GC-MS AND GC-OLFACTOMETRY ANALYSIS OF AROMA COMPOUNDS EXTRACTED FROM CULTURE FLUIDS OF *ANTRODIA CAMPHORATA*

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ABSTRACT

A comprehensive inventory of the organic components and aroma-active compounds produced by *Antrodia camphorata* during growth in submerged culture has been established by extracting culture fluids using three different organic solvent systems and subjecting the extracts to gas chromatography–mass spectrometry (GC–MS) and gas chromatography–olfactometry (GC-O). Forty-three organic components, of which esters, alcohols, acids and ketones were the most prevalent, were identified in pentane/ether (1/1, v/v) extracts. The most representative of *A. camphorata* aroma-active compounds were detected in pentane/ether and ether extracts (eleven and nine aroma-active compounds, respectively) by GC-O. Of these, ethyl acetate, γ-undecalactone, linalool and 3-hydroxy-2-butanone were assessed to be present at the highest intensity.

Keywords: Aroma analysis; Representative aroma extract; Sensory evaluation; Submerged culture.

INTRODUCTION

The basidiomycete, *Antrodia camphorata*, is a rare and expensive mushroom assigned to the family Polyporaceae (Aphyllophorales). The fungus is native to Taiwan, where it grows in the walls of the inner heartwood of *Cinnamomum kanehirai* Hay [1, 2], and has been ascribed various medicinal properties [3, 4. Our research has also revealed that submerged cultures of *A. camphorata* are highly odoriferous, suggesting that the mushroom might serve as an important source of natural aroma compounds for the food and cosmetic industries. Therefore, in the present study, we have first determined the effectiveness of three solvent systems for extracting a comprehensive inventory of organic compounds from culture fluids generated after submerged culture of *A. camphorata* mycelium under specified growth conditions. We have then used gas chromatography–mass spectrometry (GC–MS) combined with gas chromatography–olfactometry (GC-O) to identify the most important aroma active compounds.

MATERIAL AND METHODS

Fungal strain and culture conditions. *Antrodia camphorata* was obtained from the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences and grown on potato dextrose agar (PDA) slopes at 26^{O}C for 2 weeks and stored at 4^{O}C. Discs (0.5 cm) of agar containing fungal mycelium grown on PDA plates at 26 ^{O}C for 2 weeks were used as inocula for submerged cultures.

Submerged cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml basal medium consisting of (w/v): 4.0% glucose, 0.6% soybean, 0.1% K_2HPO_4, 0.05% $MgSO_4$ and 0.01% vitamin B_1. Mycelium was removed after incubation at 25 ^{O}C for 5 days and the culture fluid was retained.

Extraction of volatile compounds. Culture filtrates (100 ml) were extracted four times using three different organic solvents: diethyl ether (Extract E), a mixture (1:1, v/v) of pentane/diethyl ether (Extract P/E), and a mixture (2:1, v/v) of pentane/dichloromethane (Extract P/D). After extraction, the upper organic phases were separated, dried over anhydrous sodium sulphate, concentrated at 42 ^{O}C to 1 ml, and stored at –20 ^{O}C prior to GC–MS and GC-O analyses. All extractions were performed in triplicate.

Gas chromatography–mass spectrometry (GC–MS). Volatile components were identified by GC-MS using a Finnigan TRACE GC-MS (Thermo Quest Finnigan Co., USA) equipped with a DB-Wax capillary column (60 m × 0.32 mm). Helium (flow rate, 1.0 ml/min) was used as the carrier gas, and injection volumes were 1 µl. The column temperature was maintained initially at 40 ^{O}C for 3.5 min, followed by increases to 60 ^{O}C at a rate of 5 ^{O}C/min, from 60 to 120 ^{O}C at a rate of 6 ^{O}C/min, and from 120 to 230 ^{O}C at a rate of 8 ^{O}C/min, and then this temperature was held constant for 12 min. The electron impact energy was 70 eV and the ion source temperature was set at 230 ^{O}C. Electron impact (EI) mass spectra were recorded in the 33-450 aMU range at 1 s intervals.

Sensory evaluation of extracts. Sensory evaluation of aroma extracts obtained with the three organic solvent systems was performed to select extracts representative of the odour of *A. camphorata* culture filtrates. Samples were prepared by placing 1 ml of *A. camphorata* culture filtrate or an aroma extract in a brown flask, eliminating the solvent from the latter under a nitrogen flux, and hermetically sealing the flask prior to evaluation. The evaluation was carried out using triangular tests by a panel of 15 assessors (seven females and eight males) selected at random from university students with natural olfaction. The significance of the test was evaluated by binomial distribution using published tables [5] (Stone & Sidel, 1985).

GC–Olfactometry (GC-O). GC–O analyses were conducted using a Finnigan TRACE GC (Thermo Quest Finnigan Co., USA) equipped with a sniffing port. Column type and analysis conditions were as described above, and the temperature of the sniffing port was 250 ^{O}C. Sniffing tests on *A. camphorata* E and P/E extracts in combination with reference compounds were performed by two trained and experienced testers in paired alternate chromatographic runs conducted at 15 min intervals. Effluent from the GC containing the separated compounds was diluted with humidified air, and qualitative and semi-quantitative odour evaluation was carried out for each analyte leaving the chromatographic column during the entire GC analysis [6] (Pollien *et al*. 1997). Data were recorded only in cases where the testers assigned the same aroma attribute.

Table 1. Volatile compounds detected in aroma extracts from *Antrodia camphorata* obtained using different organic solvents

Compound	E	P/E	P/D	Compound	E	P/E	P/D
Esters				4-Hydroxybenzene ethanol	+		
Ethyl acetate	+	+	+	***Acids***			
2-Hydroxypentanoic acid ethyl ester	+	+		Acetic acid		+	
Carbitolacetate		+		4-*tert*-Butylcyclohexyl acetate		+	
2, 6, 10, 14-Tetramethyl pentadecanoic acid methyl ester		+		4-Methyl hexanoic acid		+	
Octadecyl acetate		+	+	2-Hydroxy-2-methylbutyric acid		+	
Hexadecanoic acid-1-methyl ethyl ester	+	+		Neodecanoic acid	+	+	+
γ-Undecalactone	+	+	+	3-Mercaptopropionic acid			+
Methyl formate	+			Hexadecanoic acid	+		
Phthalic acid diisobutyl ester		+	+	Caprylic acid	+		
4-Decanoic acid methyl ester			+	Tetradecanoic acid	+		
γ-Decalactone			+	2-Methylene-4-hydroxybutyric acid	+		
2, 2-Dimethyl-3-oxobutyric acid methyl ester			+	***Ketones***			
Acetic acid 3, 7, 11, 15-tetramethyl-hexadecyl ester			+	3-Hydroxy-2-butanone	+	+	+
Octadecanoic acid 2-2-hydroxyethoxy) ethyl ester			+	2 (1-Methyheptyl) cyclopentanone		+	
Alcohols				2, 5-Furandione		+	
1, 2-Propanediol		+		2-Butanone		+	
2-Butanol	+		+	1-(4-Methylphenyl)-1-pentanone	+		
3-Methyl-3-butylene-1-ol	+			4-Hydroxy-2-butanone	+		
1, 3-Butanediol	+			2-Furyl methyl ketone	+		
3-Methyl-2-pentanol		+		***Aldehydes***			
4-Methyl-2-pentanol	+			Furfural	+	+	+
3-Methyl buten-1-ol	+	+		5-Hydroxymethyl-2-furaldehyde	+	+	
3-Methyl-3-octanol	+			2, 4-Dimethyl benzaldehyde	+		

Table 1- Continue

2, 3-Butanediol	+			***Hydrocarbons***			
2-Phenylethyl alcohol	+			Heneicosane	+	+	+
Linalool	+	+	+	Undecane		+	
α-Terpineol	+	+		Docosane		+	
[E]-Nerolidol		+		1, 3, 5-Trioxacycloheptane	+	+	+
T-Cadinol	+	+		***Heterocyclic***			
Cubenol	+			2, 4, 5-Trimethyl-1, 3 dioxolane	+	+	
4-Methyl-5-thiazole ethanol		+	+	2-Methyl-1, 3 dioxolane		+	
8-Hydroxylinalool	+	+	+	4, 4-Dimethylcyclooctene		+	
7-Methyl-3-propyl-2, 6-decadien-1-ol	+	+		6-Methyl-3, 5-dihydroxy-2, 3-dihydro-4H-pyran	+	+	+
3-Methyl-2-butanol		+		Hydroquinone	+		
2-Hexyl-1-decanol		+		3, 4-Dimethoxyphenol	+		
[E] 9-Hexadecen-1-ol		+		1-Hydroxy-2-acetyl-4-methylbenzene	+		
2-Tetradecyl alcohol		+		Butylated hydroxytoluene	+		
Isobutyl alcohol		+					

(E) Ether extract, (P/E) Pentane/ether extract, (P/D) Pentane/dichloromethane extract. +, detected.

RESULTS AND DISCUSSION

Clear differences were observed in the organic components extracted from *A. camphorata* culture fluids using the three different solvent systems, and these probably reflected the different solvent polarities. The highest number of components (43) was detected in P/E extracts, while solvent E contained 40 and solvent P/D contained only 20 (Table 1). Organic components identified in P/E extracts consisted of 8 esters, 16 alcohols, 5 acids, 2 aldehydes, 5 heterocyclic compounds, 4 hydrocarbons and 5 ketenes. Solvent E extracts contained 5 esters, 15 alcohols, 5 acids, 3 aldehydes, 6 heterocyclic compounds, 2 hydrocarbons and 4 ketones.

Esters and alcohols that were predominant in both P/E and E extracts were 2-hydroxypentanoic acid ethyl ester, hexadecanoic acid-1-methylethyl ester, 3-methyl buten-1-ol, α-terpineol, T-cadinol and 7-methyl-3-propyl-2,6-decadien-1-ol. These compounds along with 5-hydroxymethyl-2-furaldehyde and 2, 4, 5-trimethyl-1, 3-dioxolane were identified in E and P/E extracts but not in the P/D extract. Octadecyl acetate, phthalic acid diisobutyl ester and 4-methyl-5-thiazole ethanol were identified in the P/E and P/D extracts, but not in the E extract. Ethyl acetate, γ-undecalactone, linalool, 8-hydroxylinalool, neodecanoic acid, 3-hydroxy-2-butanone, furfural, heneicosane, 1, 3, 5-trioxacycloheptane and 6-methyl-3, 5-dihydroxy-2, 3-dihydro-4H-pyran were identified in all three extracts. All the above compounds have hitherto not been reported in *A. camphorata*.

Sensory evaluation involving 15 assessors using triangular tests detected no differences in the aroma of E and P/E extracts compared with the original culture filtrates ($p < 0.05$). Accordingly, E and P/E extracts were chosen for GC-O analysis and quantification of the aroma-active compounds. Testers perceived thirteen aroma active compounds in these extracts. Seven of these were recorded in both extracts: ethyl acetate (sweet), γ-undecalactone (peach, sweet), linalool (citrus-like, fresh floral) and 3-hydroxy-2-butanone (buttery, milky) at strong intensities, α-terpineol (deal, clove) and furfural (almond, spicy) at medium intensities, and T-cadinol (spicy) at weak intensity. 2-Phenylethyl alcohol (soft rose, floral) and cubenol (spicy) were found only in extract E at medium and weak intensities, respectively. Isobutyl alcohol (fresh, spicy), *[E]*-nerolidol (mild floral), 4-methyl-5-thiazole ethanol (meaty, spicy) and 1,2-propanediol (floral, pollen) were all identified only in extract P/E, the first three at medium and the latter at weak intensities. [7] Chang *et al*. (2001) reported that *A. camphorata* mycelium contained high concentrations of soluble sugars and the flavour 5'-nucleotides, 5'-guanosine monophosphate (5'-GMP) and 5'-xanthosine monophosphate. Soluble sugars contribute a sweet taste and 5'-GMP a meaty flavour to mushrooms [8] (Litchfield, 1967). The related *Antrodia* species, *A. malicola* and *A. xantha*, were previously reported to have 'faintly fragrant' and 'lemon-like' aromas, respectively but the chemical components responsible were not identified [9] (Jong & Birmingham, 1993).

Although distinctive odours have long been used as taxonomic markers for mushroom identification, the potential of higher fungi for the industrial production of natural aroma compounds has remained largely unexploited. Ethyl acetate, γ-undecalactone, linalool and 3-hydroxy-2-butanone were adjudged to make a major contribution to the special aroma character of *A. camphorata* culture fluids while, interestingly, none of the C-8 alcohols (e.g.1-octen-3-ol) commonly found in other mushrooms were detected. Although γ-undecalactone has been identified earlier in fruits, to our knowledge this is the first time the compound has been reported in fungi.

Several key volatile compounds identified by GC-MS in all the extracts, e.g. 8-hydroxylinalool, neodecanoic acid and heneicosane, were not detected in the GC-O analysis. However, some of these odorants appeared in short, complex sections of the chromatogram thereby making detection and assessment of aroma characteristics more difficult. Furthermore, GC-O does not take into account matrix effects, which can have a large impact on odorant volatility and perception [10] (Ferreira *et al*. 2002).

In view of the range of compounds present in culture fluids of *A. camphorata*, the fungus has potential value as a source of food flavors in cases where floral-fresh-fruity-milky aromas are required such as chewing gums, sweets, teas, soft and energy drinks and milk products. It could also provide fragrances essential to the cosmetic industry for the manufacture of shampoos, soaps, shower gels, body lotions, deodorants and toothpastes.

This present study was conducted on extracts of culture fluids generated after submerged culture of fungal mycelium under specified growth conditions because of their highly odiferous nature and due to difficulties in obtaining sufficient quantities of mushroom fruit bodies. Future research will study the effects of different culture conditions, including the addition of precursors, on the production of flavour compounds by *A. camphorata*.

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