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Volatile flavor constituents of *Lepista nebularis* (Clouded Clitocybe)

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Abstract – Odorous wild *Lepista nebularis* (Clouded *Clitocybe*) was investigated for volatile constituents by GC/MS using organic solvent extraction and hydrodistillation. Forty-nine and 28 volatile components were identified by solvent and distillation methods, respectively, and biosynthesized from the lipidic, shikimic and terpenic pathways. The major odorous compounds identified in fresh *L. nebularis* organic extract and distillate were 2-phenylethanol (34.6 and 46.5%), benzaldehyde (4.2 and 9.2%), β-barbatene (8.7 and 4.5%), undecane (4.6 and 2.7%), indole (4.3 and 2.1%) and C-8 derivatives (6.3 and 5.9%), respectively. Monoterpenes and sesquiterpenes, i.e., linalool, linalool oxides, and (E)-nerolidol were also identified in both extracts. β-Barbatene (musty-earthy odor) and indole derivatives (fecal odor) possessed unpleasant odors. C8-Derivatives (fungal odors) and butyric acid derivatives (cheese odor) as well as 2-phenylethanol (rose odor) and benzaldehyde (almond odor) are widely used as aroma components in flavor industry due to their strong odors. Many major and minor volatile compounds contribute to the overall smell of *Lepista nebularis*.

***Basidiomycota* / β-barbatene / benzaldehyde / hydrodistillation / indole / solvent extraction / 2-phenylethanol / *Tricholomataceae* / volatile**

Résumé – Les composés volatils de *Lepista nebularis* (Clitocybe nébuleux) ont été étudiés par chromatographie en phase gazeuse couplée à la spectrométrie de masse. Quarante-neuf et 28 substances volatiles ont été respectivement identifiées à partir de l'extrait organique et de l'hydrodistillat ; elles sont biosynthétisées par la voie des lipides, des composés aliphatiques ou des terpènes. Les composés majoritaires mis en évidence, respectivement, dans les deux extraits sont le 2-phényléthanol (34,6 et 46,5 %), le benzaldéhyde (4,2 et 9,2 %), le β-barbatène (8,7 et 4,5 %), l'undécane (4,6 et 2,7 %), l'indole (4,3 et 2,1 %) et les dérivés en C-8 (6,3 et 5,9 %). Des monoterpènes et des sesquiterpènes tels que le linalol, les oxydes de linalol et le (E)-nérolidol ont également été identifiés dans les deux extraits. Le β-barbatène (odeur moisie-terreuse) et les dérivés indoliques (odeur fécale)

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possèdent des odeurs désagréables tandis que les dérivés en C8 (odeurs fongiques) et les dérivés de l'acide butyrique (odeur de fromage) ainsi que le 2-phényléthanol (odeur de rose) et le benzaldéhyde (odeur d'amandes amères) sont très largement utilisés dans l'industrie des arômes. De nombreux composés volatils, majoritaires ou minoritaires, contribuent donc à l'odeur très complexe de *Lepista nebularis*.

***Basidiomycota* / β-barbatène / benzaldéhyde / hydrodistillation / indole / extraction par solvant / 2-phényléthanol / *Tricholomataceae* / substances volatiles**

INTRODUCTION

Mushrooms are claimed by mycologists to exhibit various pleasant and unpleasant odors such as anise, coal tar, farinaceous, fruity, fungal, garlic and musty-earthy odors (Breheret *et al.*, 1999; Laatsch & Matthies, 1992; Mau *et al.*, 1994; Rapior *et al.*, 1997, 1998, 2000, 2002; Rösecke & Köning, 2000; Venkateshwarlu *et al.*, 1999; Watson & Largent, 1986). A large number of odorous molecules have been identified in the fungus kingdom and then the higher fungi have been valued as flavorful food and biotechnological sources (Hanssen & Abraham, 1987; Janssens *et al.*, 1992; Lomascolo *et al.*, 1999; Venkateshwarlu *et al.*, 2000). Nevertheless, relatively little is known from the literature about the volatile composition of *Lepista nebularis* in relation with its complex odor.

As expressed by its Latin name, which comes from *nebula* (cloud), *Lepista nebularis* (Batsch:Fr.) Harm. (Clouded Agaric, Graycap) takes its name from the clouded gray color of its thick cap (McIlvaine & Macadam, 1973). The Clouded Clitocybe was described as possessing either an intensive disagreeable odor (Lincoff, 1998; Rambsbottom, 1923) as from shrunk cabbage (Ammirati *et al.*, 1985) or exhibiting a strong complex odor rather cyanic (Claus, 1978; Mazza, 1998) farinaceous (Bigelow, 1982; Breheret, 1997; Breheret *et al.*, 1997) or even repellent (Courtecuisse, 1999). It might be noted that Bon (1997) distinguished three different taxa within *L. nebularis* (Batsch:Fr.) Harm. : var. *nebularis*, fo. *alba* (Batsch) Imai and var. *stenophylla* (P. Karst.) M. Bon. As far as odor is concerned, they might exist 2 different taxa in *L. nebularis* : the first one has adnate or slightly décurrent gills and a complex disagreeable smell when it ages, while the second with very decurrent lamellae develops quickly a strong disagreeable odor (Deconchat, personal communication). According to McKenny and Stuntz (1987), the Clouded Clitocybe tastes the way it smells.

L. nebularis is considered an edible species, sometimes causing gastrointestinal disturbance (Bresinsky & Besl, 1990) but producing biological active components (Pang *et al.*, 1994; Cherqui *et al.*, 1999).

The present study elucidates the profile of volatile compounds from wild *Lepista nebularis* (Batsch:Fr.) Harm. var. *nebularis* by organic solvent extraction and hydrodistillation using gas chromatography/mass spectrometry (GC/MS) to identify the components contributing to the mushroom complex smell.

MATERIALS AND METHODS

Wild and fresh specimens of *L. nebularis* var. *nebularis* (= *Clitocybe nebularis* (Batsch:Fr.) Kummer) were collected in the fall of 1999 in France (Hérault), wrapped in waxed paper bags, and treated immediately after collection. Fruiting bodies, representing a combination of young and mature basidiomes, were investigated for volatile components by organic solvent extraction and hydrodistillation, and analyzed by gas chromatography/mass spectrometry (GC/MS) as detailed by authors (Breheret *et al.*, 1999; Rapior *et al.*, 2000). Both extraction processes were performed from odorous mushrooms ranging from 250 to 500 g using basidiomes cut in cubes (approximately 100 mm^3). On the one hand, volatile components from Clouded Clitocybe were extracted with dichloromethane. On the other hand, *L. nebularis* was subjected to a three-hour hydrodistillation with a Likens-Nickerson apparatus using dichloromethane as solvent. Both extracts were gently concentrated to a small volume (0.5 mL) under nitrogen stream and directly analyzed (1.0 μL) in duplicate by GC/MS.

GC/MS analyses were carried out using a gas chromatograph Hewlett-Packard (5890) and a mass selective detector Hewlett-Packard (5971) with a potential of 70 eV for ionization by electron impact. Solvent extract and distillate analyses were carried out using a 25 m × 0.20 μm × 0.13 μm dimethylpolysiloxane Optima 5 (Macherey-Nagel), fused silica capillary column. The injector and detector temperatures were 200 °C and 270 °C, respectively. The column was temperature programmed as follows: 50 °C (2 min) to 200 °C (3 °C/min). The carrier gas was helium with a constant flow rate set close to 0.9 mL/min. All volatile components were identified by comparison with mass spectral library NBS (MacLafferty & Stauffer, 1989); literature spectra (Adams, 1995; Jennings & Shibamoto, 1980; National Institute of Standard and Technology, 1994) and our own data bank.

RESULTS AND DISCUSSION

The screening of fresh wild *L. nebularis* for volatile components showed the diversity of chemical structures biosynthesized from the lipidic, shikimic and terpenic pathways. Forty-nine and 28 volatile components were identified from the solvent extract and distillate of *L. nebularis*, respectively. Table I points out the major volatile constituents as 2-phenylethanol, benzaldehyde, β-barbatene, undecane and indole; they were identified from both solvent extract and hydrodistillate. Differences in GC/MS profiles were due to the experimental extraction procedures: the most hydrosoluble components, i.e., acids, phenols, nitrogen derivatives were not extracted from the distillate and then not identified from the essential oil.

In the solvent extract, the most abundant compounds identified are biosynthesized from the shikimic pathway, i.e., 2-phenylethanol (34.6%), indole (4.3%), benzaldehyde (4.2%), 2-phenylbut-2-enal (2.6%), 2-phenylethanal (2.0%) and 4-hydroxybenzaldehyde (1.4%). The other important components came from the terpenic pathway as sesquiterpenes, i.e., β-barbatene (8.7%) and (E)-nerolidol (2.0%). Alcanes (undecane, dodecane, tridecane, pentadecane and hexadecane) represented 11.5% of the volatiles biosynthesized from the lipidic pathway whereas 5.0% were butyric derivatives: 3-methylbutyric acid, 2-methylbutyric acid and butyrolactone. Moreover, while C8-compounds constitute normally the most frequently identified volatile compounds in mushrooms (Audouin *et al.*, 1989; Rapior *et al.*, 1998, 2002), only low amounts of octen-3-ol, octan-3-one octan-3-ol, (E)-oct-2-enol, and 1,5-octadien-3-ol were detected in the Clouded Agaric extract (Table I). Vanhaelen *et al.* (1980) demonstrated that low concentrations of 1,5-

Table I. Gas Chromatographic Analyses (%[a]) of fresh *Lepista nebularis* extract and hydrodistillate

Volatile compounds	RI[b]	Solvent extract	Hydrodistillate
Hexanal	798	0.3	
Octane	800	0.2	0.1
Isobutyric acid	825	0.1	
Butyric acid	856	0.1	
Butyrolactone	882	1.4	
3-Methylbutyric acid	909	2.9	0.2
2-Methylbutyric acid	917	0.5	
(E)-Hept-2-enal	928	0.3	0.8
Benzaldehyde	932	4.2	9.2
1,5-Octadien-3-ol	953	0.2	
2,3-Octanedione	957	2.1	0.2
Octan-3-one	961	0.8	2.5
Octen-3-ol	972	1.8	1.8
Pentylfurane	975	0.1	
Octan-3-ol	987	0.8	0.3
Dihydropyranone	1008	2.0	
Benzylalcohol	1014	0.2	0.3
2-Phenylethanal	1018	2.0	5.3
Hexanoic acid	1042	1.1	
(E)-Oct-2-enol	1068	0.3	1.0
Octanol	1074	0.1	
Trans-linalool oxide	1077	0.2	
Cis-linalool oxide	1089	0.2	
Linalool	1095	0.7	3.1
Undecane	1100	4.6	2.7
2-Phenylethanol	1110	34.6	46.5
(E)-Non-2-enal	1138	0.1	
2-Phenylpropenal	1142	0.3	1.0
2-Phenylethyl formiate	1161	0.6	
Dodecane	1200	2.5	3.3
2-Phenylethyl acetate	1251	0.3	0.5
2-Phenylbut-2-enal	1258	2.6	5.3
Benzoic acid	1272	1.4	
Undecan-2-one	1283	1.6	0.3
Tridecane	1300	1.4	0.4
(E,E)-Nona-2,4-dienal	1308	0.5	0.9
Indole	1312	4.3	2.1
Phenylacetic acid	1336	0.8	
α-Terpenyl acetate	1347	0.5	
Tetradecane	1400	0.2	
3-Methylindole	1401	0.3	
Phenylacetamide	1403	1.2	
β-Barbatene	1439	8.7	4.5
3-Phenylpyridine	1453	0.6	0.1
2-Phenylhex-2-enal	1477	0.7	0.6
4-Hydroxybenzaldehyde	1491	1.4	
Pentadecane	1500	1.2	0.8
(E)-Nerolidol	1562	2.0	1.0
Hexadecane	1600	1.8	0.9

[a] Relative percentage of the identified volatile component based on the GC/MS chromatographic area
[b] Retention indices due to the GC column used

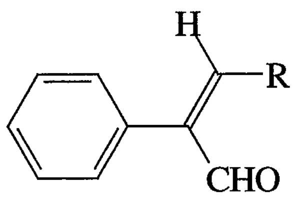
octadien-3-ol in mushrooms (Homobasidiomycetes) – 0.17% in case of *L. nebularis* – were a significant attractant for the cheese mite (*Tyrophagus putrescentiae* Schrank).

Minor volatile compounds such as monoterpenes (linalool, 0.7%; *cis*-linalool oxide, 0.2%; *trans*-linalool oxide, 0.2%) also characterized the solvent extract as previously reported in the case of *L. nebularis* (Breheret *et al.*, 1997). On the other hand, the authors (SR and JMB) also identified linalool and both linalool oxides from *Lepista nuda* (Bull.:Fr.) Cke. (unpublished results).

It should be noted that the organic solvent extract contained compounds 1, 2, 3 and 4 (Fig. 1) possessing the same basic chemical skeleton and were biosynthesized *via* a crotonisation reaction between 2-phenylethanal and various aldehydes such as methanal, ethanal, butanal and 2-phenylethanal, respectively. Compound 4 (2,4-diphenylbut-2-enal, Fig. 1) was detected among the less volatile constituents from our *L. nebularis* extract and was previously reported by Pang *et al.* (1994); 2,4-diphenylbut-2-enal was found to possess weak antifungal (*Paecilomyces varioti, Mucor miehei*) and antibacterial (*Bacillus subtilis, B. brevis*) activities.

The distillate from fresh Clouded Clitocybe consisted of 28 volatile components (Table I). The volatile pattern of *L. nebularis* was dominated by a large number of compounds possessing aromatic skeletons (> 70.0%): the investigation of volatiles indicated high amounts of 2-phenylethanol (46.5%) and benzaldehyde (9.2%) and lower amounts of 2-phenylethanal (5.3%), 2-phenylbut-2-enal (5.3%), indole (2.1%) and 2-phenylpropenal (1.0%). C8-Derivatives, frequently present in mushrooms, represented 5.9% of the *L. nebularis* distillate in the form of octane, octan-3-one, octen-3-ol, octan-3-ol and (E)-octen-2-ol. Linalool (3.1%), a monoterpene, was also detected in our study as previously reported from *L. nebularis* distillate by Audouin *et al.* (1989). β-Barbatene (4.5%) and (E)-nerolidol (1%), two sesquiterpenes, were identified for the first time from *L. nebularis* distillate.

The diversity in the volatile composition of *L. nebularis* demonstrates that there is not only one odorous molecule responsible for the overall odor of the mushroom. All the major and minor volatile compounds participate in the general aroma of the Clouded Clitocybe. Among the volatile flavor components, many C8-derivatives were identified. These are well known for their typical mush-



Compound 1*	R = H	2-phenylpropenal
Compound 2	R = CH_3	2-phenylbut-2-enal
Compound 3	R = $CH_2CH_2CH_3$	2-phenylhex-2-enal
Compound 4	R = $CH_2C_6H_6$	2,4-diphenylbut-2-enal

* E,Z isomery not determined

Fig. 1. Naturally occurring condensed aldehydes from *Lepista nebularis*.

room-like odors: more musty-earthy for octen-3-ol and octan-3-ol, and more fruity for octan-3-one (Breheret, 1997). The monoterpenes (linalool, *cis*- and *trans*-linalool oxides) are described as refreshing floral and sweet-woody, respectively (Breheret *et al.*, 1997) and the sesquiterpenes – (E)-nerolidol and β-barbatene – as woody, floral warm, and musty-earthy, respectively (Arctander, 1994). It should be noted that fragrant components with low odor threshold might significantly contribute to fungus aroma despite low contents as for N-heterocycles, i.e., indole and 3-phenylindole (skatole) characterized by fecal odors (Bauer *et al.*, 1990). Due to their strong odors, butyric acid derivatives (various cheese odors), 2-phenylethanol (rose odor) and benzaldehyde (almond odor) are widely used as sweet aroma components in food industry and/or cosmetics.

The broad spectrum of volatile components identified in *L. nebularis* contributes to its specific and complex smell. Consequently, the various odors reported for the Clouded Clitocybe by mycologists are probably due to their individual olfactory perception for each molecule detected from the mushroom aroma. Our results also show the large potential of Basidiomycota as biotechnological sources to synthesize natural components used in the flavor industry (Janssens *et al.*, 1992; Gabelman, 1994; Krings & Berger, 1998; Lomascolo *et al.*, 1999; Venkateshwarlu *et al.*, 2000).

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