APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2001; **15**: 445–456
DOI: 10.1002/aoc.189

Comparison of three methods for the extraction of arsenic compounds from the NRCC standard reference material DORM-2 and the brown alga *Hijiki fuziforme*

Doris Kuehnelt,* Kurt J. Irgolic† and Walter Goessler
Institute of Chemistry, Analytical Chemistry, Karl-Franzens-University Graz, Universitaetsplatz 1, A-8010 Graz, Austria

The NRCC standard reference material DORM-2 and the marine brown alga *Hijiki fuziforme* were extracted with water, methanol/water (9 + 1), and 1.5 M orthophosphoric acid. The extracts from DORM-2 were analyzed by HPLC–ICP-MS for arsenobetaine, arsenocholine, trimethylarsine oxide, and the tetramethylarsonium cation and the extracts from *H. fuziforme* for arsenous acid, arsenic acid, dimethylarsinic acid, methylarsonic acid, and four arsenoriboses. Almost no differences between the three extractants were observed when DORM-2 was investigated. Only arsenobetaine was slightly better extracted with 1.5 M orthophosphoric acid or methanol/water (9 + 1) than with water. The sum of all extractable compounds (arsenobetaine, the tetramethylarsonium cation, and a formerly unknown compound recently identified as the trimethyl(2-carboxyethyl)arsonium ion) accounted for 94% of the total arsenic when 1.5 M orthophosphoric acid was used, for 92% when methanol/water (9 + 1) was used, and for 87% when water was used. Significant differences in the extraction yields obtained for the alga were observed for arsenic acid and one of the arsenoriboses ('glycerol-ribose'). Orthophosphoric acid removed twice as much of this ribose from the algal material than water and three times more than methanol/water (9 + 1). Arsenic acid was 1.2 times better extracted with orthophosphoric acid than with water and ten times better than with methanol/water (9 + 1). Almost no differences in the extraction yields were found for dimethylarsinic acid and the other three riboses. Orthophosphoric acid extracted 76%, water 65%, and methanol/water 33% of the total arsenic from *H. fuziforme*.

Keywords: arsenic compounds; extraction; HPLC–ICP-MS; NRCC DORM-2; *Hijiki*; brown algae

Received 2 February 2001; accepted 22 February 2001

INTRODUCTION

The estimation of arsenic toxicity requires the quantification of the individual arsenic species in biological material. Therefore, efforts have been focused on the development of methods for the separation and detection of the arsenic compounds. Powerful separation systems coupled to element-specific detectors with low detection limits have been developed.[1] For the application of these methods to natural samples, an extraction step that makes the arsenic compounds available for analysis is necessary. Arsenic compounds should be extracted quantitatively without decomposition or chemical conversion. The most common extractants for arsenic compounds in biological material are methanol, water, and methanol/water mixtures. Sonication or mechanical agitation are often used for the extraction of biological material. Extraction with microwave-assisted heating[2] as well as the application of special extraction devices like accelerated solvent extraction (ASE) systems[3] are also described in the literature.

A variety of data about arsenic compounds in the standard reference material (SRM) DORM-1 has

* Correspondence to: D. Kuehnelt, Institute of Chemistry, Analytical Chemistry, Karl-Franzens-University Graz, Universitaetsplatz 1, A-8010 Graz, Austria.
† Deceased.

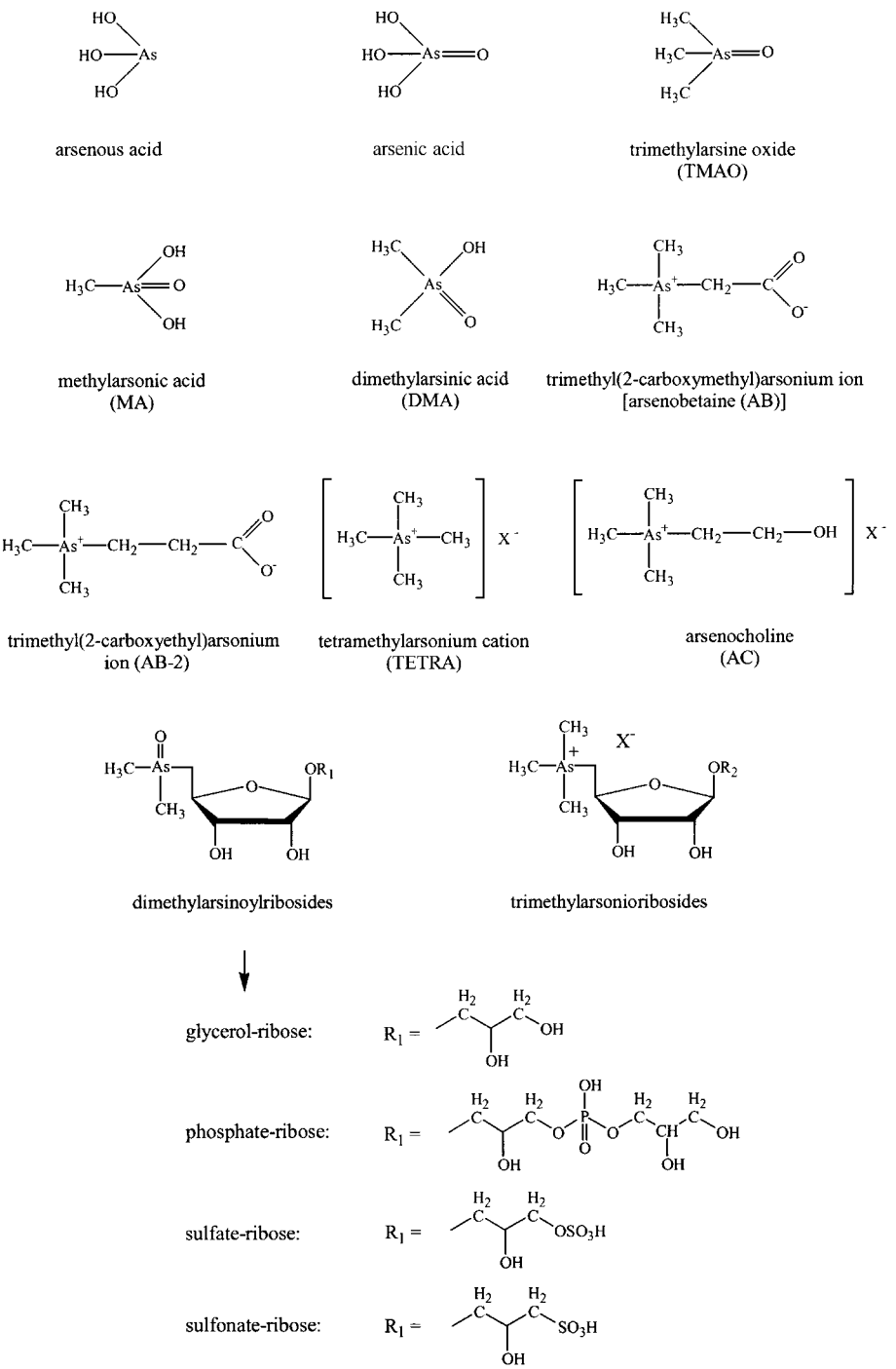

Figure 1 Arsenic compounds detected in biological samples. Arsenous acid, arsenic acid, MA, DMA, and the glycerol-, phosphate-, sulfate-, and sulfonate-riboses were investigated in the extracts of *H. fuziforme* and AB, AC, TMAO, TETRA, and AB-2 in extracts of DORM-2 by HPLC–ICP-MS.

been published.[4] Many different extractants and extraction methods have been applied to this SRM. DORM-2, the successor to DORM-1, has recently been certified for arsenobetaine (AB; 16.4 ± 1.1 mg As kg^{-1}) and the tetramethylarsonium cation (TETRA; 0.248 ± 0.054 mg As kg^{-1}), which together account for 92% of the total arsenic in this SRM.[5] However, less data exists about different extraction methods for DORM-2 than for DORM-1. Goessler *et al.*[4] extracted the arsenic compounds of DORM-2 with methanol/water (9 + 1) by 14 h of mechanical agitation and achieved an extraction yield of 93% calculated with respect to the certified total arsenic concentration (18.0 mg As kg^{-1}). They reported the presence of dimethylarsinic acid (DMA; 0.28 ± 0.01 mg As kg^{-1}), AB (16.0 ± 0.7 mg As kg^{-1}), arsenocholine (AC; 0.024 ± 0.01 mg As kg^{-1}), TETRA (0.23 ± 0.02 mg As kg^{-1}), and an unknown arsenic compound (0.16 ± 0.01 mg As kg^{-1}), which was recently identified as the arsenic-containing betaine trimethyl(2-carboxyethyl)arsonium ion (Fig. 1),[6] in DORM-2. Corr[7] performed two consecutive extractions of DORM-2 with methanol/chloroform (2 + 1) in an ultrasonic bath for 30 min and reported an AB concentration of 16.6 ± 0.6 mg As kg^{-1}. AB was also the only compound detected in DORM-2 in another study.[8] Its concentration was found to be 16.0 ± 0.6 mg As kg^{-1} after threefold extraction by 30 min sonication with methanol/water (1 + 1). Mattusch and Wennrich[9] detected arsenous acid (0.08 mg As kg^{-1}), DMA (0.28 mg As kg^{-1}), arsenic acid (0.48 mg As kg^{-1}), AB (16.5 mg As kg^{-1}), and AC (0.08 mg As kg^{-1}) in DORM-2 after extraction with water, achieving an extraction yield of 97%.

Londesborough *et al.*[10] extracted DORM-2 by shaking for 2 h with water and obtained an extraction yield of 82%. DORM-2 was found to contain arsenous acid (0.1 mg As kg^{-1}), DMA (0.3 mg As kg^{-1}), arsenic acid (0.4 mg As kg^{-1}), AB (13.5 mg As kg^{-1}), trimethylarsine oxide (TMAO; 0.4 mg As kg^{-1}), AC (0.02 mg As kg^{-1}), and TETRA (0.1 mg As kg^{-1}). Suner *et al.*[11] used mechanical agitation with methanol/water (1 + 1) for 15 min for the extraction of arsenic from DORM-2. The procedure was applied three times, resulting in an extraction yield of 92%. AB (16.3 ± 0.1 mg As kg^{-1}), AC (0.098 ± 0.002 mg As kg^{-1}), and TETRA (0.127 ± 0.001 mg As kg^{-1}) were detected.

Microwave-assisted extraction was also applied to DORM-2.[2] Microwave heating to 50 °C for 4 min resulted in the extraction of 74% of the total arsenic, when water or methanol/water (8 + 2) were used as extractants. Methanol/water (1 + 1) extracted 86% and a 5% tetramethylammonium hydroxide solution extracted 95% of the total arsenic. When methanol/water (8 + 2) was employed as extractant at 65 °C, the arsenic was quantitatively extracted within 2 min. Extraction at 80 °C resulted in lower extraction yields. Microwave-assisted heating to 80 °C with water for 2 min removed 84% of the arsenic from DORM-2. By applying 1 min heating to 65 °C and 4 min to 100 °C, 91% of the arsenic was extractable with water. AB (24.6 mg As kg^{-1}) accounted for more than 100% of the arsenic in DORM-2, when a methanol/water (8 + 2) extract obtained by heating to 65 °C for 4 min was analyzed by high-performance liquid chromatography coupled with an inductively coupled plasma mass spectrometer (HPLC–ICP-MS). A small amount of DMA was also detected.

McKiernan *et al.*[3] compared ASE with a traditional sonication method for the extraction of arsenic compounds from DORM-2. Sonication and ASE were performed three times with acetone (extracting the non-polar arsenicals) and three times with methanol/water (1 + 1) (extracting the polar arsenicals). Both fractions, as well as the extraction residue (residual arsenic), were then digested for the determination of the total arsenic concentration. In the case of ASE, 5% of the total arsenic (calculated on the certified value) was extractable with acetone, 89% with methanol/water, and 3% was found in the residue. The corresponding results for the sonication procedure were 3%, 89%, and 11%. Therefore, 94% of the total arsenic was extracted by ASE and 92% by sonication with acetone and methanol/water (1 + 1). When the arsenic compounds in the methanol/water extracts were determined by HPLC–ICP-MS, the relative peak areas of AB/AC, DMA, and one unknown compound were the same for extraction by ASE and sonication. Slightly better extraction yields for methylarsonic acid (MA) and significantly better results for arsenic acid were achieved with ASE. Another unknown compound, which was not present after sonication, was detected after ASE.

In this work, three extractants are compared for the extraction of animal (DORM-2) and algal material (*Hijiki fuziforme*). Arsenous acid, arsenic acid, DMA, MA, and four arsenosugars were investigated in *H. fuziforme* and AB, AC, TMAO, TETRA, and the trimethyl(2-carboxyethyl)arsonium ion (AB-2) in DORM-2 (Fig. 1). Extraction

with the two common extractants, water and methanol/water (9 + 1), was compared with extraction with 1.5 M orthophosphoric acid, which was recently optimized in-house for the extraction of arsenic compounds from an ant-hill sample, to investigate differences in the extraction yields as well as in the pattern of extracted arsenic compounds.

EXPERIMENTAL

Reagents, solutions, and samples

All solutions were prepared with Milli-Q (18.2 MΩ cm) water. Nitric acid [Merck (Darmstadt, Germany), p.a.] was further purified in a quartz sub-boiling distillation unit. Pyridine (p.a.), 30% hydrogen peroxide (suprapur), ammonium dihydrogen phosphate (p.a.), and 25% aqueous ammonia (suprapur) were purchased from Merck (Darmstadt, Germany), formic acid (puriss. p.a.), orthophosphoric acid (puriss. p.a.), and methanol (puriss. p.a.) from Fluka (Buchs, Switzerland). Orthophosphoric acid was appropriately diluted with water to achieve the desired molarity. The glycerol-ribose was synthesized by Dr Toshikazu Kaise (Laboratory of Environmental Chemistry, School of Life Science, University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachijoji, Tokyo 192-03, Japan). The phosphate-, the sulfonate-, and the sulfate-riboses were isolated from marine algae by Dr Kevin A. Francesconi (Institute of Biology, Odense University, DK-5230 Odense M, Denmark).[12,13] Standard solutions of the arsenic compounds were prepared as described elsewhere.[14,15] The standard reference material DORM-2 (dogfish muscle tissue) was purchased from the National Research Council of Canada (NRCC), Ottawa, Ontario, Canada. The dried alga *H. fuziforme* was obtained from Dr Toshikazu Kaise.

Instrumentation

The dry alga *H. fuziforme* was pulverized in a Retsch ZM 1000 mill (Retsch, Haan, Germany) equipped with a titanium rotor and a 0.25 mm sieve. *H. fuziforme* was digested with an MLS-1200 Mega microwave system (MLS, Leutkirch, Germany). Cellulose ester filters (Millex-GS, 0.22 μm) for the filtering of the extracts prior to HPLC were purchased from Millipore (Bedford, MA, USA).

Total arsenic was determined with a VG PlasmaQuad 2 Turbo Plus inductively coupled argon-plasma mass spectrometer (VG Elemental, Winsford, UK) equipped with a Meinhard concentric glass nebulizer type TR-30-A3.

The HPLC system for the determination of the arsenic compounds in DORM-2 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 arsenic compounds were separated on a Supelcosil LC-SCX (Supelco, Bellefonte, USA) cation-exchange column (25 cm × 4.6 mm i.d., 5 μm silica-based particles with propylsulfonic acid exchange-sites). The outlet of the HPLC column was connected *via* 60 cm of 1/16″ polyether–ether–ketone (PEEK) capillary tubing (0.25 mm i.d.) to a hydraulic high-pressure nebulizer (HHPN) (Knauer, Berlin, Germany). The VG PlasmaQuad 2 Turbo Plus ICP-MS served as 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 intensity at *m/z* 77 ($^{40}Ar^{37}Cl$, ^{77}Se) was monitored to detect $^{40}Ar^{35}Cl$ interferences on *m/z* 75. Prior to each HPLC–ICP-MS run the ion intensity at *m/z* 87 (RbCl added to the mobile phases) was optimized at the rate meter of the instrument.[16]

The HPLC system for the determination of the arsenic compounds in *H. fuziforme* consisted of a Hewlett Packard 1100 chromatographic system including solvent delivery unit, autosampler, and column heater (Hewlett Packard, Waldbronn, Germany). The arsenic compounds were separated on a Hamilton (Reno, USA) PRP-X100 anion-exchange column (25 cm × 4.1 mm i.d., 10 μm styrene–divinylbenzene particles with trimethylammonium exchange-sites) or the Supelcosil LC-SCX cation-exchange column. The outlet of the HPLC column was connected *via* 100 cm of 1/16″ PEEK capillary tubing (0.25 mm i.d.) to the Babington-type nebulizer of an HP4500 ICP-MS (Hewlett Packard, Waldbronn, Germany). The ion intensity at *m/z* 75 (^{75}As) and the ion intensity at *m/z* 77 ($^{40}Ar^{37}Cl$, ^{77}Se) were monitored. Instrumental settings are published elsewhere.[14]

The chromatograms were exported and the peak areas were determined using software written in-house.[17] The arsenic compounds were quantified with external calibration curves established with arsenous acid, arsenic acid, MA, and DMA on the Hamilton PRP-X100 column, and with AB, AC,

TETRA, and TMAO on the Supelcosil LC-SCX column. The phosphate-ribose, the sulfate-ribose, and the sulfonate-ribose were quantified with the calibration curve for DMA, and the glycerol-ribose with the calibration curve for AB, assuming that all arsenic compounds give the same response for arsenic.[14]

Determination of the total arsenic in *H. fuziforme*

Aliquots of the freeze-dried algal material (~0.2 g) were weighed to an accuracy of 0.1 mg into Teflon digestion vessels. Concentrated nitric acid (5.0 cm^3) and 30% hydrogen peroxide (0.50 cm^3) were added to each vessel. The vessels were closed, secured in the rotor, and placed into the microwave oven. The samples were digested with the following program (watts/minutes): 250/2, 0/0.5, 300/5, 0/0.5, 450/5, 0/0.5, 600/5, 500/7, 0/2 (ventilation). The digests were transferred quantitatively into 50 cm^3 volumetric flasks. An aliquot (0.250 cm^3) of a solution containing 10 $\mu g\,cm^{-3}$ of gallium 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 aqueous solutions of arsenic acid containing arsenic at 10.0, 50.0, or 100 $\mu g\,dm^{-3}$.

Extraction of arsenic compounds from DORM-2 and *H. fuziforme*

DORM-2 [~200 mg (extraction with orthophosphoric acid) or ~100 mg (extraction with water or methanol/water)] or the pulverized alga *H. fuziforme* (~500 mg) were weighed to an accuracy of 0.1 mg into screw-capped polyethylene vials. An aqueous 1.5 M solution (10 cm^3) of orthophosphoric acid, water (10 cm^3) or methanol/water (9 + 1) (10 cm^3) was added to each vial. Arsenic compounds were extracted on a cross-shaped rotor by rotating the vials at 45 rpm for 14 h (25 °C). The orthophosphoric acid extracts were neutralized with 25% aqueous ammonia, filled to 50 cm^3, centrifuged, and filtered. The water extracts were centrifuged and the supernatants were filtered. The methanol/water extracts were centrifuged. The centrifugation residues were washed three times with 10 cm^3 methanol/water (9 + 1). The combined supernatants were evaporated to dryness on a Rotavapor (Buechi, Switzerland) at room temperature under an aspirator vacuum. The residues were dissolved in 10 cm^3 water and the solutions were filtered through cellulose ester filters.

The undiluted extracts of DORM-2 were analyzed for AB, AC, TMAO, TETRA, and AB-2 by cation-exchange chromatography on the Supelcosil LC-SCX column with an aqueous solution of 20 mM pyridine at pH 2.5 (adjusted with formic acid). The extracts of the alga *H. fuziforme* were appropriately diluted with water (see Fig. 3 caption) and then analyzed for arsenous acid, arsenic acid, DMA, MA, the phosphate-, the sulfate-, and the sulfonate-riboses by anion-exchange chromatography on the Hamilton PRP-X100 column with an aqueous solution of 20 mM $NH_4H_2PO_4$ at pH 5.6 or pH 6.0 [adjusted with aqueous ammonia (25%)] as mobile phases, or for the glycerol-ribose on the Supelcosil LC-SCX column with an aqueous solution of 20 mM pyridine at pH 2.6 (adjusted with formic acid) as mobile phase.

RESULTS AND DISCUSSION

Most methods for the identification and quantification of arsenic compounds in solids (biota, soils, sediments) require that the arsenic compounds are transferred from the solid state into a solvent. This process of extraction should ideally be quantitative and must not change the arsenic compounds chemically. Whether an extraction is successful depends on the solubilities of the arsenic compounds in the chosen extractant and on the ability of the extractant to come in contact with the arsenic compounds.

During the past three decades several arsenic compounds have been definitely identified in marine and terrestrial biota (Fig. 1). Unidentified signals exist in chromatograms, indicating that additional arsenic compounds are yet to be identified. The definitely identified arsenic compounds have low molecular mass and have in their molecules functional groups (onium centers, hydroxyl groups, doubly bonded oxygen atoms) that provide solubility in polar solvents such as water and methanol. The concentrations of arsenic compounds in biota are low (micrograms to milligrams per kilogram dry mass) and even relatively insoluble arsenic compounds should dissolve in a reasonable volume (~10 cm^3) of a polar extractant. Such a dissolution will occur only when the extractant penetrates to the components in biota that contain the arsenic compounds. Biota, of course, consist of cells. Of the total fresh biota mass, water accounts for at least

70%. This water, with substances such as arsenic compounds dissolved in it, resides within cells and outside of cells. The intracellular solutions will come in contact with an extractant only when the membrane is ruptured. The extracellular solutions should be accessible to the extractant without difficulty, after diminution of the tissues. Consequently, for a quantitative transfer of the arsenic compounds present in the extracellular and intracellular aqueous solutions, a break up of the tissue and rupture of the cells is required. Frequently, biota are freeze-dried to constant (dry) mass before extraction. Removal of all water will force the arsenic compounds to crystallize and the cells to rupture. Grinding of the dry material to a fine powder will aid the break up of the biological structures. An extractant should now have easier access to the arsenic compounds and the extraction should be quantitative.

However, the arsenic compounds do not have to be present dissolved in extra- and intra-cellular water. They could be bonded to insoluble constituents of cells. Hardly anything is known with certainty about such insoluble compounds. Speculation is easiest for AC, the 2-hydroxyethyltrimethylarsonium cation, because rudimentary experimental evidence is available. AC is easily soluble in aqueous systems and in methanol. When AC is bound *via* an ester-oxygen atom to the phosphorus atom in phosphatitic acid, the resulting arsenic-containing phospholipid, the arsenic-analogue of a lecithin (arsenolecithin),[18,19] will not be soluble in water and only minimally in methanol. Arsenolecithins could be incorporated into cell membranes. Arsenoriboses could be bound to cell surfaces, as are many other simple and complex carbohydrates. Even the acidic arsenic compounds (arsenous acid, arsenic acid, MA, DMA) could be bonded to biopolymers, such as proteins containing amino acids with thiol groups. Arsenic has a high affinity to sulfur. Reactions of trivalent arsenic compounds with thiol groups of enzymes are postulated to be the cause of arsenic toxicity. Pentavalent arsenic acids can be reduced *in vivo* to trivalent compounds,[20] which in turn will react with thiols. The As-S compounds formed in these reactions (for instance, between dimethylhydroxyarsine and reduced glutathione) are expected to be stable toward hydrolysis, and especially stable when a five-membered heterocycle with the S-As-S group is obtained (for instance, from methyldihydroxyarsine and a vicinal dithiol such as lipoic acid or 2,3-dimercaptopropanol).

Many of these high-molecular-mass arsenic compounds will not be extractable into aqueous solvents. If such arsenic compounds exist in biota, extractants other than aqueous solutions must be chosen (for instance, chloroform/methanol mixtures for arsenolecithins[18,19]) or hydrolytic reactions must cleave the bonds holding the arsenic compounds to the biopolymer and regenerate the low-molecular-mass, water-soluble arsenic compounds shown in Fig. 1.

Total arsenic in *H. fuziforme*

The total arsenic concentration in *H. fuziforme* was determined by ICP-MS after microwave digestion with an HNO_3/H_2O_2 mixture. The algal material contained 87 ± 5 mg As kg^{-1} dry mass ($n = 3$). This value is in good agreement with arsenic concentrations published for marine brown algae (up to 179 mg As kg^{-1} dry mass)[21] and the arsenic concentration in *H. fuziforme* published by Yoshinaga *et al.* (66 to 75 mg As kg^{-1} dry mass)[22] and Yasui *et al.* (93 mg As kg^{-1} dry mass).[23]

Extraction of arsenic compounds from DORM-2 and *H. fuziforme*

DORM-2 and the alga *H. fuziforme* were analyzed for their arsenic compounds. The arsenic compounds in DORM-2 were shown to be AB,[2–5,7–11] DMA,[3,4,9,10] AC,[3,4,9–11] TETRA[4,5,10,11] and an unknown compound,[4] which was recently identified as AB-2.[6] Two unknown compounds were reported by McKiernan *et al.*[3] The presence of arsenous acid,[9,10] arsenic acid,[3,9,10] MA,[3] and TMAO[10] has been reported less often. *H. fuziforme* was reported to contain arsenic acid as major,[22–24] arsenous acid as minor,[23] and arsenoriboses as major arsenic compounds.[22,24] Therefore, anionic, neutral, and cationic arsenic compounds are found in these two samples. The samples were extracted with 1.5 M orthophosphoric acid, water, or methanol/water (9 + 1), analyzed for arsenic compounds, and the extraction efficiencies for the different arsenic compounds were compared.

DORM-2

Cation-exchange chromatography (Supelcosil LC-SCX, 20 mM pyridine pH 2.5) was chosen to investigate the extracts of DORM-2, because AB is known to be the major constituent of this reference material.[5] Under these conditions AB, TMAO, AC, TETRA, and AB-2 can be separated without suppression of the AB signal by coeluting sodium or potassium, also present in the extract.

Table 1 Concentration of the arsenic compounds extracted from DORM-2 in comparison with literature data (mean of three determinations)[a]

Extractant	Extraction mode	Concentration (mg As kg^{-1} dry mass)											
		Arsenous acid	Arsenic acid	MA	DMA	AB	AC	TMAO	TETRA	AB-2[b]	Sum of species	Percentage of total	Ref.
1.5 M H_3PO_4	Mechanical agitation	n.i.	n.i.	n.i.	n.q.	16.4 ± 0.8	<0.05	<0.05	0.25 ± 0.01	0.24 ± 0.01	16.9 ± 0.7	94	This work
CH_3OH/H_2O (9 + 1)	Mechanical agitation	n.i.	n.i.	n.i.	n.q.	16.1 ± 0.3	<0.05	<0.05	0.23 ± 0.01	0.20 ± 0.01	16.5 ± 0.3	92	This work
H_2O	Mechanical agitation	n.i.	n.i.	n.i.	n.q.	15.3 ± 0.3	<0.05	<0.05	0.22 ± 0.01	0.19 ± 0.01	15.7 ± 0.3	87	This work
CH_3OH/H_2O (9 + 1)	Mechanical agitation	<0.03	<0.03	<0.03	0.28 ± 0.01	16.0 ± 0.7	0.024 ± 0.01	<0.03	0.23 ± 0.02	0.16 ± 0.01	16.7	93	4
CH_3OH/H_2O (1 + 1)	Mechanical agitation					16.3 ± 0.1	0.098 ± 0.002	<0.001	0.127 ± 0.001		16.5	92	11
CH_3OH/H_2O (1 + 1)	Sonication					16.0 ± 0.6					16.0 ± 0.6	89	8
$CH_3OH/CHCl_3$ (2 + 1)	Sonication					16.6 ± 0.6					16.6 ± 0.6	92	7
CH_3OH/H_2O (8 + 2)	Microwave heating				n.q.	24.6 ± 1.4					24.6 ± 1.4	137	2
H_2O	Not reported	0.08	0.48		0.28	16.5	0.08				17.4	97	9
H_2O	Mechanical agitation	0.1	0.4		0.3	13.5	0.02	0.4	0.1		14.8	82	10
	Certified					16.4 ± 1.1			0.248 ± 0.054		16.6	92	5

[a] n.i.: not investigated; n.q.: not quantified.
[b] Quantified with the calibration curve for AB.

DMA, which elutes before AB, cannot be quantified under these conditions, because the coelution of sodium and potassium suppresses the arsenic signal.[4]

Therefore, AB, TETRA, and AB-2 were quantified in the extracts of DORM-2 (Table 1, Fig. 2). AC and TMAO were below the detection limit (50 μg As kg^{-1}). Almost no differences were observed when the three extractants were investigated. The results obtained with orthophosphoric acid and methanol/water (9 + 1) were the same, whereas a slightly lower extraction yield for AB was achieved with water. Considering a total arsenic concentration of 18.0 ± 1.1 mg kg^{-1} certified for DORM-2, all three extractants are able to extract ~90% of the total arsenic from this sample. The good extractability of the arsenic is probably caused by the fact that DORM-2 is defatted. Fat was reduced to less than 24% during the manufacturing process by extracting the material three times with acetone.[5] The concentrations obtained for AB and TETRA are in good agreement with the certified values, especially when 1.5 M orthophosphoric acid or methanol/water (9 + 1) are used for extraction (Table 1). Therefore, the extraction with 1.5 M orthophosphoric acid, which was originally optimized for the dissolution of arsenic compounds from ant-hill material — a completely different matrix — is also suitable for application to marine animals.

The concentration of AB obtained by extraction with 1.5 M orthophosphoric acid or methanol/water (9 + 1) is also in good agreement with literature data employing methanol/water or methanol/chloroform mixtures as extractants.[4,7,8,11] The slightly lower concentration of AB found by extraction with water corresponds to the results of Londesborough *et al.*[10] However, Mattusch and Wennrich reported an AB concentration of 16.5 mg As kg^{-1} dry mass obtained by extraction with water.[9] The presence of TMAO published by one working group as 0.4 mg As kg^{-1} dry mass[10] was not confirmed by our work, although this concentration is above the detection limit of our method.

AC was also below the detection limit, which is in agreement with the published concentration of 0.02 mg As kg^{-1} dry mass,[4,10] although values of ~0.09 mg As kg^{-1} dry mass were also reported.[9,11] The concentrations obtained for TETRA and AB-2 correspond to previous investigations.[4] Lower concentrations for TETRA (~0.1 mg As kg^{-1} dry mass) were found by two working groups.[10,11] As mentioned above, we did not quantify DMA, which

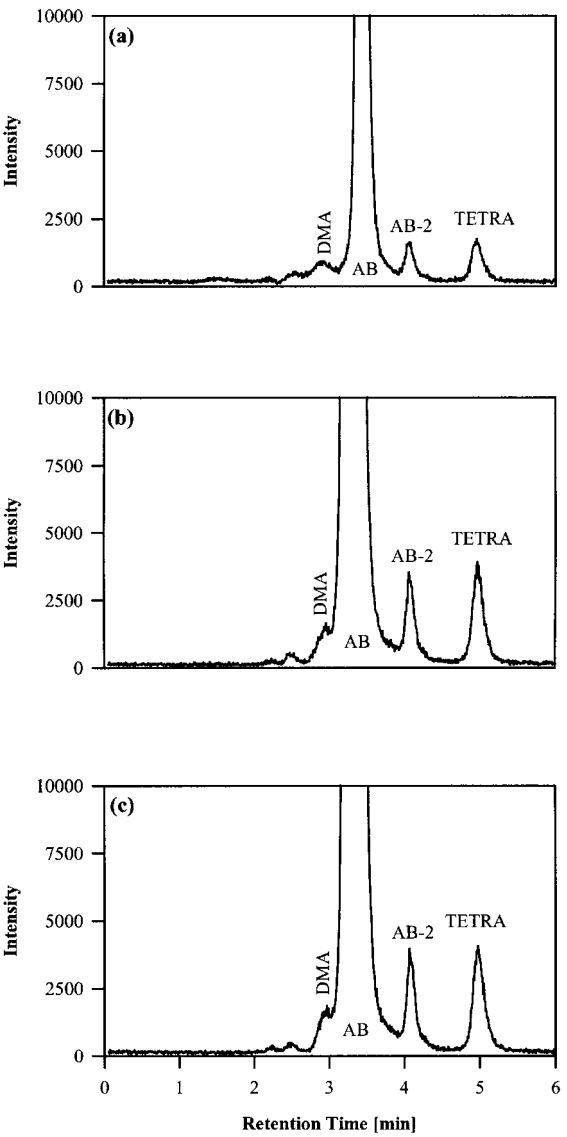

Figure 2 Chromatograms of extracts of DORM-2 on the Supelcosil LC-SCX cation-exchange column (mobile phase: 20 mM pyridine at pH 2.5; flow rate: 1.5 cm^3 min^{-1}; column temperature: 40 °C; 100 mm^3 injected). (a) Sample extracted with 1.5 M orthophosphoric acid (0.25 g sample in 50 cm^3, undiluted extract). (b) Sample extracted with methanol/water (9 + 1) (0.1 g sample in 10 cm^3, undiluted extract). (c) Sample extracted with water (0.1 g sample in 10 cm^3, undiluted extract).

Table 2 Concentration of the arsenic compounds extracted from *H. fuziforme* (mean of three determinations)

	Concentration [mg As/kg dry mass]								
Extractant	Arsenous acid/ glycerol-ribose/ DMA[a]	Glycerol-ribose[b]	DMA	Phosphate-ribose[c]	Arsenic acid	Sulfate-ribose[c]	Sulfonate-ribose[c]	Sum of species	As extracted (%)
1.5 M H_3PO_4	11.5 ± 0.2	9.8 ± 0.7	1.2 ± 0.1	2.2 ± 0.2	36 ± 1	13.2 ± 0.6	3.3 ± 0.3	66 ± 2	76
CH_3OH/H_2O (9 + 1)	6.2 ± 0.9	3.0 ± 0.1	0.8 ± 0.1	2.0 ± 0.1	3.5 ± 0.4	14.2 ± 0.9	3.2 ± 0.2	29 ± 2	33
H_2O	6.3 ± 0.3	4.3 ± 0.1	0.9 ± 0.1	2.5 ± 0.1	29 ± 1	16.3 ± 0.4	2.8 ± 0.2	57 ± 2	65

[a] Quantified with the calibration curve for arsenous acid.
[b] Quantified with the calibration curve for AB.
[c] Quantified with the calibration curve for DMA.

is also reported in the literature,[2,4,9,10] although it was present in all extracts (Fig. 2).

H. fuziforme

H. fuziforme was investigated by anion- and cation-exchange chromatography. MA, the phosphate-ribose, arsenic acid, the sulfonate-, and the sulfate-riboses can be separated on a Hamilton PRP-X100 anion-exchange column with 20 mM $NH_4H_2PO_4$ at pH 5.6 as mobile phase.[14] The glycerol-ribose and arsenous acid coelute with the solvent front, closely followed by DMA, which cannot be separated from these two compounds under these conditions. Therefore, DMA has to be identified and quantified with 20 mM $NH_4H_2PO_4$ at pH 6.0 as mobile phase.[16] Applying this mobile phase, DMA is separated from arsenous acid and the glycerol-ribose, whereas arsenic acid and the sulfonate-ribose coelute. AB, AC, TMAO, TETRA, and the glycerol-ribose can be identified and quantified by cation-exchange chromatography on the Supelcosil LC-SCX column with 20 mM pyridine at pH 2.6 as mobile phase. Under these conditions the glycerol-ribose elutes between AB and TMAO.

Significant differences in extractability were found when the extracts of *H. fuziforme* were analyzed (Table 2, Fig. 3). The glycerol-ribose was best extracted by orthophosphoric acid, which was able to remove more than twice the amount extracted by water and three times more than methanol/water (9 + 1). The extraction of arsenic acid is best performed with orthophosphoric acid. Water extracts 20% less arsenic acid from the algal material than orthophosphoric acid. Only 10% of the extraction yield achieved with orthophosphoric acid and 12% of the extraction yield achieved with water can be reached with methanol/water (9 + 1). These results correspond to the findings of Byrne et al.,[25] who reported higher extraction yields with water than with methanol/water (9 + 1) in a mushroom sample containing almost all of its arsenic as inorganic arsenic. Almost no differences were observed in the extractability of DMA, the phosphate-, the sulfate-, and the sulfonate-riboses. DMA was slightly better extracted with orthophosphoric acid than with water or methanol/water (9 + 1). Water extracted the sulfate-ribose slightly better than orthophosphoric acid or methanol/water (9 + 1). MA, AB, AC, TMAO and TETRA were below the detection limit (50 μg As kg^{-1}). *H. fuziforme* was best extracted by orthophosphoric acid (about 76% of the total arsenic extracted), indicating that this extractant is also very useful for the extraction of algae. The lowest extraction yield was achieved by methanol/water (9 + 1) (about 33%). About 65% of the total arsenic was extracted by water.

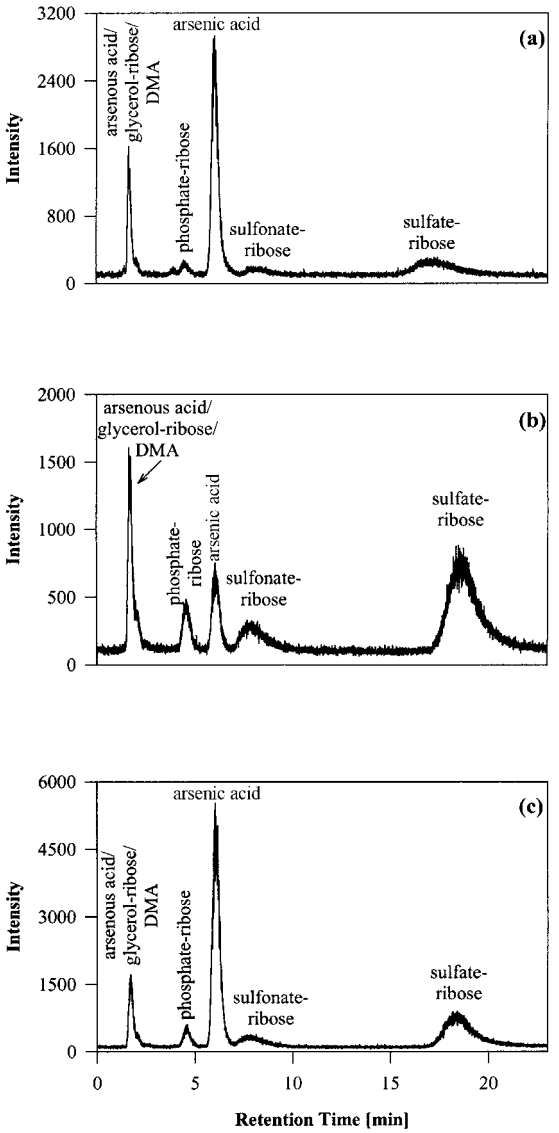

Figure 3 Chromatograms of extracts of *H. fuziforme* on the Hamilton PRP-X100 anion-exchange column (mobile phase: 20 mM $NH_4H_2PO_4$ at pH 5.6; flow rate: 1.5 cm^3 min^{-1}; column temperature: 30 °C; 50 mm^3 injected). (a) Sample extracted with 1.5 M orthophosphoric acid (0.5 g sample in 50 cm^3, extract diluted 1 + 4). (b) Sample extracted with methanol/water (9 + 1) (0.5 g sample in 10 cm^3, extract diluted 1 + 9). (c) Sample extracted with water (0.5 g sample in 10 cm^3, extract diluted 1 + 9).

The results obtained for arsenic compounds in

the extracts of *H. fuziforme* mostly correspond to literature data. Yoshinaga *et al.*[22] also reported arsenic acid as the major arsenic compound in a water extract of this alga, when they carried out preliminary studies for the preparation of a certified algal reference material. They also reported the sulfate-ribose as the major arsenoribose in the water extract. They additionally extracted the algal material with methanol/water (1 + 1) or methanol. Corresponding to our results, they found that arsenic acid was extracted better by water than by methanol/water, but they achieved slightly better extraction yields for the arsenoriboses with methanol/water. Considering that the methanol-to-water ratio influences the extraction yield, these finding cannot be compared directly with our results because Yoshinaga *et al.*[22] used methanol/water (1 + 1) whereas we used methanol/water (9 + 1). Edmonds *et al.*[24] reported that *H. fuziforme* contains 50% of its arsenic as inorganic arsenic and 50% as arsenoriboses. They isolated the arsenoriboses and also found the sulfate-ribose as the dominant compound. They additionally reported the presence of the phosphate- and the sulfonate-riboses, but they did not detect the glycerol-ribose. Another arsenoribose, which was not investigated in this work, was also found by Edmonds et al.[24]

CONCLUSIONS

Results obtained in the analysis of samples for arsenic compounds depend on the extraction procedure applied. For some compounds (glycerol-ribose, arsenic acid), significant differences in the extraction yields were achieved by varying the extractant. For some compounds (DMA, AB, TETRA, phosphate-, sulfate-, sulfonate-riboses), almost the same extraction yields were achieved with the three extractants investigated. Therefore, the extractant has to be chosen with respect to the arsenic compounds present in the sample. Additionally, the matrix, and hence the manner in which the arsenic compounds are enclosed in the matrix, also influences the extraction efficiency of an extractant. To obtain the best results, the extraction procedure has to be optimized for each kind of sample. However, 1.5 M orthophosphoric acid, an extractant found to be useful for removing arsenic compounds from ant-hill material, was also shown to be successful for use with other matrices, like marine animals and algae. Future work will also include the application of this extractant to green plants, which gave low extraction yields with water and methanol/water mixtures.[26]

Acknowledgements The authors are grateful to Dr Kevin A. Francesconi (Institute of Biology, Odense University, DK-5230 Odense M, Denmark) for the phosphate-, the sulfonate-, and the sulfate-riboses and to Dr Toshikazu Kaise (Laboratory of Environmental Chemistry, School of Life Science, University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachijoji, Tokyo 192-03, Japan) for the glycerol-ribose and the alga *H. fuziforme*.

REFERENCES

1. Szpunar J. *Analyst* 2000; **125**: 963.
2. Ackley KL, B'Hymer C, Sutton KL, Caruso JA. *J. Anal. At. Spectrom.* 1999; **14**: 845.
3. McKiernan JW, Creed JT, Brockhoff CA, Caruso JA, Lorenzana JM. *J. Anal. At. Spectrom.* 1999; **14**: 607.
4. Goessler W, Kuehnelt D, Schlagenhaufen C, Slejkovec Z, Irgolic KJ. *J. Anal. At. Spectrom.* 1998; **13**: 183.
5. National Research Council of Canada. Certification Sheet: Dogfish Muscle and Liver Certified Reference Materials for Trace Metals. National Research Council of Canada: Ottawa, 1999.
6. Francesconi KA, Khokiattiwong S, Goessler W, Pedersen SN, Pavkov M. *Chem. Commun.* 2000; 1083.
7. Corr JJ. *J. Anal. At. Spectrom.* 1997; **12**: 537.
8. Wu J, Mester Z, Pawliszyn J. *Anal. Chim. Acta* 2000; **424**: 211.
9. Mattusch J, Wennrich R. *Anal. Chem.* 1998; **70**: 3649.
10. Londesborough S, Mattusch J, Wennrich R. *Fresenius' J. Anal. Chem.* 1999; **363**: 577.
11. Suner MA, Devesa V, Rivas I, Velez D, Montoro R. *J. Anal. At. Spectrom.* 2000; **15**: 1501.
12. Edmonds JS, Francesconi KA. *Nature* 1981; **289**: 602.
13. Edmonds JS, Francesconi KA. *J. Chem. Soc. Perkin Trans 1* 1983; 2375.
14. Raber G, Francesconi KA, Irgolic KJ, Goessler W. *Fresenius' J. Anal. Chem.* 2000; **367**: 181.
15. Kuehnelt D, Goessler W, Irgolic KJ. *Appl. Organomet. Chem.* 1997; **11**: 289.
16. Kuehnelt D, Goessler W, Schlagenhaufen C, Irgolic KJ. *Appl. Organomet. Chem.* 1997; **11**: 859.
17. Koelbl G, Kalcher K, Irgolic KJ. *J. Autom. Chem.* 1993; **15**: 37.
18. Bottino NR, Cox ER, Irgolic KJ, Maeda S, McShane WJ, Stockton RA, Zingaro RA. Arsenic uptake and metabolism by the alga Tetraselmis chui. In *Organometals and Organometalloids. Occurence and Fate in the Environment*, Brinckman FE, Bellama JM (eds). *ACS Symposium Series* 82. ACS: Washington, DC, 1978; 116–129.
19. Irgolic KJ, Woolson EA, Stockton RA, Newman RD, Bottino NR, Zingaro RA, Kearney PC, Pyles RA, Maeda S, McShane WJ, Cox ER. *Environ. Health Persp.* 1977; **19**: 61.

20. Vahter M, Envall J. *Environ. Res.* 1983; **32**: 14.
21. Francesconi KA, Edmonds JS. *Adv. Inorg. Chem.* 1997; **44**: 147.
22. Yoshinaga J, Shibata Y, Horiguchi T, Morita M. *Accred. Qual. Assur.* 1997; **2**: 154.
23. Yasui A, Tsutsumi C, Toda S. *Agric. Biol. Chem.* 1983; **47**: 1349.
24. Edmonds JS, Morita M, Shibata Y. *J. Chem. Soc. Perkin Trans. 1* 1987; 577.
25. Byrne AR, Slejkovec Z, Stijve T, Fay L, Goessler W, Gailer J, Irgolic KJ. *Appl. Organomet. Chem.* 1995; **9**: 305.
26. Kuehnelt D, Lintschinger J, Goessler W. *Appl. Organomet. Chem.* 2000; **14**: 411.

