APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 11, 459–470 (1997)

Arsenic Compounds in Terrestrial Organisms II: Arsenocholine in the Mushroom *Amanita muscaria*

Doris Kuehnelt, Walter Goessler and Kurt J. Irgolic*
Institute for Analytical Chemistry, Karl-Franzens-Universität Graz, Universitätsplatz 1, 8010 Graz, Austria

Arsenic compounds were identified and quantified in the mushroom *Amanita muscaria*, collected close to a facility that had roasted arsenic ores. The powdered dried mushrooms were extracted with methanol/water (9:1), the extracts were concentrated and the concentrates were dissolved in water. The resulting solutions were chromatographed on anion-exchange, cation-exchange and reversed-phase columns. Arsenic was detected on-line with an ICP–MS detector equipped with a hydraulic high-pressure nebulizer. Arsenite, arsenate, dimethylarsinic acid and the tetramethylarsonium cation were minor arsenic compounds (~2% each of the total 22 mg kg^{-1} dry mass), and arsenobetaine, arsenocholine (~15% each) and several unidentified arsenic compounds (~60%) were the major arsenic compounds in *Amanita muscaria*. The presence of arsenocholine (detected for the first time in a terrestrial sample) was ascertained by matching retention times in the anion-exchange, cation-exchange and reversed-phase chromatograms with the retention time of synthetic arsenocholine bromide and chromatographing extracts spiked with arsenocholine bromide.

Appl. Organometal. Chem. **11**, 459–470 (1997)
No. of Figures: 4 No. of Tables: 2 No. of Refs: 31

Keywords: arsenocholine; mushroom; arsenobetaine; arsenic compounds; hydraulic high-pressure nebulizer; ICP–MS

Received 18 April 1996; accepted 8 July 1996

* Correspondence to: Kurt J. Irgolic.

INTRODUCTION

The transformation of arsenic compounds in the marine environment has been investigated in detail,[1] whereas comparatively little is known about the fate of arsenic in the terrestrial environment.[2] Arsenobetaine was found to be the main arsenic compound in a range of marine animals such as fish, crustaceans, molluscs and echinoderms.[1,2] Arsenocholine, the rather elusive member of the marine arsenic cycle and a constituent of arsenic-containing phospholipids,[3] was claimed to be present in shrimp,[4–10] in lobsters,[10] in mussels,[8,11] in the leatherback turtle[12] and in fish species.[8,10,11,13,14] Extracts prepared from fish, lobster and shrimp were investigated by a combination of fast-atom-bombardment mass spectrometry and tandem mass spectrometry.[7] Pyrolysis and gas chromatography/mass spectrometry were used to determine arsenocholine in fish.[10,13] Extracts of marine organisms were chromatographed on cation-exchange columns,[4,5,8,9] anion-exchange columns,[8,11] C_{18} reversed-phase columns[6,12,14] or gel permeation columns[12] for the determination of arsenocholine. As arsenic-specific detectors, flame[4] or graphite furnace[4,6] atomic absorption spectrometers or ICP–MS[8,9,11,12,14] were used. The arsenocholine reported to be present in the dogfish reference material DORM-1 (National Research Council of Canada)[14] was suggested to be the tetramethylarsonium ion.[15] However, recent investigations[8] suggested that both arsenocholine and the tetramethylarsonium ion are present in DORM-1.

Because arsenobetaine, the renowned marine arsenic compound, was recently detected in mushrooms for the first time in the terrestrial environment,[16,17] arsenocholine could be present as well. Simple methylated arsenic compounds are known to be formed by fungi,[2] bacteria,[2] mushrooms,[18] green plants,[19] animals[20] and

 CCC 0268–2605/97/060459–12 $17.50

humans[21] from inorganic arsenic. These methylated arsenic compounds could serve as starting materials for the biosynthesis of arsenocholine. Although arsenic concentrations in terrestrial organisms are generally lower than in the marine environment,[2] ICP–MS equipped with a hydraulic high-pressure nebulizer has the required low detection limits for the identification and quantification of traces of arsenocholine and other arsenic compounds in terrestrial biota. Because preliminary experiments had shown that extracts prepared from *Amanita muscaria* grown on soil with high arsenic concentrations contained a large number of arsenic compounds, one of which was very likely to be arsenocholine,[17] further chromatographic separations were carried out to identify as many arsenic compounds as possible and to verify the presence of arsenocholine.

MATERIALS AND METHODS

Instrumentation

The mushrooms and the humic soil were freeze-dried in an Alpha 1-4 freeze-drying system (Christ, Osterode am Harz, Germany). The freeze-dried samples were pulverized in a Retsch ZM 1000 mill (Retsch, Haan, Germany) equipped with a titanium rotor and a 0.25-mm (mushroom) or 4-mm (soil) sieve. Digestions for total arsenic determinations were performed in an MLS-1200 Mega microwave oven (MLS, Leutkirch, Germany). Total arsenic was determined with a VG Plasma Quad 2 Turbo Plus inductively coupled argon-plasma mass spectrometer (ICP–MS, VG Elemental, Winsford, UK) equipped with a Meinhard concentric glass nebulizer, type TR-30-A3.

The high-performance liquid chromatography 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) with a 100-mm^3 injection loop. The separations were performed on a Hamilton (Reno, USA) PRP-1 reversed-phase column (25 cm × 4.1 mm i.d., 10-μm styrene–divinylbenzene copolymer particles), 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), and a Supelcosil LC-SCX cation-exchange column (25 cm × 4.6 mm i.d., 5-μm silica-based particles with propylsulfonic acid exchange sites). The Supelcosil LC-SCX column was operated at 60 °C, the other columns at room temperature.

The outlet of the HPLC column was connected via a 60-cm, $\frac{1}{16}$ in (1.6 mm) PEEK (polyether-ether-ketone) capillary tubing (0.25 mm i.d.) to a hydraulic high-pressure nebulizer (HHPN) (Knauer, Berlin, Germany). The VG Plasma Quad 2 Turbo Plus ICP–MS served as the arsenic-specific detector. The ion intensity at *m/z* 75 (^{75}As) was monitored using the 'time-resolved' analysis software© Version 1a (Fisons Scientific Equipment Division, Middlesex, UK). Additionally, the ion intensities at *m/z* 77 ($^{40}Ar^{37}Cl$, ^{77}Se) and *m/z* 82 (^{82}Se) were monitored to detect possible argon chloride ($^{40}Ar^{35}Cl$) interferences on *m/z* 75. Prior to each HPLC–ICP–MS run the ion intensity at *m/z* 87 (Rb added to the mobile phases) was optimized at the rate meter of the instrument. Instrumental settings used throughout this work are summarized in Table 1. The chromatograms were exported and the peak areas were determined using software written in-house. The arsenic compounds were quantified with external calibration curves established with each of the eight compounds. Within experimental error the signals from the ICP–MS are independent of the nature of the arsenic compound (same peak area for same amount of arsenic).[22]

Reagents, standards and mobile phases

All solutions were prepared with NANOpure (18.2 MΩ cm) water. Concentrated nitric acid (Merck p.a.) was further purified in a quartz sub-boiling distillation unit. The preparation of standard solutions and the mobile phases for the anion-exchange and reversed-phase separations was described previously.[17] The mobile phase for the cation-exchange HPLC was prepared by dissolving 1.58 g pyridine to 1000 cm^3 and adjusting the pH of this solution to 3.0 by addition of formic acid (~98%, Fluka puriss. p.a.). Rubidium (as RbCl in water) was added to all mobile phases to achieve a concentration of 50 ng Rb cm^{-3}.

Calibration curves for the HPLC–ICP–MS measurements were obtained by chromatographing aliquots (100 mm^3), of solutions containing 5.00, 50.0 or 100 ng As cm^{-3} of arsenite, arse-

nate, methylarsonic acid, dimethylarsinic acid and arsenobetaine on the Supelcosil LC-SAX anion-exchange column; of arsenocholine, tetramethylarsonium iodide and trimethylarsine oxide on the PRP-1 reversed-phase column; and of arsenobetaine, arsenocholine, tetramethylarsonium iodide and trimethylarsine oxide on the Supelcosil LC–SCX cation-exchange column.

Collection of *Amanita muscaria*

The mushroom *Amanita muscaria* was collected at an old arsenic smelter site in Austria, at Poellatal in Carinthia. The collected specimens (ten mushrooms) were mechanically cleaned of soil, rinsed with tap-water and frozen at -20 °C for storage. Before analysis the mushrooms (stems and caps) were freeze-dried for 24 h at -10 °C and for 24 h at 10 °C at 0.1 mbar. The freeze-dried mushrooms were mixed, then pulverized in the mill (0.25-mm sieve), and the composite powder (about 15 g) was stored over silica gel in a desiccator. Additionally, humic soil collected at the smelter site was frozen for storage at -20 °C, freeze-dried in the same manner as the mushrooms, and pulverized in the mill (4-mm sieve). The powder (about 30 g) was stored over silica gel in a desiccator.

Determination of total arsenic in *Amanita muscaria* and humic soil

Aliquots of the freeze-dried mushroom powder (~0.2 g each) and of the humic soil powder (~0.25 g each) were weighed to 0.1 mg into Teflon digestion vessels. Concentrated nitric acid (3.0 cm^3) and 30% hydrogen peroxide (0.20 cm^3, Merck p.a.) were added to each vessel containing the mushroom powder, and concentrated nitric acid (3.0 cm^3), 30% hydrogen peroxide (1.0 cm^3) and 40% hydrofluoric acid (0.50 cm^3, Merck p.a.) were added to each vessel containing the humic soil powder. The vessels were closed, secured in the rotor and placed in the microwave oven. Mushroom samples were digested using the following digestion program: 2 min at

Table 1. Operating conditions for the HPLC–HHPN–ICP–MS system

Hydraulic high-pressure nebulizer (HHPN)	
Desolvation	
Heating	150 °C
Cooling	1.5 °C
Nebulizer gas (argon)	1.00 $dm^3\ min^{-1}$
Back-pressure	~200 bar
Inductively coupled plasma mass spectrometer (ICP–MS)	
Plasma	
Radio-frequency power	
Forward	1.4 kW
Reflected	<1 W
Argon gas flows	
Cooling gas	13.5 $dm^3\ min^{-1}$
Auxiliary gas	1.1 $dm^3\ min^{-1}$
Vacuum	
Expansion	1.6 mbar
Intermediate	$<1.0\times10^{-4}$ mbar
Analyzer	5.4×10^{-6} mbar
Ion Sampling	
Sample cone	Nickel orifice, 1.00 mm diameter
Skimmer cone	Nickel orifice, 0.75 mm diameter
Measuring parameters	
Monitored signal	^{75}As,$^{40}Ar\ ^{37}Cl$ or ^{77}Se, ^{82}Se
Time/slice	0.51 s
Total analysis time	Column-dependent (6–12 min)

250 W, 30 s at 0 W, 5 min at 300 W, 30 s at 0 W, 10 min at 400 W, 30 s at 0 W, 5 min at 500 W, 4 min at 600 W. Humic soil samples were digested using the following digestion program: 5 min at 300 W, 1 min at 0 W, 5 min at 400 W, 2 min at 0 W, 10 min at 500 W. The clear, colorless digests of the mushroom powder were transferred quantitatively into 50-cm^3 volumetric flasks. An aliquot (0.250 cm^3) of the 10 μg Ga cm^{-3} solution was added to each flask. The clear, colorless digests of the humic soil samples were transferred quantitatively into 100-cm^3 flasks, and an aliquot (1.00 cm^3) of the Ga solution was added to each flask. The flasks were filled to the mark. Total arsenic concentrations were determined in these solutions by ICP–MS with an external calibration curve established with arsenate solutions.

Extraction of the arsenic compounds from *Amanita muscaria*

Aliquots (~0.1 g each) of the freeze-dried mushroom powders were weighed to 0.1 mg into 50-cm^3 polyethylene tubes. A methanol/water mixture (9:1 v/v, 10 cm^3) was added to each tube. The tubes were shaken for 14 h. Then the mixtures were centrifuged at 2500 rpm and the supernatants were transferred into round-bottomed flasks. The residues in the centrifuge tubes were washed three times with methanol/water (9:1 v/v, 10 cm^3 each), the mixtures were centrifuged, and the supernatants were again transferred into the round-bottomed flasks. The methanol was evaporated from the supernatants on a Rotavapor (Büchi, Switzerland) at room temperature under an aspirator vacuum. To each of the brownish-yellow, gummy evaporation residues water was added to a total mass of 10.0 g. The redissolved extraction residues were centrifuged at 8000 rpm. The supernatants were filtered through 0.2-μm cellulose nitrate filters (Sartorius, Göttingen, Germany).

Determination of total arsenic in the redissolved extraction residues from *Amanita muscaria*

External calibration

To 3.00 cm^3 of each redissolved extraction residue in a 10-cm^3 volumetric flask 0.10 cm^3 of nitric acid and 0.100 cm^3 of the 10 μg Ga cm^{-3} solution were added. The flasks were filled to the mark. Arsenic was determined without mineralization in these solutions by ICP–MS using an external calibration curve (10.0, 50.0, and 100 ng As cm^{-3}) established with $Na_2HAsO_4 \cdot 7H_2O$.

Standard addition

To avoid matrix effects, arsenic in the redissolved extraction residues was also determined by the standard addition method. To 2.00 cm^3 of each of the filtered redissolved extraction residues in a 12-cm^3 polystyrene tube 2.00 cm^3 of water, 40.0 mm^3 of the 10 μg Ga cm^{-3} solution and 0.04 cm^3 of nitric acid were added. These solutions were spiked with 10.0 mm^3 or 20.0 mm^3 of a 20 μg As cm^{-3} standard solution containing $Na_2HAsO_4 \cdot 7H_2O$. Arsenic in these solutions was determined by ICP–MS without mineralization.

Determination of arsenic compounds in the redissolved extraction residues

Aliquots (100 mm^3) of the filtered, redissolved extraction residues were chromatographed on the Supelcosil LC-SAX anion-exchange, the Supelcosil LC-SCX, and the Hamilton PRP-1 reversed-phase column. For the separation on the Hamilton column an aliquot (0.400 cm^3) was spiked with 10.0 mm^3 of an arsenocholine bromide solution containing 1.00 μg As cm^{-3}. For the separation on the cation-exchange column 0.500-cm^3 aliquots were spiked with 40.0 mm^3 of the arsenocholine bromide solution and 20.0 mm^3 of a tetramethylarsonium iodide solution containing 1.00 μg As cm^{-3}. The spiked solutions (100 mm^3) were chromatographed.

RESULTS AND DISCUSSION

Total arsenic concentration in *Amanita muscaria* and humic soil

On the site on which the *Amanita muscaria* specimens and humic soil were collected on 8 September 1995, a facility for roasting arsenopyrite to arsenic trioxide had been in operation for about 500 years. The facility was closed approximately 100 years ago. The site is at an altitude of 1400 m in the narrow Poellatal in northern Carinthia, Austria. The valley is so narrow that the creek (Lieser) and the roasting facility (16 m × 18 m) take up all the reasonably level ground between the steep mountain slopes,

from which frequent rock falls bombard the site and make the soil very rocky. The ten *Amanita muscaria* specimens found not more than 30 m from the center of the roasting facility (Fig. 1) were freeze-dried and ground to a powder. The homogenized powder was used in all subsequent experiments.

The total arsenic concentration in the humic soil sample was found to be 730 ± 60 mg As kg^{-1} dry mass (average of three digestions of the humic soil powder). Therefore, the mushrooms had grown on arsenic-rich humic soil and were expected to have accumulated and biotransformed arsenic compounds. The total arsenic concentration in *Amanita muscaria* determined by ICP–MS after mineralization was 21.9 ± 0.9 mg As kg^{-1} dry mass (average from four aliquots of the composite powder). Considering the arsenic-richness of the humic soil, *Amanita muscaria* is not a good arsenic accumulator compared with other mushrooms such as some *Laccaria* species.[23] However, the arsenic concentration in *Amanita muscaria* (~ 22 mg kg^{-1}) was high enough to allow the determination of the arsenic compounds by HPLC–ICP–MS. The 100-mm^3 chromatographed aliquots of the solutions prepared by extraction of ~ 0.1 g composite powder and dilution of the extract to 10 cm^3 contain ~ 20 ng arsenic. Because 0.1 ng arsenic still produces a quantifiable signal, sufficient arsenic is present in the 100-mm^3 aliquots to detect and quantify arsenic compounds even when the arsenic is distributed among several compounds.

Total arsenic in redissolved extraction residues from *Amanita muscaria*

Total arsenic concentrations in redissolved extraction residues of *Amanita muscaria* were determined without prior mineralization by ICP–MS using external calibration curves and the standard addition method. The concentration of total arsenic in the mushroom (24.7 ± 0.3 mg As kg^{-1}, average from four redissolved extraction residues) calculated from the concentrations in the redissolved extraction residues obtained by external calibration was higher than the arsenic concentration

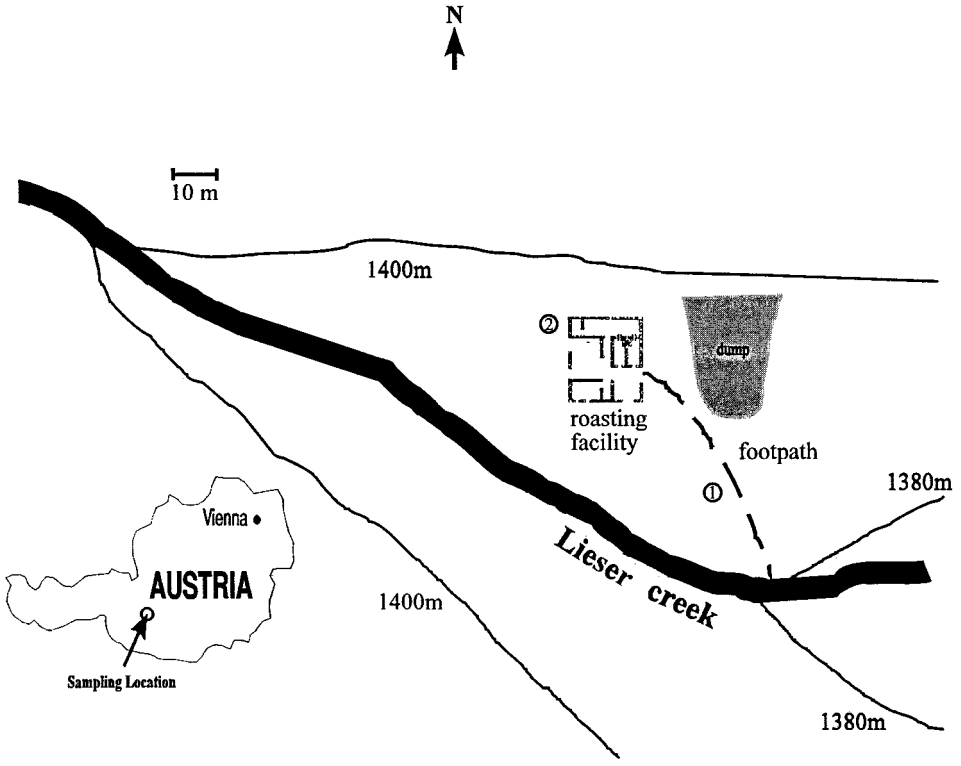


Figure 1 Location of the facility for roasting arsenic ores, where *Amanita muscaria* (1) and humic soil (2) were collected.

(21.9 ± 0.9 mg As kg^{-1}) determined directly in the mushroom after mineralization. With the standard addition method the arsenic concentration found was 22.0 ± 0.8 mg As kg^{-1} dry mass (average from four redissolved extraction residues). Therefore, the methanol/water (9:1) mixture extracts all the arsenic from the mushroom during the 14-h agitation period.

The high concentration of arsenic found with the external calibration can be attributed to the influence of organic compounds on the arsenic signal. For instance, addition of 5% methanol to an arsenic-containing solution can increase the arsenic signal by a factor of two.[24] The ICP–MS signal for elements with first ionization energies between 9 and 11 eV (arsenic 9.81 eV) are enhanced when organic compounds are present in the solution.[25] When the arsenic compounds are extracted from the mushrooms, organic compounds are co-extracted which supply sufficient carbon to cause erroneously high concentrations for arsenic.

Chromatographic separation of arsenic compounds

Ideally, the most common arsenic compounds in biological samples (arsenite, arsenate, methylarsonic acid, dimethylarsinic acid, trimethylarsine oxide, arsenocholine, arsenobetaine, tetramethylarsonium ion) should be separable on one column within a reasonably short time in a manner that allows each compound to be quantified. Although several types of columns and mobile phases were investigated,[6,8,9,11,12,14] the ideal system has not been found yet.

The chromatograms of mixtures of the eight arsenic compounds (Fig. 2) reveal that arsenocholine and tetramethylarsonium iodide leave the anion-exchange column not baseline-separated and almost with the solvent front. Employing this separation the signal of arsenocholine is a double peak with a characteristic shape.[26] The first signal ($\sim$120 s) is higher and very sharp, whereas the second signal ($\sim$130 s) is small and broad. The tetramethylarsonium ion co-elutes with the second signal of arsenocholine. Arsenobetaine and trimethylarsine oxide also have the same retention time.

On the cation-exchange column the signals for arsenite and methylarsonic acid overlap. On the reversed-phase column the arsenite signal merges with the signal for methylarsonic acid, and the arsenobetaine signal merges with the signal for dimethylarsinic acid. However, chromatography on all three columns of a solution in which all eight arsenic compounds are present will provide the desired quantitative information. When only the eight standard arsenic compounds are present, quantifications are best performed as specified in Table 2.

When compounds with neighboring signals are present in vastly different amounts, quantifications could become impossible without deconvolution of signals. Under these conditions the signals for the compounds present in small amounts could merge with the signal for the major compound. Fortunately, the signal for arsenocholine, the most interesting arsenic compound in the context of this investigation, is separated from the other seven arsenic compounds in the chromatograms obtained with all three columns (Fig. 2). Quantification can be hindered by signals from arsenic compounds that are not included in the standard mixture (Fig. 3).

A rationale for the retention behavior of the inorganic and organic arsenic compounds on the Hamilton PRP-1 reversed-phase column and the Supelcosil LC-SAX anion-exchange column was presented in an earlier publication.[17] On the Supelcosil LC-SCX column, arsenate elutes almost at the solvent front because at pH 3, the pH of the mobile phase, arsenate carries an apparent charge of $0.75-$ (75% $H_2AsO_4^-$, 25% H_3AsO_4) and is not slowed by the negatively charged sulfonate groups on the resin during the passage through the column. Arsenite (arsenous acid) and dimethylarsinic acid are uncharged. Ion-exchange processes cannot be responsible for the — albeit weak — retention of these compounds. Because the retention time of dimethylarsinic acid, a compound more hydrophobic than arsenous acid, is longer than the retention time of arsenous acid, hydrophobic interactions with the stationary phase are the likely reason for the observed elution sequence. At pH 3 about 80% of methylarsonic acid is uncharged and 20% is negatively charged (apparent charge $0.2-$). The low apparent negative charge, that would favor elution before the uncharged H_3AsO_3, and the hydrophobic character of the methyl group, that increases the likelihood of retention, combine to give methylarsonic acid a retention time slightly longer than H_3AsO_3 but shorter than dimethylarsinic acid. The signals for H_3AsO_3 and methylarsonic acid are not baseline-separated and, therefore, can be

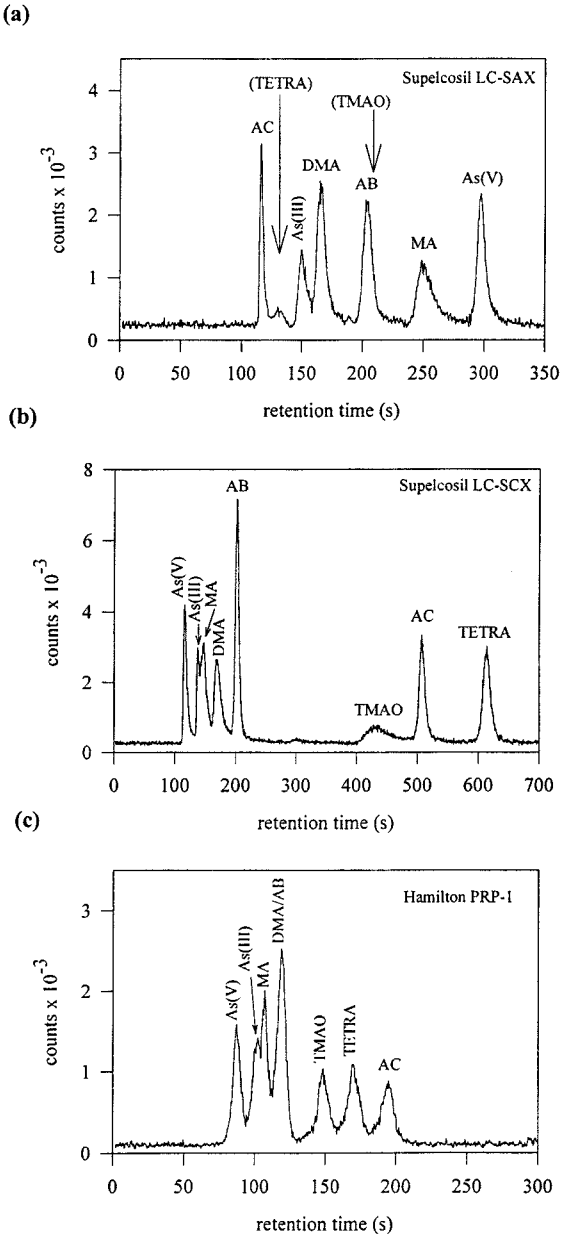


Figure 2 (a) Chromatogram obtained with a solution containing arsenate [As(V)], arsenite [As(III)], methylarsonic acid (MA), dimethylarsinic acid (DMA), arsenobetaine (AB) and arsenocholine (AC) (0.5 ng As each species) in distilled water on a Supelcosil LC-SAX anion-exchange column (mobile phase 30 mM $NH_4H_2PO_4$ with 1% methanol at pH 3.75, injection volume 100 mm^3, flow rate 1.5 $cm^3\ min^{-1}$). Tetramethylarsonium ion (TETRA) and trimethylarsine oxide (TMAO) would appear at the marked retention times. (b) Chromatogram obtained with a solution containing arsenate, arsenite, methylarsonic acid, dimethylarsinic acid, arsenobetaine, trimethylarsine oxide, arsenocholine and tetramethylarsonium iodide (0.5 ng As each species) in distilled water on a Supelcosil LC-SCX cation-exchange column (mobile phase 20 mM pyridine at pH 3.0, injection volume 100 mm^3, flow rate 1.5 $cm^3\ min^{-1}$). (c) Chromatogram obtained with a solution containing arsenate, arsenite, methylarsonic acid, dimethylarsinic acid, arsenobetaine, trimethylarsine oxide, arsenocholine and tetramethylarsonium iodide (0.5 ng As each species) in distilled water on a Hamilton PRP-1 reversed-phase column (mobile phase 10 mM 2-hydroxy-5-sulfobenzoic acid at pH 3.5, injection volume 100 mm^3, flow rate 1.5 $cm^3 min^{-1}$).

Table 2. Protocol for quantification of eight standard arsenic compounds

Chromatography	Compounds that can be quantified							
Anion-exchange	As(III)	DMA	MA	As(V)				
Cation-exchange		DMA		As(V)	AB	TMAO	AC	TETRA
Reversed-phase				As(V)		TMAO	AC	TETRA

used for the qualitative identification but not for the quantification of the two compounds. Among the remaining arsenic compounds arsenobetaine (80% cationic, 20% zwitterionic) and trimethylarsine oxide ($pK_a = 3.6$, 70% cationic, 30% uncharged) have at pH 3 an apparent positive charge of 0.8+ and 0.7+, respectively, and arsenocholine and tetramethylarsonium iodide carry a charge of 1+ irrespective of pH. The completely cationic species, therefore, have a longer retention time than the partially cationic arsenobetaine and trimethylarsine oxide. The retention sequence AB < TMAO < AC < TETRA is largely determined by the hydrophobicity, which increases from arsenobetaine to the tetramethylarsonium cation. The first three compounds all have three methyl groups. The fourth group bonded to arsenic (CH_2COOH in arsenobetaine, OH in $TMAsOH^+X^-$, CH_2CH_2OH in AC) increases in hydrophobicity, favors interactions with the hydrophobic backbone of the resin and leads to the observed retention behavior.

Arsenic compounds in *Amanita muscaria*

The chromatograms (Fig. 3) obtained with anion-exchange, cation-exchange and reversed-phase columns reveal that *Amanita muscaria* contains arsenite, arsenate, dimethylarsinic acid, arsenocholine, the tetramethylarsonium cation and arsenobetaine. Several signals (Fig. 3b) that could not be matched with signals of available standards indicate the presence of at least eight additional arsenic compounds. Methylarsonic acid and trimethylarsine oxide were not detected.

Chromatography of the redissolved extraction residues on the anion-exchange column produced a very sharp signal almost coincident with the solvent front (Fig. 3a). The shape of this signal is typical of arsenocholine.[26] Because only ten *Amanita muscaria* specimens (total mass of dry mushroom powder: 15 g) were found at the site and the mushrooms did not contain more than approximately 8 mg arsenocholine per kg dry mass, the isolation of arsenocholine was not feasible. Therefore, arsenocholine was separated from the other arsenic compounds on a cation-exchange column with 20 mM aqueous pyridine at pH 3.0 as mobile phase and on a reversed-phase column with an aqueous 10 mM solution of 2-hydroxy-5-sulfobenzoic acid (the ion-pairing reagent) at pH 3.5 as mobile phase. The strong signal in the cation-exchange chromatogram at a retention time of 500 s (Fig. 3b) and the signal in the reversed-phase chromatogram at 180 s (Fig. 3c) are in good agreement with signals obtained with synthetic arsenocholine bromide (Fig. 2).

The redissolved extraction residue was spiked with arsenocholine bromide. Cation-exchange and reversed-phase chromatography of the spiked solutions gave chromatograms (Figs. 3c, 4), in which the signals for arsenocholine were more intense than in the chromatograms of the unspiked solutions. A tetramethylarsonium iodide spike identified the signal at 600 s in the cation-exchange chromatogram (Fig. 4) as being caused by the tetramethylarsonium ion.

Although coincidence of chromatographic signals for an unknown arsenic compound with a synthetic standard does not unequivocally prove the identity of the two compounds, coincidences observed under two different separation modes with chemically unalike mobile phases increase the certainty of the identification. Because the signals assigned to arsenocholine in the cation-exchange and the reversed-phase chromatograms coincide with signals from arsenocholine bromide as standard and as spike, the presence of arsenocholine in *Amanita muscaria* can be considered to be confirmed.

The place of arsenocholine (**I**) in the marine arsenic cycle is uncertain. Experiments with algae[27–29] suggest that arsenocholine is a component of arsenic-containing phospholipids (**II**). Phosphatidylarsenocholine and a phosphatidyldimethylarsinylriboside were detected in the digestive gland of the western rock lobster, *Panulirus cygnus*.[3] Some of the unassigned

signals in the chromatogram of the arsenic compounds extracted from *Amanita muscaria* (Fig. 4) could come from these arsenolecithins (**II**). Unfortunately, synthetic standards for such lipids are not available. Only an arsenic-containing phosphonolipid (with a direct P–C bond between the P and the CH_2 of arsenocholine in **II** instead of a P–O–CH_2 bond)[30] and glycer-

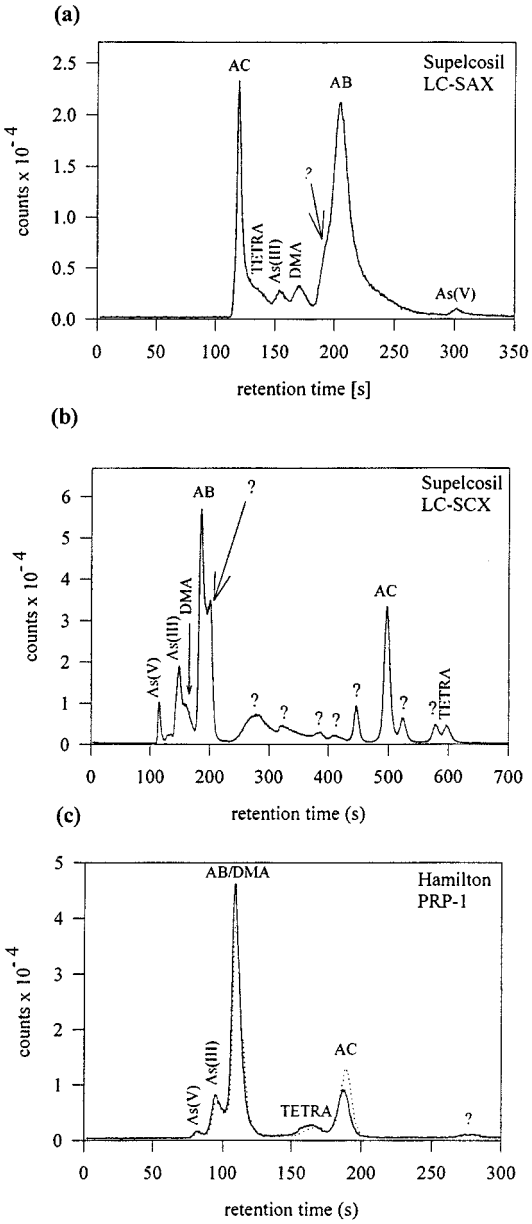


Figure 3 (a) Chromatogram obtained with a redissolved extraction residue of *Amanita muscaria* on a Supelcosil LC-SAX anion-exchange column (mobile phase 30 mM $NH_4H_2PO_4$ with 1% methanol at pH 3.75, injection volume 100 mm^3, flow rate 1.5 $cm^3\ min^{-1}$). (b) Chromatogram obtained with a redissolved extraction residue of *Amanita muscaria* on a Supelcosil LC-SCX cation-exchange column (mobile phase 20 mM pyridine at pH 3.0, injection volume 100 mm^3, flow rate 1.5 $cm^3 min^{-1}$). (c) Chromatogram obtained with a redissolved extraction residue of *Amanita muscaria* on a Hamilton PRP-1 reversed-phase column (mobile phase 10 mM 2-hydroxy-5-sulfobenzoic acid at pH 3.5, injection volume 100 mm^3, flow rate 1.5 $cm^3\ min^{-1}$). . . . . , Same redissolved extraction residue (100 mm^3) spiked with 2.4 ng As in the form of arsenocholine bromide.

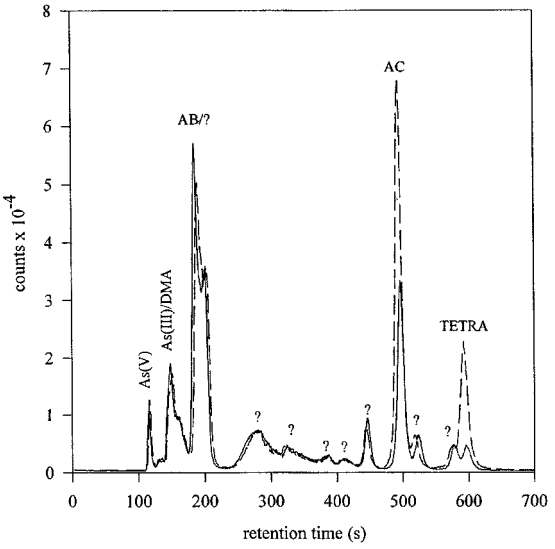
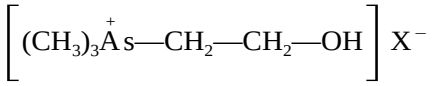
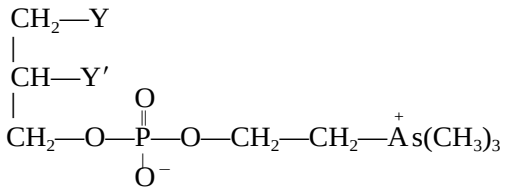


Figure 4 ——, Chromatogram obtained with a redissolved extraction residue of *Amanita muscaria* on a Supelcosil LC-SCX cation-exchange column (mobile phase 20 mM pyridine at pH 3.0, injection volume 100 mm^3, flow rate 1.5 $cm^3\ min^{-1}$). – – –, Same redissolved extraction residue (100 mm^3) spiked with 7.1 ng As in the form of arsenocholine bromide and 3.6 ng As in the form of tetramethylarsonium iodide.

ylphosphorylarsenocholine[31] (**II**, Y = Y′ = OH) have been prepared.



I



II

Y, Y′ = OH and/or RC(O)O

Quantification of arsenic compounds

The chromatograms (Fig. 3) reveal that arsenite, arsenate, dimethylarsinic acid and the tetramethylarsonium cation each account for not more than 2% of the total arsenic in *Amanita muscaria*. Arsenocholine and arsenobetaine appear to be present at approximately equal concentrations (~3 mg As kg^{-1}, ~15% each of the total arsenic). The remaining 60% of the arsenic is contained in a series of unidentified arsenic compounds that are responsible for the signals marked with question marks in Fig. 4. Signals from unidentified arsenic compounds contribute to the intensity of the signals from arsenocholine and arsenobetaine in the anion-exchange chromatogram (Fig. 3a), to the arsenobetaine signal in the cation-exchange chromatogram (Fig. 3b) and in the reversed-phase chromatogram (Fig. 3c), and thus complicate the quantification of these compounds.

The quantification of arsenite, arsenate, dimethylarsinic acid and the tetramethylarsonium cation poses no difficulties, because the signals of these compounds are not overlapped by any other signal in at least one of the chromatograms. The concentrations of these compounds in the dry mushroom are approximately 0.4 mg As kg^{-1} (Table 3).

The arsenocholine signal appears to be undisturbed in the cation-exchange (Fig. 3b) and in the reversed-phase chromatograms (Fig. 3c). Based on a calibration curve with arsenocholine bromide, the concentration of arsenocholine in *Amanita muscaria* is approximately 2.7 mg As kg^{-1} (Table 3). The signal for 'arsenocholine' in the anion-exchange chromatogram (Fig. 3a) produces a concentration of 6.2 mg As kg^{-1}. Because the tetramethylarsonium ion, the signal of which overlaps with the arsenocholine signal, contributes only ~0.4 mg As kg^{-1}, approximately 3 mg As kg^{-1} must have come from an unidentified arsenic compound.

In none of the chromatograms is the signal for arsenobetaine undisturbed. In the anion-exchange chromatogram (Fig. 3a) a shoulder is clearly discernible on the low-retention-time side of the arsenobetaine signal. The corresponding signal in the cation-exchange chromatogram (Fig. 3b) has two badly resolved maxima. In the reversed-phase chromatogram (Fig. 3c) the signal for dimethylarsinic acid (~0.4 mg As kg^{-1}), overlapping with the arsenobetaine signal, cannot be responsible for the high intensity (~8.5 mg As kg^{-1}) of the combined signal. Unidentified arsenic compounds must also contribute. When the arsenobetaine signal in the cation-exchange chromatogram is deconvoluted, a concentration of arsenobetaine of approximately 3.3 mg As kg^{-1} is obtained.

When the total arsenic concentrations are calculated as the sum of the concentrations of the arsenic species in the three chromatograms, only the sum from the anion-exchange chromatogram ($\sim$22 mg kg^{-1}) agrees with the total concentration of arsenic determined after mineralization of the mushroom powder. Consequently, the arsenic bound in the unknown compounds must contribute to, and be represented by, the signals for 'arsenocholine' and 'arsenobetaine' (Fig. 3a). The sums from the cation-exchange ($\sim$16 mg As kg^{-1}) and the reversed-phase ($\sim$14 mg As kg^{-1}) chromatograms, (Table 3; Figs 3b, 3c) are significantly smaller than the total of 22 mg kg^{-1}.

A likely explanation for this observation is the complete retention of some of the unidentified arsenic compounds on the cation-exchange and on the reversed-phase columns, with perhaps a slow bleeding of the arsenic compounds from the columns, that would produce very low-intensity, very broad signals merging with the background.

The chromatograms allow general properties of the unidentified arsenic compounds to be deduced. On the anion-exchange column approximately 3 mg As kg^{-1} elutes with arsenocholine, almost with the solvent front. Consequently, the arsenic compound(s) must be cationic at the pH (3.75) of the mobile phase or neutral (and not too lipophilic). Most of the arsenic ($\sim$12 mg kg^{-1}) in the unidentified compound(s) appears with arsenobetaine and thus should have cationic/zwitterionic character with some lipophilicity. The appearance of several signals in the cation-exchange chromatogram with retention times longer than that for arsenobetaine (Fig. 3b) suggests a positive charge for these compounds, and lipophilic properties.

Among the arsenic compounds likely to be present in biota, arsenic-containing sugars (dimethylribosylarsine oxides) and arsenic-containing, fully acylated or partially hydrolyzed phospholipids (**II**) could be present in *Amanita muscaria* and responsible for the unassigned signals in the chromatograms. Trialkylarsine

Table 3. Concentrations of arsenic compounds in *Amanita muscaria* determined by HPLC–HHPN–ICP–MS using anion-exchange, cation-exchange and reversed-phase separations

	Concentration (mg As kg^{-1} dry mass)		
Arsenic compound	LC-SAX[a]	LC-SCX[b]	RP[a]
As(III)	0.3, 0.4		
As(III)+DMA+?		2.0±0.3	
As(III)+?			1.4, 1.6
As(V)	0.2, 0.3	0.4±0.2	0.2, 0.2
DMA	0.4, 0.7		
DMA+As(III)+?		2.0±0.3	
DMA+AB+?			8.1, 8.9
AB		3.3±0.4[c]	
AB+?	14.0, 16.0	6.1±0.6	
AB+DMA+?			8.1, 8.9
AC		2.7±0.3	2.5, 2.7
AC+TETRA+?	6.1, 6.2		
TETRA		0.5±0.04	0.8, 0.7
TETRA+AC+?	6.1, 6.2		
Unknown?		7.6±0.8	0.4, 0.4
Sum	21.0, 23.6	16.4±1.9	13.4, 14.5

[a] Separate determinations using two aliquots of the mushroom powder.
[b] Average±SD from six aliquots of the mushroom powder.
[c] Arsenobetaine concentration after deconvolution from unknown signal.

oxides are known to be cations in an acidic medium and the phospholipids can be zwitterionic or cationic. Both types of compounds can vary in hydrophilicity/lipophilicity, depending on the nature of their substituents. Lack of easily accessible standards (particularly for arsenic-containing lipids) prevents the identification of the unknown arsenic compounds in *Amanita muscaria.*

CONCLUSION

Arsenocholine has been identified for the first time in a terrestrial sample, in the mushroom *Amanita muscaria.* In addition to arsenocholine, arsenobetaine, tetramethylarsonium cation, dimethylarsinic acid, arsenate and arsenite were also present. Because isolation of arsenocholine from the available, limited mass of mushroom was not possible, the presence of arsenocholine was ascertained by anion-exchange, cation-exchange and reversed-phase chromatography with three different mobile phases, and by chromatography of spiked extracts.

REFERENCES

1. K. A. Francesconi and J. S. Edmonds, *Oceanogr. Mar. Biol. Annu. Rev.* **31**, 111 (1993).
2. W. R. Cullen and K. J. Reimer, *Chem. Rev.* **89**, 713 (1989).
3. J. S. Edmonds, Y. Shibata, K. A. Francesconi, J. Yoshinaga and M. Morita, *Sci. Total Environ.* **122**, 321 (1992).
4. H. Norin and A. Christakopoulos, *Chemosphere* **11**, 287 (1982).
5. H. Norin, R. Ryhage, A. Christakopoulos and M. Sandström, *Chemosphere* **12**, 299 (1983).
6. J. F. Lawrence, P. Michalik, G. Tam and H. B. S. Conacher, *J. Agric. Food Chem.* **34**, 315 (1986).
7. B. P. Y. Lau, P. Michalik, C. J. Porter and S. Krolik, *Biomed. Environ. Mass Spectrom.* **14**, 723 (1987).
8. E. H. Larsen, G. Pritzl and S. H. Hansen, *J. Anal. At. Spectrom.* **8**, 1075 (1993).
9. E. H. Larsen and S. Stürup, *J. Anal. At. Spectrom.* **9**, 1099 (1994).
10. A. Christakopoulos, B. Hamasur, H. Norin and I. Nordgren, *Biomed. Environ. Mass Spectrom.* **15**, 67 (1988).
11. S. Caroli, F. LaTorre, F. Petrucci and N. Violante, *Environ. Sci. Pollut. Res.* **1**, 205 (1994).
12. J. S. Edmonds, Y. Shibata, R. I. T. Prince, K. A. Francesconi and M. Morita, *J. Mar. Biol. Ass. U. K.* **74**, 463 (1994).
13. H. Norin, A. Christakopoulos, L. Rondahl, A. Hagman and S. Jacobsson, *Biomed. Environ. Mass Spectrom.* **14**, 117 (1987).
14. D. Beauchemin, M. E. Bednas, S. S. Berman, J. W. McLaren, K. W. M. Siu and R. E. Sturgeon, *Anal. Chem.* **60**, 2209 (1988).
15. Y. Shibata and M. Morita, *Anal. Chem.* **61**, 2116 (1989).
16. A. R. Byrne, Z. Slejkovec, T. Stijve, L. Fay, W. Gössler, J. Gailer and K. J. Irgolic, *Appl. Organomet. Chem.* **9**, 305 (1995).
17. D. Kuehnelt, W. Goessler and K. J. Irgolic, *Appl. Organomet. Chem.*, **11**, 289 (1997).
18. A. R. Byrne, M. Tusek-Znidaric, B. K. Puri and K. J. Irgolic, *Appl. Organomet. Chem.* **5**, 25 (1991).
19. P. Nissen and A. A. Benson, *Physiol. Plant.* **54**, 446 (1982).
20. M. Vahter and E. Marafante, Metabolism of alkyl arsenic and antimony compounds. In: *Metal Ions in Biological Systems*, Vol. 29, Sigel H. and Sigel A. (eds), Marcel Dekker, New York, 1993, pp. 161–184.
21. J. P. Buchet, R. Lauwerys and H. Roels, *Int. Arch. Occup. Environ. Health* **48**, 71 (1981).
22. W. Goessler, D. Kuehnelt and K. J. Irgolic, Determination of arsenic compounds in human urine by HPLC-ICP-MS. In: *Arsenic: Exposure and Health Effects*, Chapter 3, Abernathy, C. E. (ed.), Chapman & Hall, London, in press.
23. A. R. Byrne and M. Tusek-Znidaric, *Chemosphere* **12**, 1113 (1983).
24. C. Demesmay, M. Olle and M. Porthault, *Fresenius J. Anal. Chem.* **348**, 205 (1994).
25. P. Allain, L. Jaunault, Y. Mauras, J.-M. Mermet and T. Delaporte, *Anal. Chem.* **63**, 1497 (1991).
26. R. Rubio, I. Peralta, J. Alberti and G. Rauret, *J. Liq. Chromatogr.* **16**, 3531 (1993).
27. N. R. Bottino, E. R. Cox, K. J. Irgolic, S. Maeda, W. J. McShane, R. A. Stockton and R. A. Zingaro, Arsenic uptake and metabolism by the alga *Tetraselmis chui.* In: *Organometals and Organometalloids. Occurrence and Fate in the Environment*, Brinckman, F. E. and Bellama, J. M. (eds), ACS Symp. Series No. 82, American Chemical Society, Washington DC, 1978, pp. 116–129.
28. A. A. Benson and P. Nissen, *Dev. Plant Biol.* **8**, 121 (1982).
29. K. J. Irgolic, E. A. Woolson, R. A. Stockton, R. D. Newman, N. R. Bottino, R. A. Zingaro, P. C. Kearney, R. A. Pyles, S. Maeda, W. J. McShane and E. R. Cox, *Environ. Health Persp.* **19**, 61 (1977).
30. T. Junk, G. C. Pappalardo and K. J. Irgolic, *Appl. Organomet. Chem.* **4**, 103 (1990).
31. K. A. Francesconi, R. V. Stick and J. S. Edmonds, *Experientia* **46**, 464 (1990).

