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Arsenic Compounds in Higher Fungi

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In 50 mushroom species (56 samples) from Slovenia, Switzerland, Brazil, Sweden, The Netherlands and USA, total arsenic was determined by radiochemical neutron activation analysis (RNAA). Arsenic concentrations ranged from 0.1 to 30 μg g^{-1} (dry mass). Arsenic compounds were determined in methanol extracts from the mushrooms by HPLC–ICP–MS. The aim of the study was not only to quantify arsenic compounds in mushrooms but also to uncover trends relating the methylating ability of a mushroom to its taxonomic or evolutionary status.

The main arsenic compound found in many mushrooms (various puffballs, Agaricales and Aphyllophorales) was arsenobetaine. Arsenate [As(V)] was the main arsenic species in *Laccaria fraterna* and *Entoloma rhodopolium* and arsenite [As(III)] in *Tricholoma sulphureum.* A mixture of arsenite and arsenate was present in *Amanita caesarea.* Dimethylarsinic acid (DMA) and methylarsonic acid were present in many mushrooms, but generally as minor components. In *Laccaria laccata, Leucocoprinus badhamii* and *Volvariella volvacea,* DMA was the major metabolite. Arsenocholine (AC) and the tetramethylarsonium ion were present in a few species, generally at low concentrations, except for *Sparassis crispa,* in which AC was the main compound. Trimethylarsine oxide was not found in any of the mushrooms. In some species small amounts of unknown compounds were also present. The possible taxonomic significance of the metabolite patterns and the predominance of arsenobetaine in more advanced fungal types are discussed. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

Arsenic compounds in the marine environment and their production and cycling have been intensively studied in the last 20 years. However, the fate of arsenic in terrestrial ecosystems is still largely unknown. Except for some studies on arsenic species in freshwater food chains,[1, 2] freshwater algae,[3] and fish,[4] only a few papers have been published about arsenic species in terrestrial plants such as vegetables,[5] moulds[6] and mushrooms.[7, 8] Arsenobetaine and arsenocholine have been identified in the terrestrial environment thus far only in mushrooms.[8, 9]

The arsenic compounds present in foodstuffs including edible mushrooms are obviously of concern to the consumer and the authorities, but not all arsenic compounds have the same toxicity. Inorganic compounds of arsenic are more toxic than organic derivatives. Certain organic arsenic compounds such as arsenobetaine (AB)—the major metabolite in seafood—and arsenocholine (AC) appear to be almost nontoxic.[10] Another reason for investigating arsenic metabolites in mushrooms lies in the important role of fungi in the cycling of elements in the biosphere. Saprophytic species break down decaying matter, whereas mycorrhizal types exchange nutrients and elements symbiotically with plant roots.

The main aim of this work was the identification of the arsenic compounds present in the fruitbodies of a representative selection of

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mycorrhizal and saprophytic mushrooms by high-performance liquid chromatography (HPLC) and an inductively coupled plasma–mass spectrometer (ICP–MS) as arsenic-specific detector. In a recent study of a number of arsenic-accumulating mushrooms several arsenic compounds were identified, among which arsenobetaine appears to be the major species.[8, 9] The type of arsenic metabolite seems to be fungus-dependent. Therefore, we examined a wider range of mushrooms to define the pattern in the Fungal Kingdom, and to investigate possible links between taxonomic position and the ability to accumulate arsenic compounds.

EXPERIMENTAL

Reagents and standards

$NH_4H_2PO_4 \cdot 2H_2O$, H_3PO_4, $NaAsO_2$ and $Na_2HAsO_4 \cdot 7H_2O$ (analytical grade) were purchased from Merck. Methylarsonic acid (MA, m.p. 156 °C) and dimethylarsinic acid (DMA, m.p. 190 °C) were gifts from Vineland Chemical Co. (Vineland, NJ, USA). Arsenobetaine bromide (m.p. 228 °C) was prepared from trimethylarsine and bromoacetic acid[11] and trimethylarsine oxide (TMAO) from trimethylarsine and hydrogen peroxide.[12] Tetramethylarsonium (TETRA) iodide and arsenocholine (AC) bromide were prepared in-house by literature methods.[11] Standard solutions containing 100 mg dm^{-3} arsenic were appropriately diluted with NANOpure water (18.2 MΩ cm) before analysis. Methanol for extractions, HNO_3, H_2SO_4 and H_2O_2 were of analytical grade.

Apparatus

A Triga MK II nuclear reactor with a neutron flux of 1.8×10^{12} n cm^{-2} s^{-1} in the specimen rack was used for the irradiation of samples and a coaxial HP Ge detector (resolution FWHM 1.72 keV and efficiency 20% for ^{60}Co at the 1332.5 keV gamma line) was connected to a Canberra 90 multichannel analyser system for the measurement of ^{76}As activity.

The high-performance liquid chromatographic (HPLC) system consisted of a Hewlett Packard 1050 solvent delivery unit (Hewlett Packard, Waldbronn, Germany) and a Rheodyne 9125 six-port injection valve (Rheodyne, Cotati, USA) equipped with a 100-mm^3 injection loop. Separations were performed on a Supelcosil LC-SAX (Supelco, Bellefonte, USA) anion-exchange column (25 cm × 4.6 mm i.d., 5-μm silica-based particles with quaternary aminopropyl exchange sites). To confirm and quantify the presence of TMAO, AC and TETRA, a Supelcosil LC-SCX cation-exchange column was used with a 20 mM aqueous solution of pyridine, pH adjusted to 3 with HCOOH, as mobile phase. The VG Plasma Quad 2 Turbo Plus ICP–MS with a hydraulic high-pressure nebulizer (HHPN) (Knauer, Berlin, Germany) served as the arsenic-specific detector.[9] The chromatograms were exported and the peak areas were determined with external calibration curves using software written in-house.

Collection of mushrooms

Most mushrooms were collected at sites in Slovenia and Switzerland; a few were collected in Brazil, The Netherlands, Sweden and the USA, or obtained from mushroom collections. Mushrooms from Slovenia were freeze-dried and ground in a Fritsch Pulverisette 14 Rotor Speed Mill and stored in polyethylene containers. Other mushrooms were dried in a stream of air at 50 °C, ground and stored dry in polyethylene or glass containers. Most mushrooms, except for a few of the most common ones, were identified by experts.

Determination of total arsenic

Total arsenic was determined by radiochemical neutron activation analysis (RNAA), employing mineralization of the sample with a mixture of HNO_3 and H_2SO_4 followed by addition of H_2O_2 to complete the destruction, boiling, addition of KI, extraction of arsenic tri-iodide into toluene and measurement of the ^{76}As activity at 559 keV.[13]

Identification of arsenic compounds

Preparation of extracts

Aqueous extracts were prepared using a slightly modified literature procedure:[14] 0.1–0.5 g of the dry, homogeneous powder was mixed with 10–30 ml of MeOH/H_2O (9 : 1). The mixture was shaken for 12 h, and then dried on a rotary evaporator without heating. The residue was suspended in 10 ml of water. Aliquots of this suspension were centrifuged, filtered through a

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0.22-μm membrane filter and chromatographed. Mushrooms with higher proportions of arsenite and arsenate were also extracted with water alone, which is a better extractant for these arsenic compounds.[8]

Separation and quantification of arsenic compounds with HPLC-ICP-MS

The extracted arsenic compounds were separated according to a modified literature procedure.[15] The anionic compounds (arsenite, DMA, MA, arsenate) and arsenobetaine were separated on a Supelcosil LC-SAX anion-exchange column with an aqueous solution of $NH_4H_2PO_4$ (20 or 30 mM), pH adjusted to 3.75 with H_3PO_4, as a mobile phase. The cationic compounds (TMAO, AC, TETRA) were separated on a Supelcosil LC-SCX column with a 30 mM aqueous solution of pyridine, pH adjusted to 3.0 with formic acid, as mobile phase. The concentrations of arsenic compounds were determined by comparing their peak areas with the calibration curves for the same compounds. The detection limits were around 0.5 ng As ml^{-1} in the aspirated solutions.

RESULTS AND DISCUSSION

Selection of mushrooms

Total arsenic concentrations are generally low in terrestrial plants. However, several mushrooms such as *Laccaria amethystina*,[7, 8, 16–20] *Laccaria fraterna*,[19] *Sarcosphaera coronaria*,[8, 19] *Macrolepiota procera*[21] and *Agaricus* sp.[8, 22, 23] are known to accumulate arsenic to high concentrations (2000 μg g^{-1} in *Sarcosphaera coronaria*[8, 19]). Previously we identified the arsenic compounds present in most of these mushrooms.[7, 8] In the present study, we were interested not so much in accumulators but rather in the pattern of arsenic compounds in a wider range of genera of mushrooms and hence in relationships between fungal taxonomy or evolutionary status and the arsenic compounds present. Because low concentrations of individual arsenic compounds in complex mixtures are difficult to quantify accurately, the total arsenic concentration in the dry mushrooms should not be lower than about 0.5 mg kg^{-1}. Consequently, the selection of mushrooms is slightly biased towards species with higher rather than lower concentrations of arsenic. In 56 mushroom samples (50 species) collected, the total arsenic concentrations ranged from 0.1 to 30 μg g^{-1} dry mass. In 41 samples (38 species, Table 1) arsenic compounds were determined. The highest concentrations of arsenic were found in *Laccaria fraterna* (30 μg g^{-1}). Of the mushrooms with arsenic concentrations above 1 μg g^{-1}, only *Geastrum* sp., *Amanita muscaria*, *Leucocoprinus badhamii* and *Thelephora terrestris* are inedible or toxic. Table 1 is arranged to show the generic relationships between the mushrooms investigated.

Identification and quantification of arsenic compounds

For the identification of arsenic compounds, the dried, powdered mushrooms were extracted with methanol/water (9:1) and the extract evaporated. The residue was suspended in water, centrifuged and filtered. In most cases more than 80% of the total arsenic in the mushrooms was found in the extracts. Arsenic compounds in most mushrooms investigated thus far[8] were extracted almost quantitatively by methanol/water (9:1), but water alone is a better extractant for arsenite and arsenate. Therefore, if substantial proportions of these two compounds were found in the methanol extract, a second analysis using an aqueous extract was performed. Exceptionally, methanol/water (9:1) extracted only 2.5% of the arsenic in *Thelephora terrestris*; about 8% was extracted with water alone and 18% with CCl_4. Arsenic in *Thelephora terrestris* must be bound strongly to tissue constituents or be present in a form not soluble in methanol/water.

Arsenic compounds in mushrooms were first separated and identified (Fig. 1) using a Supelco LC-SAX column (mobile phase 30 mM $NH_4H_2PO_4$, pH 3.75). On prolonged use of this column the retention times became shorter and the separation deteriorated. Performance could be restored by using a more dilute buffer (20 mM) as mobile phase.

In some cases small amounts of compounds with retention times longer than 300 s were also found. These unidentified compounds are shown in Tables 2–7 as 'unknown'. Two 'unknowns', with retention times of 335 and 460 s, present in several mushrooms (Tables 3–5), are very likely to be anionic.

If present, AC and TETRA are eluted together from the LC-SAX column at about 130 s before the As(III) peak (Figs 1, 2). If no peak was found

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Table 1 Total arsenic concentrations in mushrooms determined by radiochemical neutron activation analysis

Mushroom	As ($\mu g\ g^{-1}$)[a] dry mass	Mushroom	As ($\mu g\ g^{-1}$)[a] dry mass
Basidiomycetes		*Entoloma rhodopolium*	0.55 ± 0.06
Order: Aphyllophorales		Family: Lepiotaceae	
Family: Steraceae		*Macrolepiota procera*	0.42 ± 0.04
Thelephora terrestris	15.9 ± 1.0	*Leucocoprinus badhamii*	2.9 ± 0.7
Family: Clavariaceae		Family: Agaricaceae	
Ramaria pallida	3.7	*Agaricus abruptibulbus*	3.49 ± 0.45
Sparassis crispa (1)	1.03 ± 0.10	*Agaricus bisporus*	1.00 ± 0.03
(2)	0.57 ± 0.04	*Agaricus campester*	1.32 ± 0.17
Family: Cantharellaceae		*Agaricus elvensis*	2.43 ± 0.29
Gomphus clavatus	4.47 ± 0.20	*Agaricus fuscofibrillosus*	2.54, 2.81
Order: Agaricales		*Agaricus lilaceps*	1.78 ± 0.08
Family: Polyporaceae		*Agaricus macrosporus*	3.32 ± 0.36
Albatrellus cristatus	7.72 ± 1.02	*Agaricus silvicola*	6.24 ± 0.49
Albatrellus ovinus	0.24, 0.27	*Agaricus subrutilescens*	10.8 ± 0.04
Albatrellus pes-caprae	0.77 ± 0.02	Family: Amanitaceae	
Family: Tricholomataceae		*Amanita phalloides*	0.55
Laccaria fraterna (1)	11.2, 11.8	*Amanita magniverrucata*	0.50
(2)	30.0, 29.6	*Amanita muscaria*	3.11 ± 0.24
Laccaria laccata		*Amanita caesarea*	0.45
var. *pallidifolia* (1)	0.66 ± 0.08	*Amanita rubescens*	0.12
(2)	4.26 ± 0.15	Gastromycetales	
Lyophyllum conglobatum	0.63, 0.63	Order: Gastrales	
Tricholoma inamoenum	0.39, 0.36	Family: Lycoperdaceae	
Tricholoma pardinum	0.63 ± 0.06	*Calvatia excipuliformis*	0.72 ± 0.06
Tricholoma sulphureum	0.26 ± 0.01	*Calvatia utriformis*	0.79 ± 0.07
Family: Pluteaceae		*Lycoperdon echinatum*	1.23 ± 0.10
Volvariella volvacea (1)	0.82 ± 0.08	*Lycoperdon perlatum*	2.81 ± 0.24
(2)	1.05, 1.07	*Lycoperdon piriforme*	0.46 ± 0.09
Family: Entolomataceae		*Geastrum sp.*	3.12 ± 0.20

[a] At least three separate determinations were performed when the standard deviation is given.

in this region, limits of detection for AC and TETRA were calculated. If a peak was obtained, the extracts were also chromatographed on the cation-exchange column (Supelcosil LC-SCX). The chromatogram of a standard solution containing eight arsenic compounds from the LC-SCX column is shown in Fig. 3.

A typical example of a chromatogram from the anion-exchange separation for *Amanita muscaria* (Fig. 2A) shows, as well as the major peaks, an initial AC/TETRA peak and two small peaks of unknown arsenic compounds at long retention times. The separation was then repeated on the cation-exchange column (Fig. 2B). The poorly separated signals for the anionic compounds and for AB are followed by well-resolved signals for the cations AC and TETRA and an unknown arsenic compound.

Additional chromatograms (Fig. 4) illustrate that the pattern of arsenic compounds differs greatly among mushrooms. For instance, *Gomphus clavatus* (Fig. 4D) has AB as the major arsenic compound and two small unidentified, probably cationic, arsenic compounds, whereas *Sparassis crispa* (Fig. 4C) has AC and an unidentified arsenic compound as the major arsenic-containing components.

The detailed results are presented in Tables 2–7. The unknown compounds were quantified by means of the calibration curves of the arsenic compounds with neighbouring peaks. It is evident that the sum of the arsenic compounds obtained from the chromatograms agreed with the total arsenic concentrations determined by RNAA within ±20% for 21 of the 41 mushrooms investigated. Incomplete extraction of the arsenic compounds can be considered responsible for the 16 cases in which the sum of the species was more than 20% lower than the RNAA total. Only in four cases were the sums of

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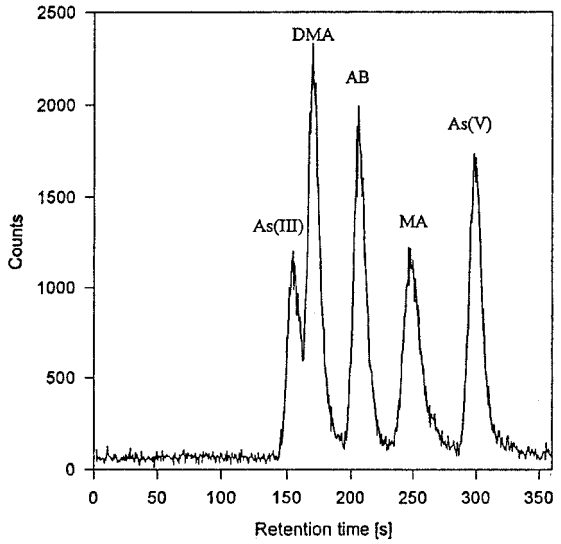

Figure 1 Separation of As(III), DMA, MA, AB and As(V) in standard solutions (5 ng g^{-1} each) on a Supelco LC-SAX anion-exchange column, mobile phase 20 mM $NH_4H_2PO_4$, pH 3.75, flow rate 1.5 ml min^{-1}, injection volume 100 μl. If present, AC and TETRA are eluted in front of the arsenite, and TMAO between AB and MA.

the species more than 20% higher than the totals.

Taxonomy and arsenic compounds

In arsenic-accumulating mushroom species several arsenic compounds were previously identified;[7,8] for instance, methylarsonic acid in *Sarcosphaera coronaria*, dimethylarsinic acid in *Laccaria amethystina*, and arsenobetaine in two representatives of the genus *Agaricus*. *Sarcodon imbricatus* (Hydnaceae) contained a mixture of arsenite and arsenate, MA, DMA and AB, whereas *Entoloma lividum* (Entolomataceae) only inorganic arsenic. The patterns of arsenic compounds were characteristic of the mushroom species, as demonstrated by analysis of samples collected at various localities. Arsenic accumulators were identified in widely different families of fungi, and their way of metabolizing arsenic seemed to vary with their taxonomic position. We decided, therefore, to investigate this matter further on a number of fungi with total arsenic concentrations higher than 0.1 μg g^{-1} dry mass belonging to a wide range of families and genera within the orders Aphyllophorales, Agaricales and Gastrales.

The arsenic compounds identified in the various taxonomic groups of mushrooms are listed in Tables 2–7. The most frequently encountered metabolite is arsenobetaine. In only nine of the 41 types of mushrooms was AB not detected. In most of the other mushrooms AB was the predominant arsenic compound. Apparently, many fungi are able to detoxify inorganic arsenic by conversion to this harmless metabolite. There does not seem to be any relation between the ability to metabolize arsenic and the biology of the fungi, e.g. saprophytic species were as apt to produce AB as mycorrhizal fungi.

Although the number of fungi analysed is too small to permit definite conclusions on a possible relationship between arsenic metabolism and taxonomic position, there is little doubt that the ability to synthesize AB was only acquired during the higher stages of fungal evolution. We find formation of AB most expressed in repre-

Table 2 Arsenic compounds in mushrooms belonging to the order Gastrales

	Arsenic (μg g^{-1})		Arsenic compounds (%[b]) from LC-SAX						
Mushroom	Total[a]	Sum of species	As(III)	DMA	AB	MA[c]	As(V)	Unknown[c]	AC/TETRA[c]
Calvatia excipuliformis	0.72±0.06	0.8	4	20	72	<	4	<	<
Calvatia utriformis	0.79±0.07	0.5	Trace	9	85	<	6	<	<
Lycoperdon echinatum	1.23±0.10	0.3	Trace	12	78	<	10	<	<
Lycoperdon perlatum	2.81±0.24	3.6	Trace	5	88	7	Trace	<	<
Lycoperdon piriforme	0.46±0.09	0.5	8	Trace	62	Trace	30	<	<
Geastrum sp.	3.12±0.20	2.9	2	2	94	Trace	2	<	<

[a] Total arsenic in Tables 2–7 was determined in duplicate (both values given) or triplicate (average±SD) by RNAA. All concentrations are based on dry mass.
[b] Percentage: 100 (μg As g^{-1} for arsenic compound)/(sum of species in μg As g^{-1}).
[c] < means below detection limit (<0.1%).

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Table 3 Arsenic compounds in mushrooms belonging to the Lepiotaceae and Agaricaceae families

Mushroom	Arsenic ($\mu g\ g^{-1}$)		Arsenic compounds (%) from LC-SAX						Arsenic compounds (%) from LC-SCX		
	Total	Sum of species	As(III)	DMA	AB	MA	As(V)	Unknown[d]	AC	TETRA	Unknown[d]
Macrolepiota procera	0.42±0.04	0.1	Trace	Trace	100	Trace	Trace	<	<	<	<
Leucocoprinus badhamii	2.9±0.7	2.6	Trace	47	49	Trace	2	2 (460 s)	Trace	Trace	<1 (220 s)
Agaricus abruptibulbus	3.49±0.45	3.0	1	3	88	5	3	<	<	<	<
Agaricus bisporus	1.00±0.03	0.8	<	27	55	6	12	<	<	<	<
Agaricus campester	1.32±0.17	1.3	Trace	Trace	96	Trace	<	<	<	4	<
Agaricus elvensis	2.43±0.29	2.6	2	4	84	2	4	4 (335 s)	<	<	<
Agaricus fuscofibrillosus	2.54, 2.81	2.7	Trace	4	95	<	1	<	<	<	<
Agaricus lilaceps	1.78±0.08	2.3	6	24	66	<	3	1 (460 s)	<	<	<
Agaricus macrosporus	3.32±0.36	2.4	<	8	85	4	3	<	<	<	<
Agaricus silvicola	6.2±0.5	7.2	1	4	90	<	3	2 (460 s)	<	<	<
Agaricus subrutilescens	10.8±0.4	10.4	0.5	1.5	96	1	1	<	<	<	<

See footnotes to Table 1.
[d] For unknown compounds retention times are given in parentheses.

sentatives of the Gastrales, mainly puffballs and the related *Geastrum* sp. (Table 2), which are indeed at the top of the evolutionary ladder.[24] At an earlier stage we find this metabolite also in the more accomplished gilled fungi, notably *Agaricus* species (Table 3; Fig. 4A). *Agaricus* mushrooms have many biochemical features in common with Lycoperdaceae, such as formation of urea,[25] concentration of silver,[17, 26] selenium[27] and mercury, and biosynthesis of methylmercury.[28, 29] It would be interesting to analyse *Endoptychum agaricoides*, a rare species constituting a 'bridge' between the Gasteromycetes and the Agaricaceae. Other evolutionarily advanced gilled fungi are the Cortinariaceae, of which a few genera are also clearly linked to the Gasteromycetes. Two arsenic-rich *Cortinarius* species, *Telamonia bivela* and *Phlegmacium melliolens*, collected in a former mining area, were found to contain virtually only AB (Goessler *et al*., unpublished results).

Such links are less evident in the Amanitaceae (Table 4), but at least in the Fly agaric, *Amanita muscaria* (Fig. 2), AB is the predominant metabolite together with smaller amounts of the unusual precursor arsenocholine (AC). Essentially the same pattern was reported for *Amanita muscaria* by Kuehnelt *et al*.[9]

The Aphyllophorales are an enormously heterogeneous group, especially from the morphological point of view. Many of its representatives grow on wood, a substrate poor in arsenic, and were therefore not investigated. *Sarcodon imbricatus*, the only representative analysed earlier,[8] contains AB (58%), but apparently the biosynthesis is not very efficient, since

Table 4 Arsenic compounds in mushrooms belonging to the Amanitaceae family

Mushroom	Arsenic ($\mu g\ g^{-1}$)		Arsenic compounds (%) from LC-SAX						Arsenic compounds (%) from LC-SCX		
	Total	Sum of species	As(III)	DMA	AB	MA	As(V)	Unknown	AC	TETRA	Unknown
Amanita phalloides	0.55[e]		Trace	Trace	Trace	Trace	Trace	<	<	<	<
Amanita magniverrucata	0.50[e]		Trace	Trace	<	Trace	Trace	<	<	<	<
Amanita muscaria	3.1±0.2	3.4	13	6	72	<	3	1 (335 s) 1 (460 s)	2	2	<1 (350 s)
Amanita caesarea	0.5[a]	0.5	32	13	<	<	38	<	<	17	<
Amanita rubescens	0.1[a]		Trace	Trace	<	<	<	<	<	<	<

See footnotes to Tables 2 and 3.
[e] Total arsenic determined by ICP–MS.

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Table 5 Arsenic compounds in mushrooms belonging to the order Aphyllophorales

	Arsenic ($\mu g\ g^{-1}$)		Arsenic compounds (%) from LC-SAX						Arsenic compounds (%) from LC-SCX		
Mushroom	Total	Sum of species	As(III)	DMA	AB	MA	As(V)	Unknown	AC	TETRA	Unknown
Thelephora terrestris	15.9±1.0	1.5	49	<	<	8	43	<	<	<	<
Sparassis crispa 1	1.03±0.10	0.8	<	<	31	<	3	<	66	<	<
2	0.57±0.04	0.3	Trace	Trace	Trace	<	Trace	<	45	Trace	45 (330 s)[c] 10 (350 s)
Gomphus clavatus	4.47±0.20	4.2	<	2	87	Trace	Trace	<1 (335 s)	8	1	1 (220 s) 1 (350 s)
Albatrellus cristatus	7.7±1.0	7.8	<	1	91	<	<	5 (335 s)	2	1	<1 (220 s) <1 (350 s)
Albatrellus ovinus	0.24, 0.27	0.2	12	Trace	64	Trace	24	<	Trace	Trace	Trace (220 s) Trace (350 s)
Albatrellus pes-caprae	0.77±0.02	1.1	Trace	<	94	Trace	6	<	<	<	<
Ramaria pallida[f]	3.7	3.0	[g]	3	81	2	[g]		13 (not resolved)		

See footnotes to Tables 2 and 3.
[f] Determined by ion exchange–instrumental neutron activation as described previously.[8]
[g] 0.03±0.03 As(III) and As(V), signals not resolved.[8]

Table 6 Arsenic compounds in mushrooms belonging to the Tricholomataceae family

	Arsenic ($\mu g\ g^{-1}$)		Arsenic compounds (%) from LC-SAX						
Mushroom	Total	Sum of species	As(III)	DMA	AB	MA	As(V)	Unknown	AC/TETRA
Laccaria fraterna 1	11.2, 11.8	10.4	7	<	<	<	93	<	<
2	30.0, 29.6	24.6	6	<	<	<	94	<	<
Laccaria laccata 1	0.66±0.08	0.3	Trace	55	Trace	Trace	45	<	<
2	4.26±0.15	2.9	2	88	9	Trace	1	<	<
Tricholoma inamoenum	0.39, 0.36	0.1	Trace	100	<	Trace	<	<	<
Tricholoma pardinum	0.63±0.06	0.8	11	21	63	<	5	<	<
Tricholoma sulphureum	0.26±0.11	0.3	72	28	<	<	Trace	<	<
Lyophyllum conglobatum	0.63, 0.63	0.2	<	34	66	<	Trace	<	<

See footnotes to Tables 2 and 3.

Table 7 Arsenic compounds in the Pluteaceae and Entolomataceae families

	Arsenic ($\mu g\ g^{-1}$)		Arsenic compounds (%) from LC-SAX						
Mushroom	Total	Sum of species	As(III)	DMA	AB	MA	As(V)	Unknown	AC/TETRA
Volvariella volvacea 1	0.82±0.08	0.7	Trace	78	10	8	4	<	<
2	1.05, 1.07	0.5	Trace	94	Trace	Trace	6	<	<
Entoloma rhodopolium	0.55±0.06	0.3	28	17	<	<	55	<	<

See footnotes to Tables 2 and 3.

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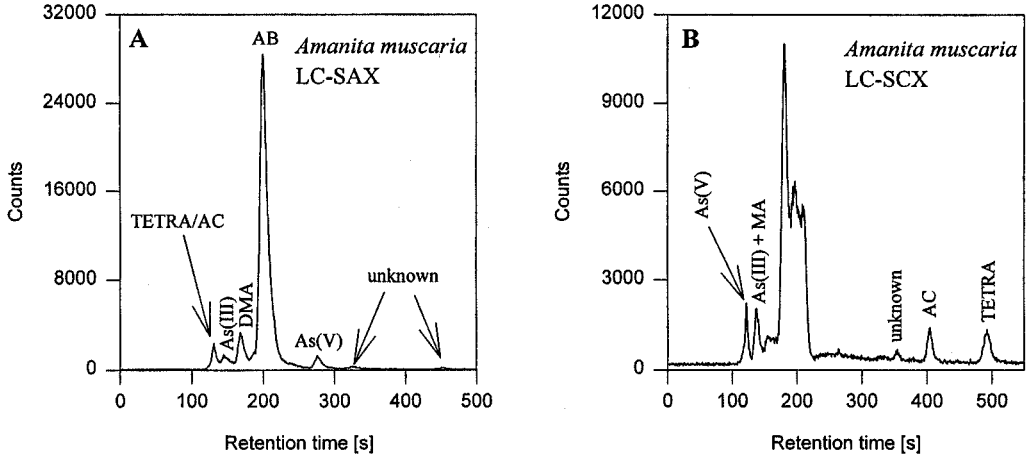

Figure 2 (A) Anion- and (B) cation-exchange separation of arsenic compounds in the extract from *Amanita muscaria* (same conditions as in Figs 1 and 3).

many other arsenic compounds are present at significant levels. Table 5 shows that members of the *Albatrellus* family (which are terrestrial polypores), *Ramaria pallida* and the cauliflower-shaped *Sparassis crispa* contain AB and several other organic and inorganic arsenic compounds. In *Sparassis crispa* arsenocholine is the predominant compound (Fig. 4C). In spite of their widely different morphology, Aphyllophorales are considered close to gilled fungi. In *Gomphus clavatus*, an intermediary species between Clavariales and Cantharellaceae (the latter having rudimentary gills), AB accounts for 89% of the arsenic and AC for 8% (Fig. 4D). On the other hand, in *Thelephora terrestris*, belonging to the more primitive crust and parchment fungi, AB is absent. Most of the arsenic is present as inextractable compounds (90%), and the extractable part consists mainly of arsenite and arsenate (Table 5).

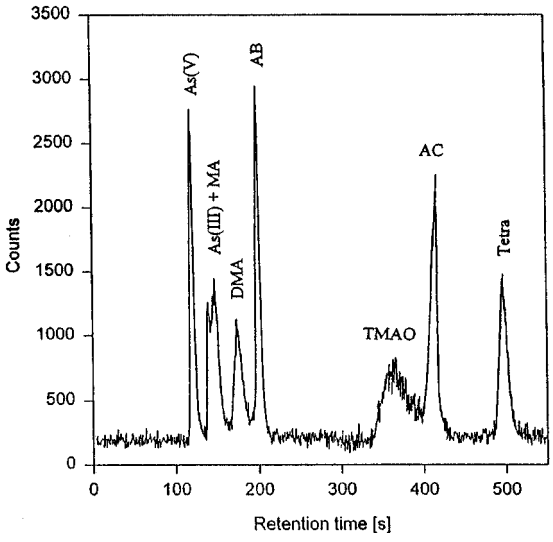

Figure 3 Separation of As(III), DMA, MA, As(V) from cations AB, TMAO, AC and TETRA in a standard solution (5 ng As g^{-1} each except TMAO, 10 ng g^{-1}) on a Supelco LC-SCX cation-exchange column, mobile phase 30 mM pyridine, pH 3.0 (adjusted with HCOOH), flow rate 1.5 ml min^{-1}, injection volume 100 μl.

Tricholomataceae also form a large group ranging from more 'primitive' genera such as *Pleurotus* to more developed ones, such as *Tricholoma* sensu strictu, *Collybia*, *Lepista* and *Lyophyllum*. Although we analysed too few fungi to warrant any firm conclusion, it is interesting to note that in the family Tricholomataceae only three of the six genera investigated contained AB (Table 6). When the edible oyster mushroom *Pleurotus* sp. was cultivated, it was found that this mushroom avidly took up inorganic arsenic from its substrate, but was able to convert only 1% of it to MA.[30] Since we had demonstrated earlier that in nature *Laccaria amethystina* consistently formed DMA almost exclusively (90%),[7,8] additional species of this difficult genus were investigated. In *Lacaria fraterna*, which can also take up considerable quantities of arsenic,[19] arsenate was the main arsenic compound and no methylated arsenicals were detected (Table 6). Perhaps this is because *Laccaria fraterna* was collected in Brazil, where it has the Eucalyptus tree as a mycorrhizal partner. European representatives of the genus usually live in symbiosis with less exotic trees.

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In the very common *Laccaria laccata*, the pattern of arsenic compounds was noticeably different in two collections: one sample had approximately equal percentages of DMA (55%) and arsenate (45%) and only a trace of AB, whereas the other had DMA (88%) as the predominant compound, only 1% of extractable arsenic as arsenate, but 9% as AB (Table 6). Here, an error in botanical identification cannot be excluded, but *Laccaria laccata* is also quite a variable species. Perhaps the presence of AB is less surprising if we consider that the genus *Laccaria* has a link to the Hydnangiaceae, which are hypogaeous Gasteromycetales.

In the genus *Tricholoma* sensu strictu, *Tricholoma sulphureum* has arsenite (72%) as the main arsenic compound. In *Tricholoma inamoenum* DMA is almost the only arsenic compound present, but in *Tricholoma pardinum* the major metabolite is AB (63%)! Interestingly, the latter species is so different from other tricholomas that it has been placed in a special subgenus of which it is the only member. Moving higher up in the Tricholomataceae we observe that in *Lyophyllum conglobatum* AB (66%) is the main compound (Table 6). This is also the case in two members of the arsenic-rich genus *Collybia* (Tricholomataceae), collected at an old smelter site in Austria.[9]

We also analysed a few reddish-spored fungi, belonging to the Pluteaceae and the Entolomataceae, which are rather difficult to classify (Table 7). It is generally recognized that Pluteaceae are the more evolved ones and indeed, in *Volvariella volvacea*, arsenic is largely present as DMA (Fig. 4B), whereas in *Entoloma rhodopolium* mainly

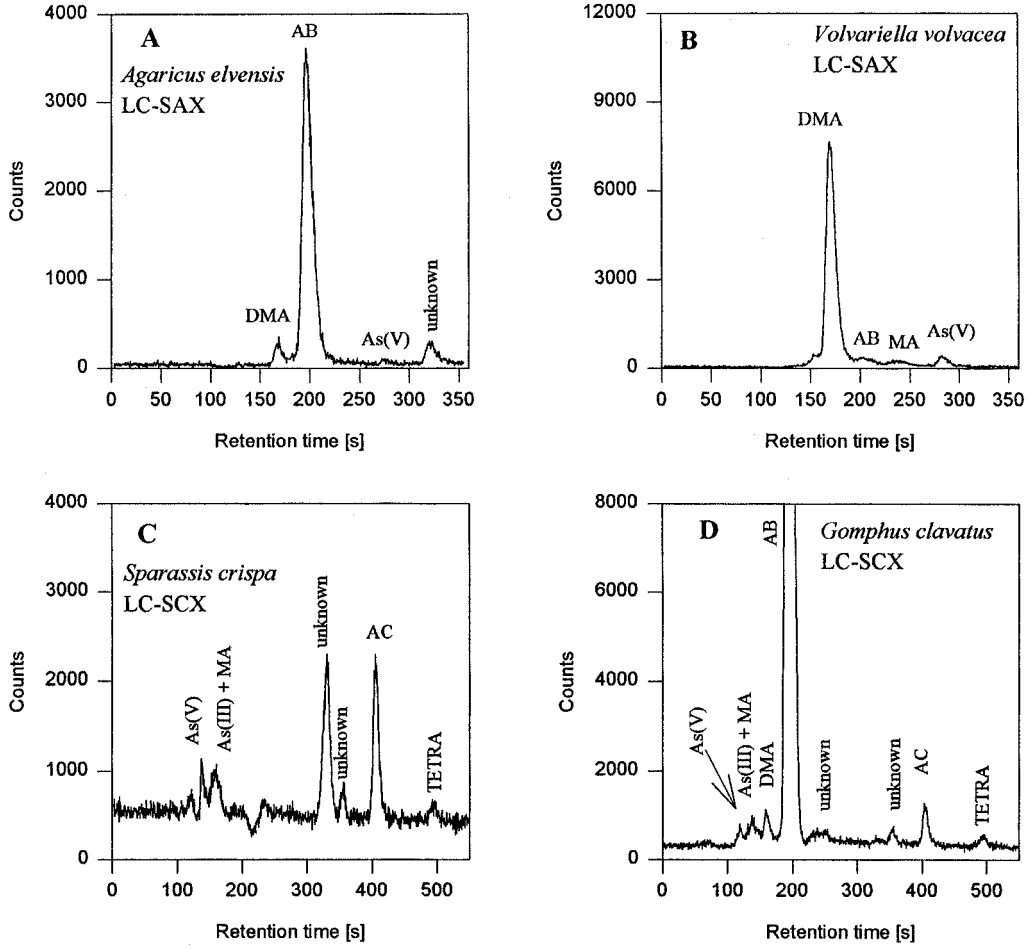

Figure 4 Anion- and cation-exchange chromatograms of mushroom extracts from *Agaricus elvensis*, *Volvariella volvacea*, *Sparassis crispa* and *Gomphus clavatus* (conditions for A and B as in Fig. 1; for C and D as in Fig. 3).

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inorganic arsenic was found, just like in *Entoloma lividum*, analysed earlier.[8]

Finally, we should point out that in discussing our results we assumed that the arsenic compounds are actively synthesized by the mushrooms (probably in the mycelium and transported to the fruitbody). However, it cannot yet be ruled out that alternatively the mushroom might preferentially accumulate or transport certain metabolites which could be synthesized in the soil, e.g. by microorganisms[31] or microfungi. However, against this the presence of AB in soil or pore water has not yet been reported; furthermore, TMAO, a common metabolite of methylation by microorganisms, was not found in any mushroom in our work. Other experiments using cultivation of mushrooms and fungal cultures grown on agar plates spiked with various arsenic compounds are in progress[30], and should cast more light on this question. In this connection it is of interest that Fischer *et al*.[32] recently reported evidence that mushrooms were capable of methylation of mercury to methylmercury, which was confirmed in experiments using cultures of the saprophytic fungi *Coprinus comatus* and *Coprinus radians*.

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