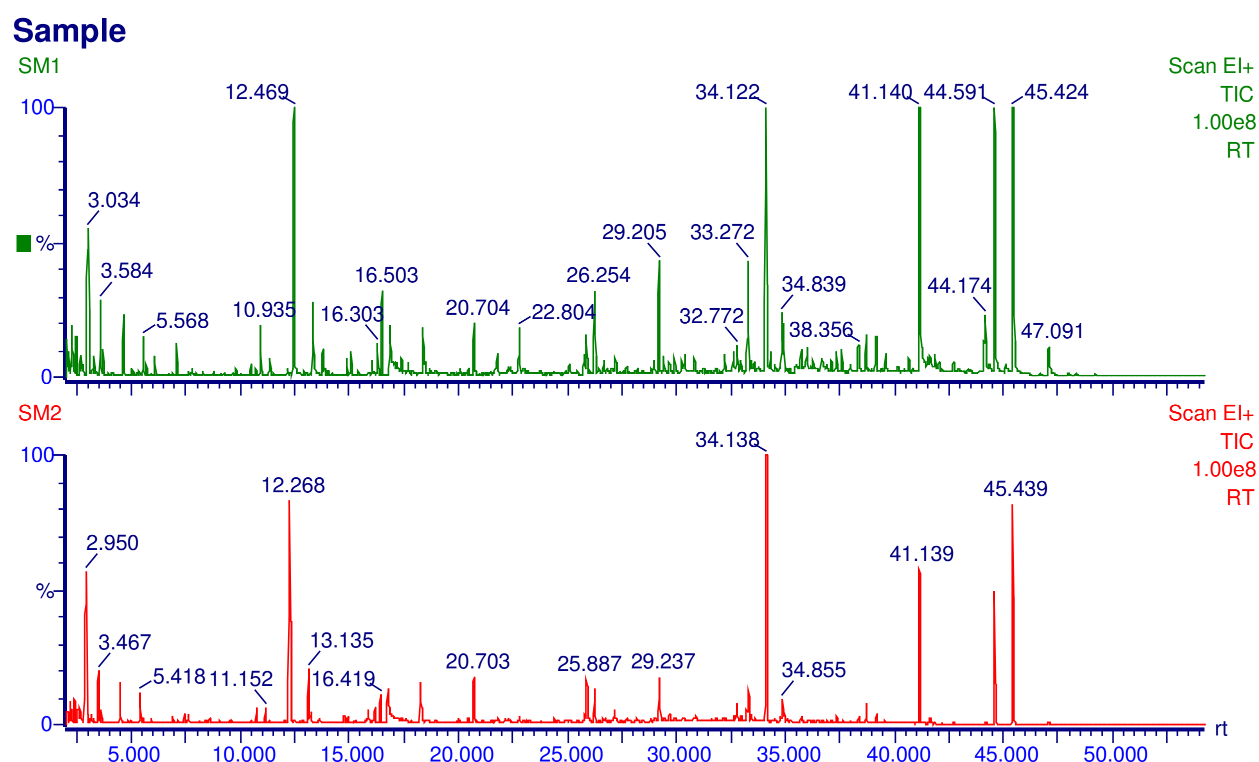

Figure 26. Endogenous enzymatic effects on volatile compositions of fruiting bodies of *F. hepatic*

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8. CURRICULUM VITAE

Shimin Wu

Male, born on May 18, 1970 in Hubei province, the People's Republic of China

Education and Studies

1985-1988	Hanchuan key senior middle school; Hubei province, China
1988-1992	Undergraduate in Huazhong Agricultural University; Wuhan, China Major: Food Science; Degree: Bachelor
1995-1998	Postgraduate in former Wuxi University of Light Industry (present Southern Yangtze University); Wuxi, China Major: Engineering of Oil and Vegetable Protein; Degree: Master
2003-2005	PhD student in University of Hannover, Hannover, Germany Major: Food Chemistry

Practices in Companies or Factories

1991	Trainee undergraduate in state-owned factories producing sugar, alcoholic drinks, and canned foods; Wusan, China
1996	Trainee graduate in "Chinese-Israeli Joint Ltd. of Oils and Fats"; Kunsan, China
1998	Supervisor of trainee undergraduates in seven different companies producing foods and drinks; Huangshi, China
2001	Supervisor of trainee undergraduates in five different foods and drinks companies; Xiaogan, China

Professional Experiences

1992-1995	Faculty in Department of Chemical Engineering, Jinmen University; Jinmen, China
1998-2003	Faculty in School of Food Science and Technology, Huazhong Agricultural University; Wuhan, China
2003-2005	Scientific researcher for doctorate at the "Institut für Lebensmittelchemie" (in English: Institute of Food Chemistry) led by Prof. Dr. Dr. R. G. Berger, Universität Hannover (in English: University of Hannover), Germany