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GC-MS studies of the chemical composition of two inedible *Agaricus* species (Section *Xanthodermatei*) in Bulgaria

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Abstract

The study is first report on the chemical composition of two inedible *Agaricus* species, namelly *Agaricus moelleri* Wasser and *A. phaeolepidotus* (F.H. Møller) F.H. Møller. This GC-MS studies on the volatile fractions and butanol extracts resulted in the identification of 42 and 40 compounds for *A. moelleri* and *A. phaeolepidotus*, respectively, including amino acids, fatty acids and their esters and sugar alcohols. The most abundant constituent in the volatiles and butanol were phenol and urea respectively, as well as identified the presence of ergosterol and two Δ7-sterols. The crystalline compound 5α,8α-epidioxi-24(ξ)-methylcholesta-6,22-diene-3β-ol was isolated for the first time from *A. phaeolepidotus*. The results obtained contribute to the knowledge of the chemical composition of species belonging to the genus *Agaricus*, and provide some explanation for the reported mild toxicity of *A. moelleri* and *A. phaeolepidotus* can be explained by a high phenol content, similar to that found in other *Xanthodermatei* species. The species are described and illustrated on the basis of Bulgarian specimens.

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Introduction

Mushrooms are macrofungi with a distinctive fruiting bodies that are consumed as a delicacy, particularly for their specific aroma and texture. They constitute an integral part of the normal human diet, presenting an increasing share, and both fresh and preserved fruiting bodies of plenty of species can be culinary-processed in different manners. Mushrooms have also been reported as source of pharmacologically active compounds, to which activities like antimicrobial, antitumor, cytostatic, hypoglycemic, anti-inflammatory or antioxidant are attributed (Kalač, 2009, 2012; Lindequist *et al*., 2005), as well as has been found to have strong analgesic, anti-inflammatory and antipyretic activity (Suseem and Mary Saral, 2011, 2013; Suseem *et al*., 2011; Ribeiro *et al*., 2011).

The genus *Agaricus* represents a large, diverse, cosmopolitan genus with c. 200 taxa worldwide and approximately 60 of them occuring in Europe (Cappelli, 1984; Bas, 1991; Calvo-Bado *et al.,* 1999; Kirk *et al.,* 2001, 2008). The genus includes the most economically important and commercially cultivated mushroom in the world (*Agaricus bisporus*), as well as many other inedible species (Calvo-Bado *et al.,* 2000).

The section *Xanthodermatei* Singer comprises only inedible species, including *Agaricus moelleri* and *A. phaeolepidotus* are known to possess unpleasant odours and induce gastrointestinal problems if consumed (Wasser, 1980; Cappelli, 1983, 1984; Lacheva, 2006). So far e made chemical analysis only two inedible *Agaricus* species (*Agaricus placomyces* and *A. pseudopratensis*) from section *Xanthodermatei* (Petrova *et al.,* 2007).

GC-MS is widely used method to identify different substances within a test sample (Manjamalai, 2011). In search of new drugs from mushrooms GC-MS techniques have been widely used. Smiderle *et al*., (2006) isolated and identified polysaccharide such as xylomannans and β-glucan from the edible mushroom *Flammulina velutipes* by using GC-MS and NMR techniques. According to Suseem and Mary Saral (2013) GC-MS studies on *Pleurotus oeus* mushroom reveal the presence of fatty acids and their esters and their strong antibacterial activity. Using Ion Trap detector in GC-MS, Ribeiro *et al*., (2008, 2009) identified free amino acid composition and thirty fatty acids from twelve wild edible mushroom species, as well as amino and fatty acids of wild edible mushroom species belonging to the genus *Boletus* was characterized by using HP-GC mass selective detector (Dembitsky *et al*., 2010).

In this article, we report the results of the GC-MS analyses of volatile and polar compounds, in addition to the sterol fraction obtained from the fruiting bodies of *A. moelleri* and *A. phaeolepidotus*. Studied is the chemical composition of these mushrooms using GC-MS.

Material and methods

Collection, determination and keeping of the mushroom material

The fruiting bodies of fungi were collected from author in Bulgaria, namely *A. moelleri* (Thracian Lowland, Plovdiv distr., above Benkovski village) and *A. phaeolepidotus* (Thracian Lowland, Plovdiv town, Bunardjik Park).

Air-dried studied specimens of the fungi are kept in the Mycological collection of the Agricultural University, Plovdiv (SOA).

The samples are documented with color photographs, concise description. Fruiting bodies of both species were photographed with SONY Cyber-shot 5.1Mpix. and CANON PowerShot A460 5.0Mpix in standard JPEG format.

The determination of the species and taxonomic decisions have been made in conformity with the researches of Wasser (1980), Cappelli (1984), Parra (2005), Lacheva (2006).

Studies of the gas chromatography–mass spectrometry analysis (GC–MS) of fruiting bodies were conducted in laboratory of the Organic and Analytical Chemistry, University of Plovdiv.

Preparation of extracts

The fresh mushrooms fruiting bodies were cut into small pieces and consecutively extracted with ethanol, ethanolchloroform (1:1) and chloroform (*A. moelleri* – 96 g; *A. phaeolepidotus* – 236 g). The extracts were combined, water was added and the chloroform layers were removed and evaporated to dry residue (2,2 g for *A. moelleri* and 1,3 g for *A. phaeolepidotus*).

Isolation and analysis of volatile compounds

A portion of the chloroform extract was subjected to a four-hour distillation-extraction on Lickens-Nickerson apparatus (Hendriks *et al.,* 1981). The volatile compounds (Table 1) were extracted from the distillate with diethyl ether, and analysed by GC-MS on a Gas Chromatograph HP 6890 series GC and Mass Spectrometer HP 5973. Compounds were separated on a HP5-MS capillary column (30m×0.25mm, internal diameter) of DB-225, held at 50°C during injection and then programmed at 40°C min^{-1} to 220°C. The carrier gas was helium, at a flow rate of 0.9 µL min^{-1}, and the injection volume was 1 µL. In mass spectrometry electron-impact ionization was performed at electron energy of 70eV.

Table 1. Volatile constituents of *A. moelleri* and *A. phaeolepidotus* (% of the total ion current, GC-MS)

Compound	A. moelleri *(%)*	A. phaeolepidotus *(%)*
Alcohols and phenols		
Benzyl alcohol	-	1.2
Phenol	50.8	90.2
Aldehydes		
Hexanal	1.0	-
Undecenal	0.5	-
2-decenal	0.2	-
2,4-decadienal	-	-
Acids		
Acetic acid	1.4	1.4
Hydroxy acetic acid	0.5	0.5
Hexadecanoic acid	5.5	5.5
Pentadecanoic acid	-	-
9,12-Octadecadienoic acid	2.5	8.6
9-Octadecanoic acid	3.7	1.8
Ethers		
Hydroquinone monopropyl ether	3.3	3.0
Fatty acid esters		
Acetic acid, phenyl ester	0.5	-
Hexadecanoic acid, ethyl ester	6.2	9.6
Octadecanoic acid, ethyl ester 18:0	0.5	1.2
Octadecenoic acid, ethyl ester 18:1	1.2	-
Tetradecanoic acid, ethyl ester	0.2	-
9,12-Octadecadienoic acid, ethyl ester	10.7	9.7
Ketones		
2-undecanone	0.2	-
Terpenoids		
Squalene	0.3	

*The ion current generated depends on the characteristics of the compound concerned and is not a true quantification.
** In parenthesis: extent of matching (as a percentage) of MS data with the literature values, where the matching is not 100%

Isolation, identification and analysis of the polar fraction

A portion of the n-butanol extract (5 mg) was dissolved in 50 µL of dry pyridine, before the addition of 75 µL of bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). The mixture was heated at 80°C for 30 min and analyzed by GC/MS. The silylated extract was investigated by GC/MS using the same instrument described above, with a capillary column HP-5 (23m×0.2mm, internal diameter). A helium carrier gas was used. The temperature programmed at 100-315°C at a rate of 5°C min^{-1}, with a 10 min hold at 315°C.

Compound Identification

Identification was carried out by searching commercial library databases. The components were identified by comparison with Computer Library attached to the GC-MS instrument. Some components remained unidentified, however, owing to both a lack of authentic samples and library spectra of the corresponding compounds.

Isolation and identification of sterols

A Gas Chromatograph (HP 5890 Series GC) linked to a Mass Spectrometer (HP 5972A MSD) was employed. Compounds were separated on a SPB-50 capillary column (30m×0.32mm, internal diameter). The chloroform extracts was subjected to column chromatography on silica gel with an *n*-hexane – acetone gradient (30:1–5:1) to produce several fractions. The third set of fractions, after further purification by preparative TLC (silica gel G, *n*-hexane – acetone 10:1), yielded sterol mixtures (25 mg from *A. moelleri* and 9.6 mg from *A. phaeolepidotus*), which were analysed by GC-MS. A helium carrier gas was used with a temperature programme set at 270°C-290°C at a rate of 4°C min^{-1} with a 20 min hold. The ion source was set at 250°C with the ionisation was performed at electron energy of 70eV. From the fourth set of preparative TLC fractions (silica gel, *n*-hexane – methyl ethyl ketone 10:1) obtained from both species 5α,8α-epidioxi-24(ξ)-methylcholesta-6,22-diene- 3β-ol was isolated (11.6 mg from *A. moelleri* and 91.4 mg from *A. phaeolepidotus*) and characterised through comparison of its EIMS, 1H- and 13C-NMR spectra, according to literature data (Gauvin *et al.,* 2000; Petrova *et al*., 2007).

Results and discussion

GC–MS analysis is a powerful tool for qualitative and quantitative analysis of various compounds present in natural products, and the technique has been widely applied in medical, biological and food research (Ribeiro *et al*., 2007; Suseem and Mary Saral, 2013).

According to our knowledge, no investigations have been reported on the constituents of crude extracts of these mushroom species. Keeping this in view, the present study has been undertaken to identify the essential bioactive compounds which are present in the petroleum ether extracts of *A. moelleri* and *A. phaeolepidotus* by GC-MS.

The volatile fractions were obtained from the fresh fruiting bodies and analysed as described in the experimental section. The results obtained are outlined in Table 1. The most abundant constituent of the volatiles in both species was phenol (over 50% in *A. moelleri* and over 90% in *A. phaeolepidotus*). Besides phenol, the main groups of compounds in the volatile fractions were fatty acids and their esters. Our findings offer the first analytical proof of the presence of phenol in these two species. Recently, it has been suggested that species with higher evolutionary positions in the section *Xanthodermatei* demonstrate higher phenol contents (Callaci *et al.,* 2005). For this reason it could therefore be surmised that *A. phaeolepidotus* is a more recently differentiated species than *A. moelleri*. Such a supposition is contained in article by Petrova *et al*., (2007) in terms of other two inedible *Agaricus* species of section *Xanthodermatei*. However, this is only a preliminary

hypothesis that will require further more detailed study. The phenol levels detected are not hugely surprising, given the numerous reports of the mushrooms phenollike odour, in addition to their belonging to the *Agaricus* section *Xanthodermatei*, which is characterised by this typical unpleasant odour (Wasser, 1980; Lacheva, 2006). It has been suggested that the production of phenol originates from an evolutionary ancestral biochemical shift, also demonstrated by the *Agaricus* species of the section *Xanthodermatei* in defence mechanisms (Del Signore *et al*., 1997; Callaci *et al.,* 2005).

Table 2. Constituents of the polar fraction of *A. moelleri* and *A. phaeolepidotus* (% of the total ion current, GC-MS)

Polar fraction	***A. moelleri* (%)**	***A. phaeolepidotus* (%)**
Alcohols		
Catechol	0.3	1.2
Ethylene diol	0.2	-
Hydroquinone	-	1.4
1,3-butane diol	1.1	1.6
Phenol	0.8	1.2
Aminoacids		
Alanine	1.4	1.5
Glycine	0.4	0.4
Isoleucine	0.5	1.8
Leucine	2.8	1.5
Phenylalanine	2.5	2.3
Proline	1.0	0.4
Threonine	0.9	0.5
Triptophan		1.2
Tyrosine		1.2
Valine	2.5	3.3
N – containing compounds		
9-H-purine-6-OH (hypoxanthine)	0.4	-
Uracyl	-	0.1
Urea	31.6	29.5
Xanthine	0.1	0.1
Acids		
Butanedioic acid	1.0	1.0
Hydroxybutanedioic acid	1.5	2.6
2-Butenedioic acid	0.6	0.6
Glutamic acid	0.4	-
Panthotenic acid	-	0.3
9,12-Octadecadienoic acid	0.1	0.1
Hexadecanoic acid	-	0.2
H_3PO_4	1.7	1.0
Sugars and sugar alcohols		
Disaccharide	-	1.4
Glucitol	33.6	9.8
Manitol	-	-

*The ion current generated depends on the characteristics of the compound concerned and is not a true quantification.

** In parenthesis: extent of matching (as a percentage) of MS data with the literature values, where the matching is not 100%

The chemical profiles of the fatty acids, the amount (%) of the individual components obtained and gas chromatographic-mass spectral data carried out for both *Agaricus* species are summarized in Table 1. The results revealed that Hexadecanoic acid, ethyl ester was found as major component followed by 9,12-Octadecadienoic acid, ethyl ester; Octadecanoic acid, ethyl ester were found as the major components in the diethyl ether extract of both species. The present study also indicates the presence of Octadecenoic acid, ethyl ester, Acetic acid, phenyl ester and Tetradecanoic acid, ethyl ester only in the fruiting bodies of *A. moelleri*.

The results of polar fraction are presented in Table 2. The polar fractions obtained from the fungal species investigated were analysed by GC-MS after silylation. The presence of phenolics is clearly evident, as is the higher amount of phenol observed in *A. phaeolepidotus*. This finding supports the suggestion of the defensive role of phenolics in section *Xanthodermatei* (Del Signore *et al*., 1997; Stoop and Mooibroek, 1999; Petrova *et al*., 2007).

Proteins of carpophores of both species were found to contain 10 amino acids, with especially high amounts of phenylalanine, valin, alanine and leucine. Another characteristic was the presence of the aromatic amino acids tryptophan and tyrosin, but only in *A. phaeolepidotus* (over 1%). The most abundant constituent of the polar extracts was urea. This finding once again confirms that fungi of the family Agaricaceae accumulate substantial amounts of urea in their fruiting bodies (Wasser, 1980; Wagemaker *et al.,* 2005, 2006; Petrova *et al*., 2007).

Petrova *et al*., (2007) investigated the sterol composition of the two *Agaricus* species and indicating that in *A. pseudopratensis* ergosterol detected in trace amounts as opposed to *A. placomyces*. According to our investigation as expected the predominant sterol in *A. placomyces*, was ergosterol, although two others were detected, each possessing a Δ7 double bond. In *A. phaeolepidotus* only the latter two sterols were present in substantial quantities, with ergosterol detected in trace amounts.

Fig. 1. Macroscopic features: a – basidiomata of *A. moelleri*, b – basidiomata of *A. phaeolepidotus* at different stages of development *in situ* (photos by Maria Lacheva).

Sugar alcohols are amongst the major soluble carbohydrates found in fungi (Lewis and Smith, 1967; Kalač, 2012). Using in GC-MS, Petrova *et al*., (2007) identified two sugar alcohols (glucitol and manitol) in inedible species *A. pseudopratensis*. According to this study in the butanol fractions of two species was found to contain only glucitol (33.6% in *A. moelleri* and 9.8% in *A. phaeolepidotus*). Petrova *et al*., (2007)

isolate the crystalline compound from the extracts of two inedible *Agaricus* species and identify it as 5α,8α-epidioxi-24(ξ)-methylcholesta-6,22-diene-3β-ol. Using column and preparative thin-layer chromatography isolated the same crystalline compound only *A. phaeolepidotus* through comparison of its spectral data with those reported in the literature. The compound was first isolated from this species and might be regarded as an artefact produced from the oxidation of the corresponding Δ5,7 sterol (Gauvin *et al*., 2000; Petrova *et al*., 2007).

Conclusion

So far, the chemical composition of these species has not been studied. Proteins contained 10 amino acids, with especially high amounts of of phenylalanine, valin, alanine and leucine. From the essential amino acids predominate valine and leucine. Among the six identified fatty acids, Octadecenoic acid was found which has the property of anti-inflammatory and antiarthritis (Sussem and Mary Saral, 2013).

Carbonyl compounds which are contained only in *A. moelleri* and totally absent from *A. phaeolepidotus* can serve as taxonomic marker such as benzoic acid, down the aromatic amino acids – tryptophan and tyrosine only in *A. phaeolepidotus* and the presence of higher concentrations of glucitol in *A. moelleri*, may also have taxonomic significance for the two species.

This study revealed a high level of chemical composition which is characteristic of fatty acid esters extracted from these mushrooms. The results can help characterise the species investigated, indicate the presence of some biologically active compounds and shed light upon their reported mild toxicity. The results obtained add to the knowledge on the chemical composition of species belonging to the genus *Agaricus*, and help provide further explanation for their reported mild toxicity, which we attribute to their high phenol content similar to that of other species from section *Xanthodermatei* of the genus *Agaricus* (Gill and Strauch, 1984; Petrova *et al*., 2007).

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