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Arsenobetaine and Other Arsenic Species in Mushrooms

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Arsenic species in arsenic-accumulating mushrooms (*Sarcosphaera coronaria*, *Laccaria amethystina*, *Sarcodon imbricatum*, *Entoloma lividum*, *Agaricus haemorrhoidarius*, *Agaricus placomyces*, *Lycoperdon perlatum*) were determined. HPLC/ICP MS and ion-exchange chromatography–instrumental neutron activation analysis (NAA) combinations were used. The remarkable accumulator *Sarcosphaera coronaria* (up to 2000 mg As kg^{-1} dry wt) contained only methylarsonic acid, *Entoloma lividum* only arsenite and arsenate. In *Laccaria amethystina* dimethylarsinic acid was the major arsenic compound. *Sarcodon imbricatum* and the two *Agaricus* sp. were found to contain arsenobetaine as the major arsenic species, a form which had previously been found only in marine biota. Its identification was confirmed by electron impact MS.

Keywords: arsenic species; mushrooms; methylarsonic acid; dimethylarsinic acid; tetramethylarsonium ion; arsenobetaine; arsenite; arsenate; HPLC/ICP MS; IC/INAA

1 INTRODUCTION

In contrast to a wealth of data on arsenic compounds in marine systems, hardly any information is available on terrestrial biota. Total arsenic concentrations are generally low in terrestrial plants, but certain higher fungi can accumulate this element. Known accumulators include *Laccaria amethystina*[1–6] and *Laccaria fraterna*[4] (*ca* 100 mg kg^{-1} dry weight), *Agaricus* sp.,[5,7] *Ramaria pallida* and *Macrolepiota procera*,[8,9] *Lycoperdon perlatum*,[5,8–10] and especially *Sarcosphaera coronaria*,[4] where up to 2000 mg kg^{-1} (dry weight) were found. Recently we showed[11] that the arsenic accumulated in the edible mushroom *Laccaria amethystina* is almost all in the form of scarcely toxic dimethylarsinic acid (DMA).

Quite apart from its scientific interest in relation to the cycling of arsenic in the environment, the arsenic compounds present in edible mushrooms are obviously of concern to the consumer (and the regulating authorities), because arsenic compounds vary considerably with respect to their toxicity. Inorganic compounds of arsenic are more toxic than organic derivatives. Certain organic arsenic compounds such as arsenobetaine (AB) and arsenocholine appear to be not toxic at all.

The aim of the work reported in this paper was the identification of arsenic compounds in arsenic-accumulating mycorrhizal and saprophytic mushrooms. Because the identification of arsenic compounds is to a certain extent methodologically dependent, the results should be confirmed by more than one technique. Hence, high-performance liquid chromatography (HPLC) coupled to an inductively coupled plasma–mass spectrometer (ICP–MS)[12] and ion-exchange chromatography with detection of arsenic by instrumental neutron activation were used.[13]

2 EXPERIMENTAL

2.1 Reagents and standards

$NaH_2PO_4 \cdot 2H_2O$, H_3PO_4, $NaAsO_2$ and $Na_2HAsO_4 \cdot 7H_2O$ of p.a. quality 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 (AB, m.p. 228 °C) was prepared from trimethylarsine and bromoacetic acid.[14] Trimethylarsine

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oxide (TMAO) was prepared from trimethylarsine and hydrogen peroxide.[15] Standard solutions containing 100 mg dm^{-3} or 1 mg g^{-1} arsenic were then prepared. Before analysis these stock solutions were diluted with nanopure water (18.2 MΩ cm^{-1}). Methanol, 0.5 M HCl, 6 M HCl, 0.75 M NH_3, 6 mM phosphate buffer, pH 6.8 [mixture of equal parts of KH_2PO_4 and $(NH_4)_2HPO_4$, all p.a. grade, strong cation-exchanger Dowex 50W × 8 (100–200 mesh) and strong anion-exchanger Dowex 1 × 8 (200–400 mesh) were used.

2.2 Apparatus

A Triga MK II nuclear reactor produced a neutron flux of 1.8×10^{12} n cm^{-2} s^{-1} in the specimen rack or 4×10^{12} n cm^{-2} s^{-1} in the pneumatic transfer system.

A coaxial HP Ge detector, resolution FWHM 1.72 keV and efficiency 20.1% for ^{60}Co at the 1332.5 keV gamma line, was connected to a Canberra 90 multichannel analyser system.

The HPLC system consisted of a Milton Roy CM 4000 multiple solvent delivery unit and an anion-exchange Supelcosil LC-SAX column (250 mm × 4.6 mm i.d.; spherical 5 μm particles of silica with basic quaternary aminopropyl exchange sites). In some cases a Hamilton PRP™-X100 anion-exchange column was employed. A 100-μL loop in conjunction with a Rheodyne six-port injection valve was used. The connection to the ICP–MS was made via a 700-mm steel capillary directly to the nebulizer.

The inductively coupled plasma (ICP) mass spectrometer (MS) used as an arsenic-specific detector was a VG PlasmaQuad 2 Turbo Plus (VG Elemental, Winsford, Cheshire, UK). A Meinhard concentric glass nebulizer, type SB-30-A3, and a double pass Scott-type spray chamber, water-cooled (0 °C), were used. For ion sampling, nickel sample cones with an orifice of 1.00 mm diameter and nickel skimmer cones with an orifice of 0.75 mm diameter were used. The gas flow rates were set to 13.5 dm^{-3} min^{-1} for cooling gas, 1.1 dm^{-3} min^{-1} for auxiliary gas, and 0.73 dm^{-1} min^{-1} for nebulizer gas. The incident power during analysis was 1.40 kW. The reflected power was less than 5 W.

A Finnegan MAT 8430/SS 300 double-focusing mass spectrometer was used to acquire electron impact mass spectra for confirmation of the identify of AB.

2.3 Sampling

Mushroom samples were collected from sites in Switzerland (seven samples) and Slovenia (four samples). The Swiss samples were dried in a stream of air at 50 °C and stored dry in polyethylene or glass containers. Samples from Slovenia were analysed fresh or stored frozen at −20 °C until analysis.

2.4 Determination of total arsenic

Total arsenic was determined by radiochemical neutron activation analysis (RNAA) including total decomposition of the sample, extraction of arsenic tri-iodide into toluene and measurement of the ^{76}As activity at 559 keV,[16] and in the samples from Switzerland by hydride generation–atomic absorption spectroscopy (HG AA) after total decomposition by nitric acid and dry ashing with magnesium nitrate.[17]

2.5 Identification of arsenic compounds

2.5.1 Preparation of extracts

Aqueous extracts were prepared using a slightly modified procedure from the literature.[18] Fresh samples (around 10 g) were homogenized and a 5–10-fold quantity of methanol was added; in the case of dry samples (around 1 g), 10% H_2O–90% MeOH was added. The mixture was sonicated in an ultrasonic bath for 1 h and then filtered (Schleicher & Schuell, 589 black band filter paper). Extraction was repeated three or four times, then the extracts were combined and evaporated gently to dryness. The residue (so-called 'orange gum') was washed with ether and then dissolved in water. Aliquots of this solution were chromatographed. Exceptionally, *Entoloma lividum* was extracted with water alone (see Section 3 below).

2.5.2 Ion-exchange chromatography with instrumental neutron activation analysis (IC–INAA)

The ion-exchange separation method was developed[13] following literature reports.[11,19,20]

Aqueous extracts were applied to a cation-exchange column (Dowex 50W × 8, 100–200 mesh, 6 × 240 mm) and successively eluted with 15 ml of 0.5 M HCl, 10 ml water, 15 ml of 0.75 M NH_3, 20 ml 3 M NH_3, 20 ml of water, 50 ml of 0.5 M HCl, and 20 ml of 6 M HCl. The positions of the peaks were determined using aqueous solu-

Table 1 Methanol extraction of arsenic from arsenic-accumulating mushrooms

Sample	Total As[a] (μg g^{-1})	Methanol-extractable As[a] (μg g^{-1})	
		Water-soluble	Ether-soluble
Sarcosphaera coronaria	1. 339 ± 17 (4)	321	—
	2. 2120	2100	—
	3. 15[b] (2)	16.1 ± 1.5 (5)	—
Laccaria amethystina	1. 3.4[b] ± 0.3 (3)	2.5	0.2
	2. 40.5	36.1	3.3
Sarcodon imbricatum	1. 0.9	0.7	0.2
	2. 23.8 (2)	21.3	—
Agaricus haemorrhoidarius	8.8 (2)	6.3	0.1
Agaricus placomyces	8.6 (2)	6.3	0.1
Entoloma lividum	38.9 ± 3.3 (6)	40.0[c]	—

[a] Mean ± SD with number of replicates in parentheses. [b] Fresh weight basis; others dry weight. [c] Aqueous extraction without methanol.

tions of pure arsenic compounds. The first fraction from 4.5 to 13.8 ml contained inorganic arsenic, the second (13.8–28 ml) MA, the third (50–60 ml) AB and DMA, and the last one (135–150 ml) the tetramethylarsonium (TMA) ion.

The third fraction from the cation column was freeze-dried, dissolved in 1 ml of phosphate buffer and applied to an anion-exchange column (Dowex 1 × 8, 200–400 mesh, phosphate form, 7 × 500 mm) and eluted with 6 mM phosphate buffer, pH 6.8. The first fraction, from 9 to 16 ml, contained AB [and any trimethylarsine oxide (TMAO)] and the second fraction, from 19 to 32 ml, DMA.

As a detection method, instrumental neutron activation analysis of the fractions was used: aliquots (3 ml) were sealed in 5-ml polythene ampoules and irradiated for 2–4 h. After about one day's decay, the ampoules were measured by gamma spectrometry, and arsenic quantified from the ^{76}As peaks (550 keV) of the sample and standard.

2.5.3 High-performance liquid chromatography–inductively coupled plasma mass spectrometry

HPLC/ICP MS

The arsenic compounds extracted (methanol, water) as described for the IC–INAA method were separated according to a modified literature procedure.[12] An aqueous solution of NaH_2PO_4 (30 mmol dm^{-3}) was mixed with a few drops of an aqueous solution of H_3PO_4 (1.5 mol dm^{-3}) to a pH of 3.75. This solution was used as mobile phase at a flow rate of 1.5 ml min^{-1}. Before analysis, the solutions were filtered through a 0.2-μm membrane filter and then chromatographed on the Supelcosil LC-SAX anion-exchange column. In the case when the Hamilton PRP^{TM}-X100 anion column was used [for better chromatograms of As(III) and As(V)], the mobile phase was a 30 mmol dm^{-3} NaH_2PO_4/Na_2HPO_4 buffer at pH 6.

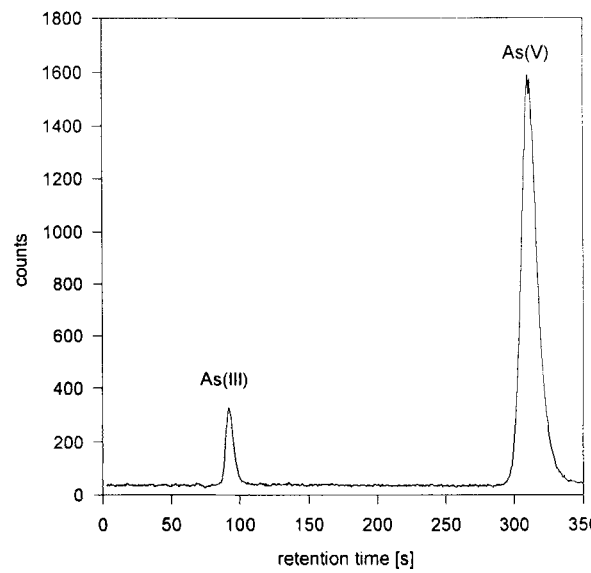

Figure 1 Separation of an aqueous extract (100 μl) of *Entoloma lividum* on a Hamilton PRP^{TM}-X100 anion-exchange column with a 30 mmol dm^{-3} phosphate buffer of pH 6 at a flow rate of 1.5 ml min^{-1}.

Table 2 Arsenic species in mushrooms from Switzerland (μg As g^{-1} dry weight)[a] determined by the ion exchange–INAA method

Sample	Total As	Inorg. As	MA	TMA ion	AB/TMAO	DMA
Sarcosphaera coronaria (Puidoux, Vaud)	HG AA: 360 RNAA: 332 ± 10 (3)	<1(5)	331 ± 18 (7)	<1.1 (3)	<2.5[b] (3)	
Sarcosphaera coronaria (St Luc, Valais)	HG AA: 2120 RNAA: —	1.6 ± 0.2 (3)	2090 ± 126 (5)	<1.5 (3)	6.2 ± 0.2[b] (4)	
Sarcodon imbricatum (Vevey market)	HG AA: 23 RNAA: 24.6	0.44 ± 0.25 (7)	3.03 ± 1.04 (7)	1.58 ± 0.23 (8)	10.4 ± 0.9 (6)	2.37 ± 0.60 (7)
Sarcodon imbricatum (Champex, Valais)	HG AA: 0.9 RNAA: 0.86	<0.02; <0.06	<0.07; <0.08	0.34; 0.23	0.27; 0.29	0.10; 0.15
Agaricus haemorrhoidarius (Grangettes, Villeneuve)	HG AA: 8.2 RNAA: 9.3	<0.25; <0.18	<0.27; <0.23	<0.19; <0.08	5.7; 6.8	0.94; 0.43
Agaricus placomyces (Grangettes, Villeneuve)	HG AA: 8.1 RNAA: 9.2	<0.22; <0.08	0.68; 0.30	<0.28; <0.31	6.5; 6.7	<0.40; <0.54
Entoloma lividum (Jura)	HG AA: 37 RNAA: 39.3 ± 3.5 (5)	41.2 ± 2.1 (4)	<1.6 (4)	<0.12; <0.08	<0.6 ± 0.2[b] (3)	

[a] Mean ± SD with number of replicates in parentheses. Where two results are shown they refer to two samples.
[b] Sum of AB/TMAO + DMA; fraction not subjected to anion-exchange separation of these species.

Table 3 Arsenic species in Slovenian mushrooms (μg As g^{-1} fresh weight)[a] determined by the ion exchange–INAA method

Sample	Total As (RNAA)	Inorg. As	MA	TMA ion	AB/TMAO	DMA
Sarcosphaera coronaria (Pokljuka)	15(2)	0.11; 0.11	12.6; 13.3	—	0.12; 0.15[c]	
Laccaria amethystina (Slivna)	3.4 ± 0.3 (3)	0.002; 0.001	0.007; 0.008	—	0.14; 0.06	2.4; 2.3
Laccaria amethystina[b] (Volčji potok)	40.5	<0.069; <0.039	<0.106; <0.078	—	<1.1; 0.26	34.2; 34.9
Lycoperdon perlatum (Mali Slatnik)	0.23	0.009; 0.011	0.005; 0.031	—	0.18: 0.19[c]	

[a] Mean ± SD with number of replicates in parentheses. Where two results are shown they refer to two samples.
[b] Dry weight basis; others fresh weight.
[c] Sum of AB/TMAO + DMA; fraction not subjected to anion-exchange separation of these species.

Table 4 Comparison of the results of the quantitation of arsenic compounds by the ion exchange–INAA and by HPLC–ICP MS (μg Ag g^{-1} dry weight)[a]

Sample	Method	As(III)	As(V)	MA	TMA ion	AB	DMA
	IC–INAA	<0.2 (2)[b]		<0.27; <0.23	<0.19; <0.08	5.7; 6.8	0.43; 0.94
Agaricus haemorrhoidarius	HPLC/ICP–MS	<0.8; <1.0	<0.8; <1.0	Trace n.q.[c]	n.d.[c]	7.0; 8.7	<0.8; <1.0
Agaricus placomyces	IC–INAA	<0.2 (2)[b]		0.30; 0.68	<0.28; <0.31	6.5; 6.7	<0.40; <0.54
	HPLC/ICP–MS	<0.8; <0.9	<0.8; <0.9	Trace n.q.	n.d.	7.9; 8.6	<0.8; <0.9
Sarcodon imbricatum (Vevey market)	IC–INAA	0.44 ± 0.25 (7)[b]		3.0 ± 1.0 (7)	1.6 ± 0.2 (8)	10.4 ± 0.9 (6)	2.4 ± 0.6 (7)
	HPLC/ICP–MS	<1.0; <1.0	1.4; 1.5	Trace n.q.	Trace n.q.	10.8; 12.5	1.2; 1.5
Entoloma lividum	IC–INAA	41.2 ± 2.1[b] (4)		<1.6 (4)	<0.12; <0.08	<0.6[d] (3)	
	HPLC/ICP–MS	2.3	31	<0.08			<0.08

[a] Mean ± SD with number of replicates in parentheses. Where two results are shown they refer to two samples.
[b] Total inorganic arsenic.
[c] Trace n.q., small peak in the chromatogram but not quantified (see Fig. 2); n.d., not detected.
[d] Sum of AB + DMA; fraction not subjected to anion-exchange separation of these species.

2.5.4 Electron impact mass spectrometry (EI MS)

The first fraction from the anion-exchange column containing AB (see Section 2.5.2 above) was rechromatographed on the cation and and anion columns. The final, purified and freeze-dried product contained about 5–10 μg arsenic.

EI mass spectra were acquired with a Finnegan MAT 8430/SS300 double focusing MS at 70 eV. The samples were inserted into the ion source with a solid probe, which was heated at a rate of 2 °C s^{-1} to 300 °C. The source temperature was 180 °C and the acquired mass range was from 20 to 800 Da.

3 RESULTS AND DISCUSSION

In mushroom samples collected from sites in Switzerland and Slovenia the total arsenic concentrations ranged from 0.9 to 2115 μg g^{-1} dry weight. The highest values were found in samples of *Sarcosphaera coronaria*. Of the mushrooms studied, *Sarcosphaera coronaria*, *Agaricus placomyces* and *Entoloma lividum* are inedible or toxic species.

For the identification of arsenic compounds, as described above the mushrooms were first extracted with a mixture of 90% methanol–10% water and the solvent was then evaporated. The

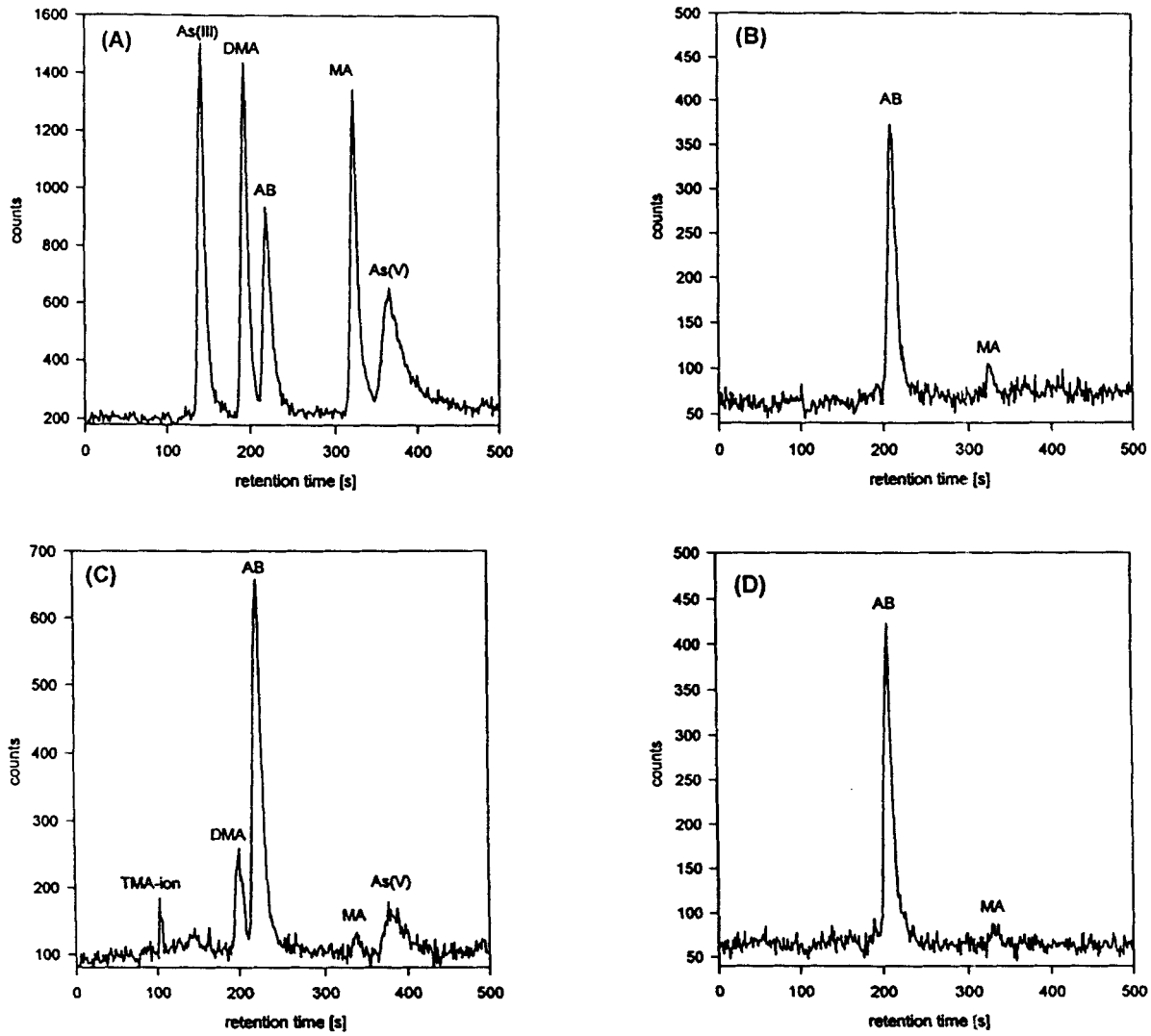

Figure 2 HPLC/MS determination of arsenic species in (A) a mixture of 10 ng each of As(III), As(V), MA, DMA and AB; (B) in the extract from *Agaricus placomyces*; (C) in the extract from *Sarcodon imbricatum*; and (D) in the extract from *Agaricus haemorhoidarius* on a Supelcosil LC-SAX anion-exchange column with 30 mmol dm^{-3} phosphate buffer of pH 3.75 as mobile phase at a flow rate of 1.5 ml min^{-1}.

residue was washed with diethyl ether (to separate any lipid-soluble arsenic compounds) and dissolved in water, in which it was completely soluble. From 80 to 100% of the total arsenic in the mushrooms was found in the final aqueous extracts (Table 1). Generally, only a small percentage of the arsenic was present in the ether wash. An interesting case is *Entoloma lividum*: with pure methanol almost no arsenic was extracted from the dried sample; a mixture of 90% methanol and 10% water extracted about 65% of the arsenic, whereas all the arsenic could be extracted with water alone.

Arsenic compounds in *Entoloma lividum* could not be determined by INAA in the fraction containing inorganic arsenic because of the high activity of ^{24}Na. Therefore total arsenic in this fraction was determined radiochemically.[16]

The efficiency with which various arsenic compounds are extracted from different matrices by methanol is an under-researched area. Here it is worth pointing out that because each of the mush-

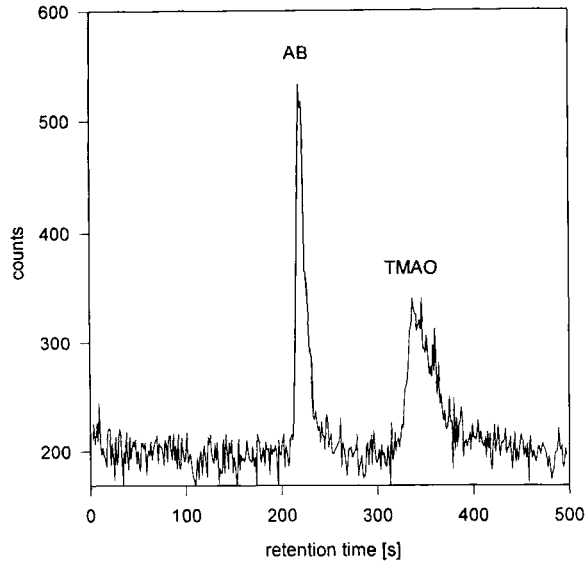

Figure 3 Separation of arsenobetaine and trimethylarsine oxide (10 ng As each in 100 µl of an aqueous solution) with conditions as in Fig. 2.

rooms in Table 1 (except *Sarcodon imbricatum*) contained, as will be shown below, only one major arsenic compound [MA, DMA, AB or As(III) and As(V)], the high recoveries of arsenic found in the extraction procedure, though expressed as total arsenic, meant that high or near-quantitative extraction of these arsenic compounds was assured in the procedure. The case of *Entoloma lividum* suggested that an appreciable amount of water should also be present in the methanol for effective extraction of As(III) and As(V).

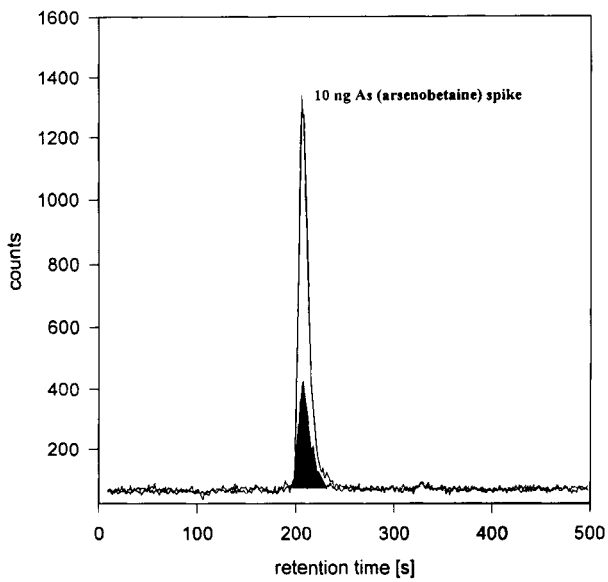

Figure 4 HPLC/MS determination of arsenobetaine in *Agaricus haemorrhoidarius* with and without a 10 ng standard addition (conditions as in Fig. 2).

Arsenic compounds were initially identified in these mushroom extracts by ion exchange combined with instrumental neutron activation analysis. The results are shown in Tables 2 and 3. In the three samples of *Sarcosphaera coronaria* MA is the major arsenic compound and in *Laccaria amethystina*, DMA. DMA as the major arsenic compound in *L. amethystina* was identified previously.[10] *Entoloma lividum* contained only inorganic arsenic, which was shown by HPLC/ICP MS to be a mixture of arsenite (8%) and arsenate (92%) (Table 4, Fig. 1). This sample had been air-dried at 50 °C and stored at room temperature; hence a conversion of arsenite to arsenate during drying and storage cannot be excluded.

Most interesting, however, was the presence of AB in *Sarcodon imbricatum*, *Agaricus placomyces* and *Agaricus haemorrhoidarius* as the major arsenic compound, and the presence of the TMA ion in *Sarcodon imbricatum*, as well as MA, DMA and arsenate (Table 2, Fig. 2). Since the ion exchange–neutron activation analysis technique could not separate AB from TMAO, the presence of AB was established by HPLC/ICP MS (Fig. 2).

The chromatogram of a mixture of standard arsenic compounds and the chromatograms of extracts from the three AB-containing mushroom species are shown in Fig. 2. A comparison of the results from the two laboratories using the two independent methods is given in Table 4, showing reasonable agreement.

The presence of TMAO at low concentrations in the mushroom samples containing AB and MA is unlikely but cannot be excluded. Although the cation/anion column–INAA system does not separate AB from TMAO, it does give unequivocal separation of MA (Tables 2, 3). The HPLC/ICP MS system (Supelcosil column) separates AB from TMAO (Fig. 3). Unfortunately, TMAO and MA have almost the same retention times under these conditions (Figs. 2, 3). However, based on the joint results of the two methods, TMAO can be present only—if at all—at concentrations smaller than the concentrations of MA found by HPLC.

The quantitation of arsenic compounds by ion-exchange separation combined with INAA and

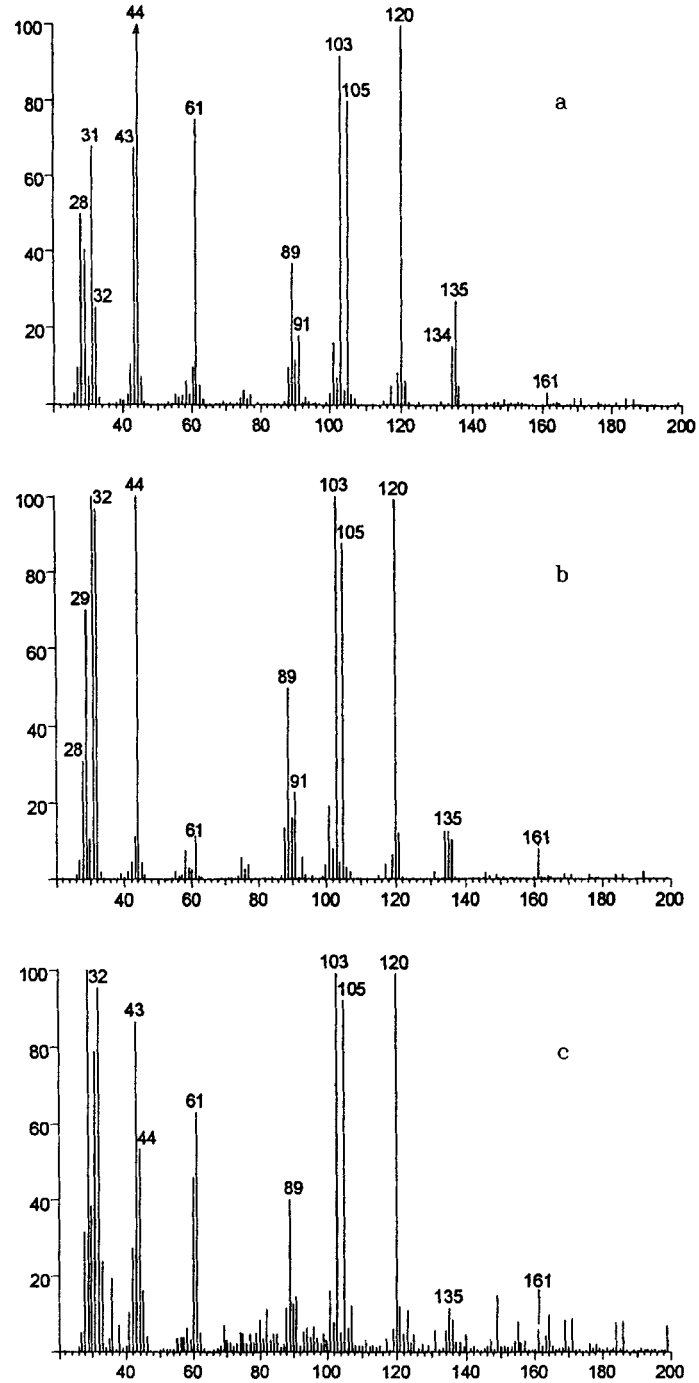

Figure 5 Electron impact mass spectra of (a) arsenobetaine standard; (b) and (c) purified arsenobetaine-containing fractions after ion-exchange separation from *Sarcodon imbricatum* and *Agaricus haemorrhoidarius*, respectively.

the determination of total arsenic were checked by the analysis of the certified reference material Dogfish Muscle DORM-1 from the National Research Council of Canada, which presently is the only reference material certified for arsenic species. Total arsenic was $17.2 \pm 1.1\ \mu g\ g^{-1}$ (certified $17.7 \pm 2.1\ \mu g\ g^{-1}$), AB $15.9 \pm 0.5\ \mu g\ g^{-1}$ (published 15.7[21] and $15.65\ \mu g\ g^{-1}$[22]), and DMA

$0.61 \pm 0.12\ \mu g\ g^{-1}$ (literature values $0.47\ \mu g\ g^{-1}$ and $0.60\ \mu g\ g^{-1}$). The HPLC results for AB were obtained using standard additions (Fig. 4).

Because the identification and quantitation of AB in these three mushrooms by chromatography should be verified by an independent method, the purified AB fraction, which did not contain any arsenocholine according to the chromatographic results, was analysed by EI MS. Figure 5(b) and (c) shows EI mass spectra of the AB fractions from samples of *Sarcodon imbricatum* and *Agaricus haemorrhoidarius*. In Fig. 5(a) the mass spectrum of a synthetic AB standard is shown. The major peaks correspond to the fragments $As(CH_3)_3^+$ ($m/z = 120$), $As(CH_3)_2^+$ ($m/z = 105$), and $AsCH_2^+$ ($m/z = 89$). The appearance of these EI spectra is rather similar to those reported for AB separated from fish by Beauchemin *et al.*[21] To the best of our knowledge, this work with mushrooms revealed for the first time the presence of AB in terrestrial organisms.

In general, the uptake of trace metals and metalloids by fungi is very complex, with many possible factors to consider.[23,24] However, a better insight into this phenomenon will only be obtained by studying more than just total concentrations in fruiting bodies. Data on trace element compounds and binding modes to biologically important molecules including metallothioneins and other proteins, such as phytochelatins, are required. Processes occurring in the mycelium and the soil must also be considered.

In further research arsenic compounds in soil solutions or soil extracts should be determined to establish whether soil fungi, bacteria or other microorganisms are capable of changing arsenic compounds in the soil. It is known that methylation of arsenic can be accomplished in soil by soil fungi under aerobic conditions; under anaerobic conditions organisms such as methanobacteria might be involved. The variety of arsenic compounds so far identified in different fungi make it likely that the conversion of inorganic to organic arsenic compounds occurs in the fungi and not in the soil. Such a conversion is more likely to occur in the mycelium than in the short-lived fruiting body; in the case of mycorrhizal fungi their occurrence in the host plant should also be studied.

In view of the global importnce of fungi as decomposers and in their mycorrhizal associations with plant roots, the study of arsenic compounds in fungi is of potential importance for an understanding of the cycling of arsenic in the environment. Challenter's[25] discovery of the mode of methylation of arsenic to trimethylarsine by moulds and soil fungi approximately 60 years ago initiated the study of the arsenic cycle in nature. Much has been learned since, but much more remains to be discovered through studies of terrestrial organisms.

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