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Studies of the uptake and binding of trace metals in fungi
Part II. Arsenic compounds in *Laccaria amethystina*

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Caps of the edible mushroom *Laccaria amethystina* collected during September and October at forested sites in the vicinity of the town of Domzale in Central Slovenia, Yugoslavia, were found by neutron activation analysis (NAA) and hydride generation to have total arsenic concentrations between 109 and 200 mg As kg^{-1} (dry mass). The extraction of fresh, frozen or freeze-dried caps with cold Tris buffer at pH 7.6, or with boiling water, transferred 60–70% of the arsenic into the aqueous phase. Sephadex gel permeation chromatography indicated that the arsenic compounds in these extracts were not associated with proteins or other organic compounds of molecular mass larger than 4 000 Dal.

Cation-exchange chromatography coupled with NAA, hydride generation, and reverse-phase chromatography with arsenic-specific detection (HPLC ICP) showed that dimethylarsinic acid is the major arsenic compound in the extracts. Methylarsonic acid and arsenate account for no more than 10% each of the total arsenic.

Keywords: Mushroom, *Laccaria amethystina*, total arsenic, dimethylarsinic acid

INTRODUCTION

During the past two decades the number of papers dealing with the uptake of trace elements in general and of heavy metals in particular by fungi grew considerably.[1] However, the propensity of mushrooms to accumulate heavy metals was hardly used at all in quantitative assessments of environmental metal burdens.[2,3] Little is known about the nature of the interactions between trace elements and fungal molecules of biological importance. Copper-containing metallothioneins were isolated from cultured mycellia of the molds *Neurospora crassa*[4,5] and *Agaricus bisporus*.[6] The amino-acid sequences in these metallothioneins were very similar to the sequences in mammalian metallothioneins.

In 1983 arsenic concentrations of the order of magnitude of 100 mg kg^{-1} dry mass were found in the fruiting body of the mushroom *Laccaria amethystina*, an edible violet-colored mushroom common in European deciduous forests in autumn.[7] In contrast, the mean arsenic concentration in 27 other species of basidomycetes has been reported to be just over 1 mg kg^{-1}.[8] Preliminary experiments indicated that the arsenic compounds in the mushroom are stable, involatile, easily extracted with water, and not bound to proteins.[7]

This paper describes the results of experiments carried out to identify the arsenic compounds in *Laccaria amethystina*.

EXPERIMENTAL

Materials and instrumentation

All reagents and solvents used were at least reagent grade. Sodium arsenite ($NaAsO_2$), sodium arsenate ($Na_2HAsO_4.7H_2O$), and dimethylarsinic acid [$(CH_3)_2AsOOH$] were purchased from Aldrich Chemical Co. Methylarsonic acid [$CH_3AsO(OH)_2$] was a gift from Vineland Chemical Co., Vineland, New Jersey, USA. Irradiation of arsenic-containing samples with

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neutrons was carried out at the Triga Mark II reactor at the Reactor Centre of the 'J. Stefan' Institute, Ljubljana. The hydride generation system with a dc-helium plasma detector was described earlier.[9] A Waters Associates liquid chromatograph was coupled to an ARL 34 000 inductively coupled argon plasma emission spectrometer (ICP-ES).[10]

Collection and preparation of *Laccaria amethystina*

Fungi were collected during the months of September and October at forested sites near the town of Domžale in Central Slovenia (northwest Yugoslavia). Traces of soil and foreign matter were removed from the collected specimens. The caps of the mushrooms were separated from the stems. The stems were discarded, because their arsenic concentrations had been found to be low and their woody structure made it difficult to homogenize them in a blender. The caps were stored in a freezer at −25°C. Several caps were freeze-dried and then powdered.

Extraction of arsenic compounds from mushroom caps

Frozen caps were thawed and then cut into small pieces. The pieces (typically 20 g) were placed in a Teflon-lined Sorvall blender. Ice-cold aqueous buffer at pH 7.6 (2.0 cm^3 per g of caps) prepared by dissolving 1.58 g (10 mmol) Tris–HCl [$(HOCH_2)_3CNH_2.HCl$] and 19.1 mg (0.1 mmol) phenylmethanesulphonyl chloride (a protease inhibitor) in 1 dm^3 of distilled water was added. The mixture was homogenized at the highest blender setting (1000 rpm) for 15 min. The homogenate was filtered through a 250-μm nylon mesh. The filtrate, kept at 4°C, was then immediately centrifuged (Sorvall Superspeed RC-2-13 centrifuge) at 10 000 rpm for 45 min. The supernatant was separated from the solid residue (sediment). Portions of this sediment were also freeze-dried and analyzed for total arsenic.

Gel chromatography of the supernatant

An aliquot (2.5 cm^3) of the supernatant was loaded onto a Sephadex G-75 column (60 cm × 1.6 cm). Tris–HCl buffer at pH 7.6 (10 mmol dm^{-3}) was used as the mobile phase at a flow rate of 13.8 $cm^3\,h^{-1}$. Fractions of 5.0 cm^3 were collected. The absorbance of each fraction was determined at 254 and 280 nm with a Perkin–Elmer Lambda-3 instrument. Arsenic was determined in each fraction by radiochemical neutron activation analysis. The column was calibrated with blue dextran (2 000 000 Da), bovine serum albumin (67 000 Da), chymotrypsinogen A (25 000 Da), and cytochrome *c* (12 300 Da). Only the fractions from 110 cm^3 to 145 cm^3 contained arsenic. For subsequent cation-exchange chromatography the arsenic-containing fractions were combined and concentrated by freeze-drying. An aliquot of the concentrate (1.0 cm^3) was treated with 5 mol dm^{-3} hydrochloric acid until the aliquot was 0.5 mol dm^{-3} with respect to hydrochloric acid. This solution was placed onto the cation-exchange column.

Cation-exchange chromatography of the supernatant and the pooled, arsenic-containing fractions from the gel chromatography

A Dowex 50X8 (100–200 mesh, H^+ form) column (24 cm × 0.8 cm) was prepared by slurrying the resin with water, decanting the water containing the fines, and filling the remaining slurry into a glass tube with a frit and a stopcock at one end. Aqueous 0.5 mol dm^{-3} HCl, water, 1.5 mol dm^{-3} aqueous ammonia, water and 0.5 mol dm^{-3} aqueous HCl, were passed through the column in the sequence given. The natural flow rate of the column was between 18 and 24 $cm^3\,h^{-1}$. An aqueous solution of dimethylarsinic acid (1.0 cm^3) containing 10 mg of the compound per cm^3 was sealed into a polythene vial. The sealed tube was placed in the pneumatic transfer system and exposed to neutrons (4×10^{12} n $cm^{-2}\,s^{-1}$) for 20 min. About 0.1 cm^3 of the irradiated solution now containing arsenite, methylarsonic acid and dimethylarsinic acid produced by the irradiation, was diluted with 10 cm^3 0.5 HCl, and 0.5 cm^3 of this, corresponding to a total amount of approximately 50 μg arsenic, was placed onto the column. To elute the compounds, 0.5 mol dm^{-3} aqueous HCl (15 cm^3), water (10 cm^3), 0.75 mol dm^{-3} ammonia (15 cm^3), and 3.0 mol dm^{-3} ammonia (20 cm^3) were passed through the column in sequence.[11] Fractions of appropriate volume (1.0–5.0 cm^3) were collected. The ^{76}As-activity of each fraction was determined with a well-type NaI–Tl-activated scintillation detector connected to a multichannel analyzer.

An aliquot (1.0 cm^3) of the supernatant or of the concentrated arsenic-containing fractions

from the gel chromatography was placed onto the Dowex column. The column was treated with the same mobile phases in the same sequence as described for the standard solution of the arsenic compounds. Based on the retention volumes determined for the arsenic compounds in the standard solution, the following fractions were collected: 4–13 cm^3 (arsenite), 14–25 cm^3 (methylarsonic acid), 26–45 cm^3 and 46–60 cm^3 (dimethylarsinic acid). Arsenic was determined in these fractions by radiochemical neutron activation analysis.

Determination of arsenic by neutron activation analysis

Samples of the mushroom caps (0.1–0.5 g), of the solid residue (0.1–0.5 g) from the centrifugation of the extracts from the caps, and standards were sealed into polythene vials and irradiated for 0.5 h at a flux of 4×10^{12} n cm^{-2} s^{-1}. Arsenic was determined by gamma-ray spectroscopy using the 559-keV ^{76}As line with an HP Ge-detector/multichannel analyzer system.

Aliquots (1.0 cm^3) of the supernatant and of the fractions from the gel or ion chromatography were sealed into polythene vials and irradiated for 12 h at a flux of 2×10^{12} n cm^{-2} s^{-1}. The samples were then wet-ashed in the presence of an arsenic carrier. The arsenic was then converted to arsenic tri-iodide, separated from the matrix by extraction into toluene, and determined by gamma-ray spectroscopy.[12]

Identification of arsenic compounds by HPLC ICP

Dried powder (1.000 g) from mushroom caps was boiled with distilled water (50 cm^3) for 2 h. After most of the water had evaporated, the residue was treated with distilled water (20 cm^3). The mixture was briefly boiled and then filtered hot into a 100-cm^3 volumetric flask. The filter cake weighed 735 mg after drying. The filtrate was diluted to 100 cm^3 with distilled water. An aliquot of this solution was placed on a Hamilton PRP-1 reverse-phase column that had been conditioned by passage of the initial mobile phase for 30 min. Solutions of hexadecyltrimethylammonium bromide in water (0.002 mol dm^{-3}, for the first 6 min, 0.002 mol dm^{-3} with 2.5 cm^3 glacial acetic acid per 100 cm^3 solution after the sixth minute) were used as the mobile phases at a flow rate of 90 cm^3 h^{-1}. The column effluent was routed to the nebulizer of an ARL 34 000 inductively coupled argon plasma emission spectrometer that served as the arsenic-specific detector by monitoring the 189.0-nm arsenic line.[10] The integration time was set at 3 s. For the identification of the peaks in the chromatogram, another aliquot was spiked with arsenite, arsenate, methylarsonic acid and dimethylarsinic acid. The spiked aliquot was chromatographed under the same conditions.

Determination of total arsenic and identification of arsenic compounds by hydride generation

The samples (mushroom caps, freeze-dried extracts) were boiled for 1 h with a mixture of concentrated sulphuric (15 cm^3, 18 mol dm^{-3}) and nitric (30 cm^3, 15 mol dm^{-3}) acids in a 250-cm^3 beaker covered with a watch glass. To avoid overheating a few glass beads were added to the cold mixture before the beaker was placed on the hot plate. When white fumes of sulphur trioxide (SO_3) appeared, the solution was cooled and then transferred into a 50-cm^3 volumetric flask. The beaker was rinsed several times with a few cubic centimeters of distilled water. The washings were poured into the volumetric flask. The flask was shaken to homogenize the solution and then filled to the mark with distilled water. Aliquots (0.1–1.0 cm^3) were pipetted into the reduction vessel of the hydride generation system. Arsenate was reduced at pH 1 with sodium borohydride and the generated arsine passed into the dc-helium plasma.[9]

For the identification of arsenic compounds the freeze-dried extracts were dissolved in water to produce 25 cm^3 of solution. A sample of the powdered caps (1.000 g) was boiled with distilled water (50 cm^3) for 50 min. The mixture was filtered, and the filtrate diluted to 100 cm^3 with distilled water. Aliquots of this solution (0.1–1.0 cm^3) were reduced with sodium borohydride in an acetate-buffered system to check for the presence of arsenite, and in an oxalic acid-buffered system to determine total inorganic arsenic and methylated arsenic compounds.[9]

RESULTS AND DISCUSSION

Several batches of *Laccaria amethystina* were collected at several sites at different times. Some caps were stored frozen and others were freeze-

dried and powdered. The fresh mushrooms consisted of 80–90% water. The total arsenic concentrations were determined in these samples by instrumental neutron activation analysis. In addition, aliquots of the powders were mineralized by boiling them with a mixture of concentrated sulphuric and nitric acids. Total arsenic was then determined in the digests by the hydride generation technique.[9] The arsenic concentrations on a dry-mass basis ranged from 109 to 200 mg kg^{-1} as reported previously.[7] The ability of this mushroom to accumulate arsenic is very likely the reason for these high arsenic concentrations. No evidence exists for a contamination of the soil with arsenic compounds. These high arsenic concentrations might be deleterious to persons consuming these mushrooms, should the arsenic be present as arsenite. These high arsenic concentrations were also found in solutions obtained by boiling powdered caps with concentrated nitric acid and analyzing these solutions with a sequential inductively coupled argon plasma atomic emission spectrometer. A semi-quantitative search for other elements using the SAMI scan feature of the instrument revealed the presence of traces of boron, barium, chromium, copper, iron, manganese, silicon and strontium, and of higher concentrations of aluminum, calcium, potassium, magnesium, sodium, zinc, phosphorus and sulphur.

For the identification of the arsenic compounds, fresh or frozen caps were extracted with cold Tris buffer in a blender; powdered caps were extracted with boiling water in a beaker and also with boiling concentrated nitric acid. The determinaton of total arsenic in these extracts by neutron activation analysis and by hydride generation revealed that 60–70% of the arsenic in the caps had been extracted (from the NAA data). The arsenic compounds in these extracts were identified by cation-exchange chromatography, by reverse-phase liquid chromatography, and by hydride generation.

To check on the association of the arsenic compounds with proteins and other high-molecular-mass molecules, samples of fresh or frozen mushroom caps were mixed with Tris–HCl buffer at pH 7.6 containing a protease inhibitor, and homogenized in a blender. The filtered and centrifuged homogenates were chromatographed on a Sephadex G-75 column with Tris buffer as the mobile phase. Fractions of 5.0 cm^3 were collected. The arsenic concentrations in these fractions were determined by radiochemical neutron activation analysis. In addition, the absorbances of the fractions were measured at 280 and 254 nm. A representative chromatogram (Fig. 1) consists of four peaks generated by the spectrophotometric detector. Only the peak with the retention volume of 127 cm^3 coincides with the peak generated by arsenic-specific detection. All of the arsenic in the aliquot of the extract placed on the gel chromatography column was recovered in the fractions between 110 and 145 cm^3. This peak corresponds to compounds of molecular mass lower than 4000 Da, typically consisting of cytosol amino-acids, partially degraded proteins, and dipeptides. The arsenic compounds could be present in these fractions unassociated or associated with low-molecular-mass organic compounds. However, the arsenic compounds are not associated with proteins.

A Dowex 50X8 cation-exchange column in the H^+ form was calibrated for the elution of inorganic arsenic, methylarsonic acid and dimethylarsinic acid. Arsenite and arsenate have the same retention times under these conditions. The arsenic compounds were eluted with the mobile phases (Fig. 2) used by Tam and co-workers.[11] Normally, the calibration is performed by chromatography of known arsenic compounds, collecting suitable fractions, and analyzing these fractions by appropriate techniques. Such a procedure is tedious and time-consuming. Alternatively, the arsenic compounds could be labelled with radioactive $^{73/74}As$ or ^{76}As. Such radiolabelled compounds are expensive or must be prepared from radiolabelled arsenate.[13] A much less expensive and simpler radiolabelling technique was used to produce the required radiolabelled arsenic compounds. Irradiation of aqueous solutions of dimethylarsinic acid or methylarsonic acid with neutrons produced ^{76}As by the (n, γ) reaction. During this process most of the As–C bonds were broken (Szillard–Chalmers effect) as expected. However, sufficient organic arsenic compounds survived the irradiation, or As–C bonds were reformed, to produce concentrations of ^{76}As-labelled dimethylarsinic acid and methylarsonic acid usable for calibration purposes. Irradiated solutions of dimethylarsinic acid were, therefore, used for the calibration. Arsenic was determined in the fractions by gamma-ray spectroscopy. The chromatogram is displayed in Fig. 2. The fractions from 25 cm^3 to 40 cm^3 should contain arsenobetaine according to the results from the literature.[14, 15]

The supernatant from the centrifuged extract

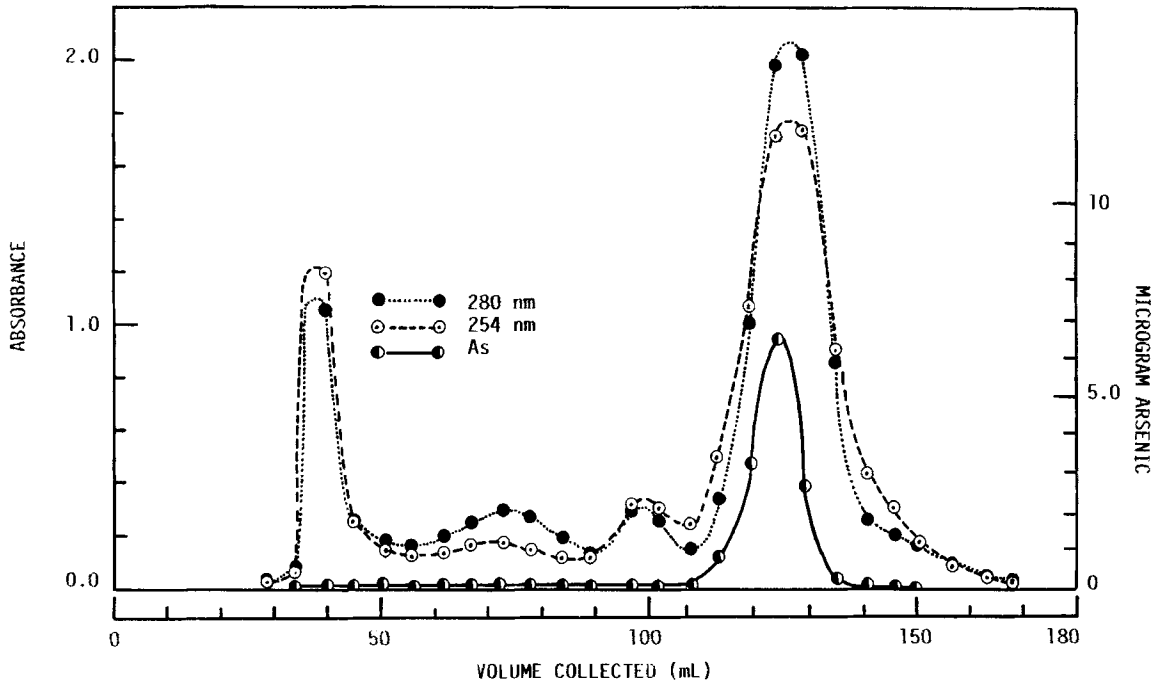

Figure 1 Gel permeation chromatogram of the supernatant from the centrifugation of a sample of mushroom caps homogenized in Tris–HCl buffer at pH 7.6 (Sephadex G-75; mobile phase Tris–HCl, 10 mmol dm^{-3}, pH 7.6; flow rate 18.3 $cm^3 h^{-1}$; 5-cm fractions collected).

obtained from the mushroom caps was directly subjected to cation-exchange chromatography. Additionally, the arsenic-containing fractions from the gel chromatography were pooled and concentrated by freeze-drying and chromatographed on the calibrated cation-exchange column. This concentration step was necessary to ensure that the column, which can take only a small volume of sample, is loaded with the quantities of arsenic compounds required for their detection. The four fractions defined in Fig. 2 were collected. Arsenic was determined in these fractions by radiochemical NAA. In all experiments the fraction corresponding to dimethylarsinic acid contained almost all of the arsenic applied to the column (Table 1).

The hydride-generation technique provides a more direct identification of arsenic compounds, because only a rather limited number of reducible and sufficiently volatile compounds can be detected. When small volumes of the extracts were analyzed, the only peak present in the chromatograms indicated the presence of dimethylated arsenic. When larger aliquots were reduced, arsenate was also detected. A representative analysis of an aqueous extract from powdered mushroom caps detected 160 mg As kg^{-1} in the form of dimethylated arsenic and 1.5 mg As kg^{-1} in the form of arsenate. Small amounts of monomethylated arsenic were undetectable under the experimental conditions. The small monomethyl signal would disappear under the large peak from dimethylated arsenic. A similar analysis of an extract obtained by boiling powdered caps with concentrated nitric acid gave evidence for dimethylated arsenic only. Arsenate was present only in traces. This result shows the high stability of dimethylarsinic acid even under strongly oxidizing conditions.

An extract from mushroom caps obtained by boiling the powder with water was analyzed by HPLC with an ICP–OES as the arsenic-specific detector.[10] The good separation of arsenic compounds achievable with this system makes it possible to determine traces of an arsenic compound in the presence of large concentrations of other arsenic compounds. The chromatogram shows the presence of dimethylarsinic acid and small concentrations of methylarsonic acid and arsenate. Approximately 80% of the arsenic is present as dimethylarsinic acid and 10% each in the form of methylarsonic acid and arsenate. The identity of the compounds was confirmed by spiking the extracts with synthetic arsenic compounds and

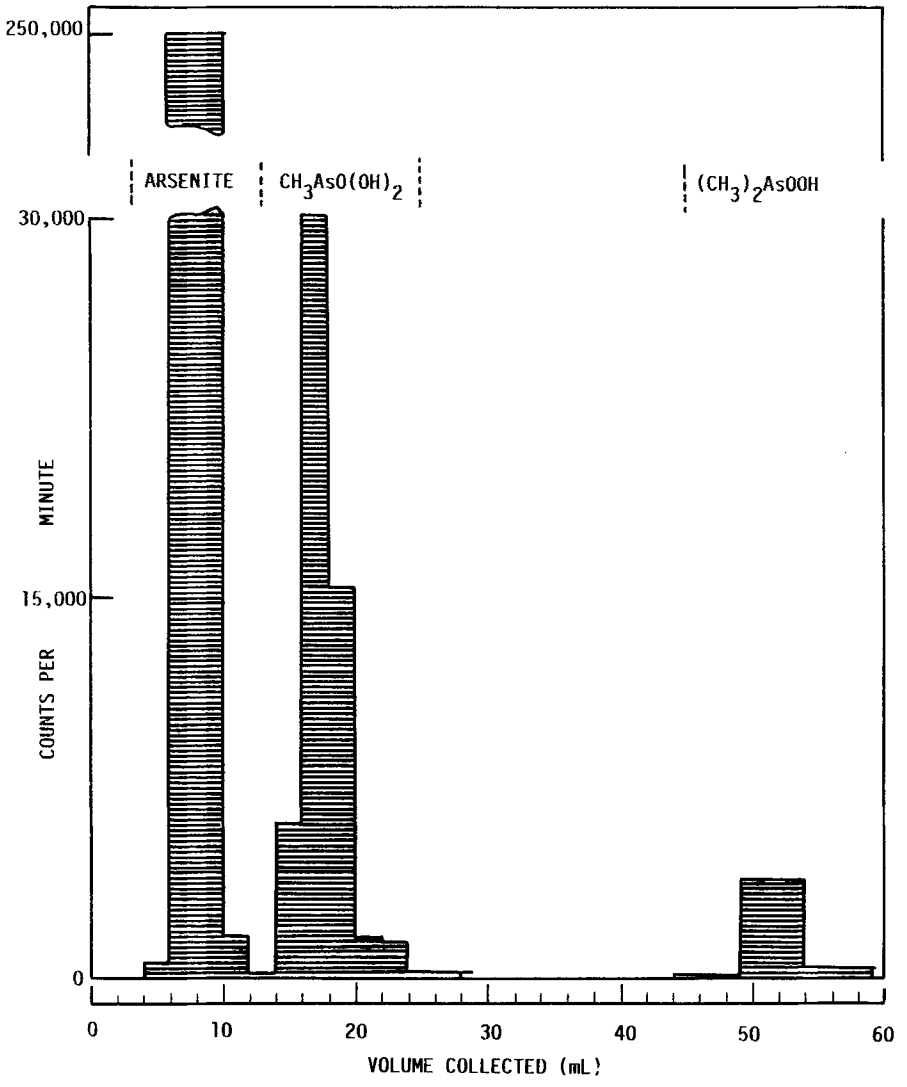

Figure 2 Cation-exchange chromatogram of a neutron-irradiated aqueous solution of synthetic dimethylarsinic acid containing, after irradiation, arsenite, methylarsonic acid, and dimethylarsinic acid (Dowex 50X8, H^+-form; mobile phases 0.5 mol dm^{-3} HCl (15 cm^3), water (10 cm^3), 0.75 mol dm^{-3} NH_3 (15 cm^3), 3.0 mol dm^{-3} NH_3 (20 cm^3), in sequence; flow rate 18–24 cm^3 h^{-1}.

chromatographing the spiked aliquot. Arsenite was not detected in the samples (Fig. 3).

The multi-technique approach to the identification of arsenic compounds in caps of the mushroom *Laccaria amethystina* clearly proved that dimethylarsinic acid is the major arsenic compound. Methylarsonic acid and arsenate are present only in traces. Arsenite was not detected in any of the samples. Because no particular precautions were observed to prevent the oxidation of any arsenite in the samples to arsenate, a small concentration of arsenite could have been present. A planned investigation of soils in which the mushrooms grow and the porewater in the soil together with mushrooms could not yet be carried out, because the dry weather conditions during the past two years prevented the growth of the mushrooms. Such investigations could provide information about the site of biomethylation of inorganic arsenic, which could be accomplished either by soil bacteria or by the mushroom in its fungal tissue or mycellium. *Laccaria amethystina* is a mycorrhizal fungus generally living in symbiosis with oak trees as the hosts. Therefore, tree tissues could also take part in the biotransformation of arsenic compounds.

Table 1 Arsenic mass balance (μg As) for the fractions from the cation-exchange chromatography of the supernatant obtained by homogenizing caps of *Laccaria amethystina* in Tris–HCl buffer followed by centrifugation and for the arsenic-containing fraction obtained by gel chromatography of the supernatant on Sephadex G-75

	Arsenic (μg)				
Sample	Total on column	Inorganic	CH_3As	$(CH_3)_2As$	$(CH_3)_3As$
Supernatant	12.3	0.045	0.014	14.0	0.020
Sephadex As Peak	2.1	0.014	0.005	2.1	0.002
Blanks[a]	—	$\leqslant$0.006	$\leqslant$0.003	$\leqslant$0.005	$\leqslant$0.005

[a] Blanks of elution reagents.

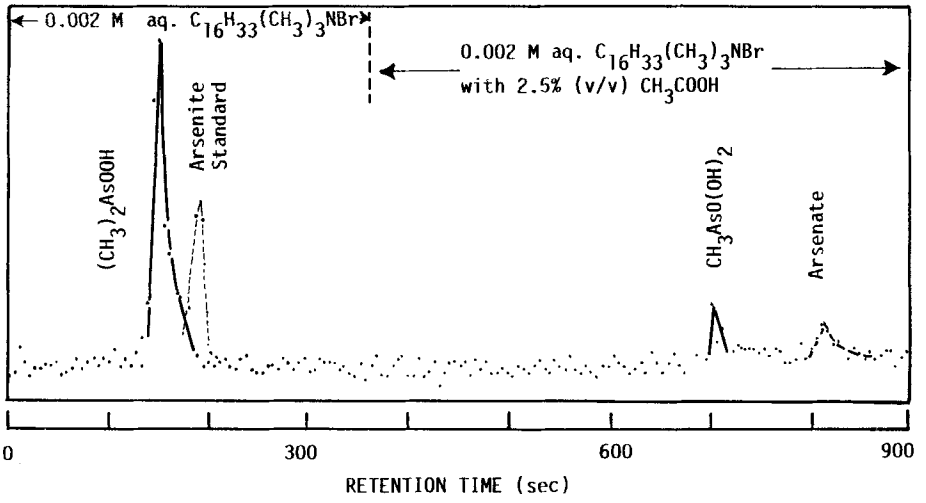

Figure 3 HPLC ICP chromatogram of the extract obtained by boiling powdered mushroom caps with water (Hamilton RPR-1 reverse-phase column; flow rate 90 $cm^3 h^{-1}$; injection volume 1.0 cm^3; integration time 3 s; detector ICP 189.0 nm.

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