APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2003; **17**: 684–692

Speciation Analysis **AOC**

Identification of arsenic species in sheep-wool extracts by different chromatographic methods

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Received 16 December 2002; Accepted 19 March 2003

Sheep on the island of North Ronaldsay (Orkney, UK) feed mostly on seaweed, which contains high concentrations of dimethylated arsenoribosides. Wool of these sheep contains dimethylated, monomethylated and inorganic arsenic, in addition to unidentified arsenic species in unbound and complexed form. Chromatographic techniques using different separation mechanisms and detectors enabled us to identify five arsenic species in water extracts of wool. The wool contained 5.2 ± 2.3 μg arsenic per gram wool. About 80% of the arsenic in wool was extracted by boiling the wool with water. The main species is dimethylarsenic, which accounted for about 75 to 85%, monomethylated arsenic at about 5% and the rest is inorganic arsenic. Depending on the separation method and condition, the chromatographic recovery of arsenic species was between 45% for the anion exchange column, 68% for the size exclusion chromatography (SEC) and 82% for the cation exchange column. The SEC revealed the occurrence of two unknown arsenic compounds, of which one was probably a high molecular mass species. Since chromatographic recovery can be improved by either treating the extract with CuCl/HCl (CAT: 90%) or longer storage of the sample (CAT: 105%), in particular for methylated arsenic species, it can be assumed that labile arsenic–protein-like coordination species occur in the extract, which cannot be speciated with conventional chromatographic methods. It is clear from our study of sheep wool that there can be different kinds of 'hidden' arsenic in biological matrices, depending on the extraction, separation and detection methods used. Hidden species can be defined as species that are not recordable by the detection system, not extractable or do not elute from chromatographic columns. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: arsenic; sheep; seaweed; analysis; methyl arsenic; wool extract

INTRODUCTION

Arsenic is a ubiquitous element, well known for its toxic properties. These are strongly dependent on the arsenic species and vary over a wide range from extremely toxic arsine, with an LD_{50} value of 3 mg per kilogram bodyweight in mice to arsenobetaine with an LD_{50} value of 10 g per kilogram bodyweight in mice.[1] Besides their acute toxicity, arsenic species can be genotoxic and carcinogenic. Therefore, it is essential to determine the arsenic species present before statements regarding the health risks posed by a given matrix can be made. Determination of molecular species is also essential for metabolic studies with different arsenic species. In most studies, where chromatographic separation of arsenic species is used, no attempt is made to look at chromatographic recovery of arsenic species. Normally, it is assumed that water-soluble arsenic species elute without problem from a high-performance liquid chromatography (HPLC) column, whether or not they are separated. During studies concerning the metabolism of arsenosugars in sheep and inorganic arsenic in fern, however, we found considerable losses of arsenic species on the column, and these were often greater than 50%.[2,3]

For the metabolic studies we used 'seaweed-eating' sheep from North Ronaldsay, a very old breed of sheep kept for meat and wool production on this island. They have developed the ability to live entirely from seaweed, mostly *Laminaria* spp., which washes ashore. *Laminaria* spp. contain

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89.7 ± 0.3 μg arsenic per gram dry weight, mainly in the form of arsenosugars. Besides a dimethylarsenic moiety, these arsenic species contain a ribofuranoside moiety with different side chains. More than 13 different arsenosugar species are currently known. They are assumed to have low acute toxicity and to pose no health risks.[4] The sheep take up the arsenosugars readily, metabolize and excrete them.[5] A small portion of the arsenic is stored in the tissue, in particular in the wool of these sheep.[5] The main urinary excretion product is sodium cacodylate (DMA(V)) and the main excretion pathway is via the kidneys into the urine.[2,5] During our studies, we found reduced chromatographic recoveries and unknown arsenic species for most of the matrices studied (urine, faeces and wool). When separating arsenic species found in human hair or urine reference material, the chromatographic recovery was nearly 100%. In order to study this problem in more detail, we focused on the factors that influenced recovery and identification of arsenic species in wool. The aim of this study was to identify the different arsenic species extractable from the wool of North Ronaldsay sheep using different chromatographic methods, to test the recovery rate from different columns and the factors influencing the quantification of different species.

MATERIAL AND METHODS

Chemicals

The arsenic standards used for identification of species and quantification of chromatograms were prepared from arsenic trioxide (As_2O_3; As(III)), sodium arsenate ($Na_2HAsO_3 \cdot 7H_2O$; As(V), DMA(V) (Strem, USA), disodium monomethyl arsonate (MMA(V)), arsenobetaine (AsB), arsenocholine (AsC), dimethylarsinoyl-glycerol-ribose (Sug.-OH) and tetramethylarsonium iodide (TMA^+). Stock solutions of 1000 mg l^{-1} of these arsenicals were prepared by dissolving the appropriate amount of solid in 1% (v/v) nitric acid, except for As(III), which was dissolved in sodium hydroxide solution and then neutralized. Trivalent monomethylarsonous acid (MMA_{red}: MMA(V) treated with reduction medium) and dimethylarsinous acid (DMA_{red}: DMA(V) treated with reduction medium) were prepared from DMA(V) and MMA(V) according to Reay and Asher.[6] The purities of these species were checked chromatographically by anion exchange chromatography. The reduction of DMA(V) gave one peak (termed DMA_{red}), whereas the separation of the MMA solution treated with the reduction medium produced two peaks eluting at 1.4 and 4.3 min (condition AI 1 PRP X100 Hamilton, 30 mM phosphate, pH 6.0) in addition to non-reduced MMA(V). The peak at 1.4 min is MMA(III), whereas the peak eluting at 4.3 min is not yet identified (termed MMA_{red}). Sodium bisulfite, sodium thiosulfate and sulfuric acid used for the reduction of pentavalent arsenicals, as well as cuprous chloride (CuCl) and hydrochloric acid (37–38%), were of analytical grade. Phosphoric acid (85% p.a.), ammonia (25% p.a.), ammonium carbonate (mixture of ammonium carbonate and ammonium hydrogencarbonate contains 30–33% ammonia, 90%), pyridine (>99% p.a.) and formic acid (100% p.a.) were used for preparing the buffers for the chromatography. All chemicals used were, if not stated otherwise, from BDH, UK. Throughout all experiments, deionized water (18 MΩ, Elga UK) was used. Phosphate and pyridine buffers were prepared by diluting the appropriate amount of phosphoric acid or pyridine in 1 l of water and adjusting the pH with ammonia solution or formic acid respectively. For the carbonate buffer, 3.2 g ammonium carbonate was dissolved in 1 l (pH 8.0). As standard reference, material human urine NIST SRM 2670 (elevated level; NIST, Gaithersburg, USA) was used to check speciation and recovery from the columns.

Collection and preparation of samples

During summer 2000, wool was collected from sheep living at North Ronaldsay. Lanolin was extracted from the wool with hexane. The wool was soaked three times for 15 min in hexane, rinsed thoroughly with water and dried. The fat-free wool was then extracted with water (*ca* 0.1 g wool per millilitre) by boiling for 6 h at 100 °C. The extract was then separated from the fibre, filtered (0.45 μm, Nylon Iso-Disc™ Filters, Sulpelco USA) and stored at 4 °C until analysis. The optimization of the extraction procedure and stability test of the extracted species is published elsewhere.[7] The water extract was injected directly onto different HPLC columns. The arsenic in each extract was determined quantitatively determined by inductively coupled plasma mass spectrometry (ICP-MS), as was the total arsenic concentration in the wool after microwave digestion using nitric acid and hydrogen peroxide with rhodium as an internal standard.[7] Since no CRM material for wool is available, the NIST SRM 2670 was used as in other studies[8] to control the quality of the arsenic speciation in an organic matrix.

Speciation by HPLC

Columns were fitted in a commercial HPLC system (LKB, Bromma, Sweden) fitted with a 20 μl sample loop, flow rate 1.0 ml min^{-1}. The column outlet was connected via a T-piece with a second pump that supplied a 10 μg l^{-1} caesium solution as an internal standard. The T-piece was connected directly to the ICP-MS nebulizer via a 30 cm Teflon tube. In addition to m/z 75 for arsenic, m/z 77 (for chloride interference) and m/z 133 caesium were monitored with a dwell time of 500 ms. Peak areas were used for quantification using the WINFASS program.[9] Since the response of ICP-MS to different arsenic species is identical, only DMA(V) was used for quantification. The columns were a Sulpelco LC-SCX (250 mm × 4.6 mm) for cation exchange, a Hamilton PRP X100 (100 × 4.1 mm) for anion exchange and an Asahipak GS220 (300 × 7.6 mm) as a size exclusion chromatography (SEC) column for small molecules. The latter column is made of a polyvinyl alcohol matrix and has considerable anion exchange properties. The mobile phase for the cation exchange column was a 20 mM pyridine buffer at pH 3.0 adjusted with formic acid (referred to as CAT). For the anion exchange (AI) and SEC column, different mobile phases were tested. We tested 30 mM ammonium phosphate buffer at pH 6.0 (referred to as AI 1 and SEC 1) and 20 mM carbonate buffer at pH 8.0 (referred to as AI 2 and SEC 2). All separations were done at room temperature. Each column was equilibrated with at least 300 ml mobile phase. Each sample was injected twice for the determination of retention times and recovery. DMA(V) standards between 10 and 100 µg l^{-1} for the quantification of the chromatograms were prepared by diluting the stock solution with water. The concentration in the standards was checked against a 1000 mg l^{-1} arsenic standard for ICP-MS (BDH, UK).

Arsenic determination by ICP-MS

A Spectromass 2000 spectrometer (Spectro Analytical Instruments, Kleve Germany) fitted with a Meinhard nebulizer and water-cooled cyclonic spray chamber was used for ICP-MS. The instrument was optimized for arsenic intensity each day and either used with an autosampler for quantitative determination of arsenic or connected with a 30 cm Teflon tube to the outlet of the HPLC column.

Species identification by electrospray ionization mass spectrometry (ESI-MS)

An Agilent HP1100 series LC/MSD (Agilent Technologies, UK) fitted with an atmospheric pressure ionization-electrospray (API-ES) spray head was used to identify the methylated arsenic species. These measurements were done using the anion exchange column with 20 mM carbonate buffer, since phosphate buffer is not useful for ESI-MS and the anion exchange column offered the best separation efficiency. The flow rate of the HPLC was 0.5 ml min^{-1}. ESI-MS was carried out in the positive scan mode (m/z 50 to 500) with a fragmentor voltage of 100 V, nebulizer pressure of 40 psi, drying gas (nitrogen; nitrogen generator, Whatman, UK) with a 12 l min^{-1} flow rate at 350 °C and a capillary voltage of 4000 V. The mass calibration and other parameters were checked regularly and optimized when necessary using the standard solution supplied by Agilent.

RESULTS AND DISCUSSION

Results from preliminary experiments showed that we could expect at least five different arsenic species in the water extract of wool.[7] For the identification of these species, efficient separation and different chromatographic methods are necessary. During recent years, various 'standard' methods for the separation of arsenicals in biological material have emerged, namely anion exchange chromatography using phosphate or carbonate buffer and cation exchange chromatography using pyridine buffer.

The analysis of the urine NIST SRM 2670 (elevated level) using all five separation conditions showed that the urine

Table 1. Retention times of arsenic standard species and for arsenic species in wool extract (in square brackets) for each separation condition ($n = 10$) (MMA_{red} standard shows two peaks)

	Retention time (min)				
Species	CAT	AI 1	AI 2	SEC 1	SEC 2
As(III)	3.2 ± 0.0 [with MMA(V)]	1.0 ± 0.1 [0.9 ± 0.1]	1.0 ± 0.1 [1.0 ± 0.0]	10.8 ± 0.4 [10.6 ± 0.5]	10.5 ± 0.4 [10.3 ± 0.3]
As(V)	2.6 ± 0.1 [2.7 ± 0.1]	2.5 ± 0.1 [2.3 ± 0.1]	7.2 ± 0.2 [7.0 ± 0.3]	6.2 ± 0.1 [6.2 ± 0.2]	5.7 ± 0.1 [5.8 ± 0.1]
DMA(V)	4.4 ± 0.1 [4.5 ± 0.0]	1.2 ± 0.1 [1.1 ± 0.0]	1.5 ± 0.1 [1.5 ± 0.0]	6.9 ± 0.1 [7.0 ± 0.0]	6.3 ± 0.1 [6.2 ± 0.0]
MMA(V)	3.4 ± 0.1 [3.5 ± 0.0]	1.5 ± 0.1 [1.5 ± 0.0]	2.0 ± 0.1 [2.0 ± 0.2]	6.3 ± 0.1 [with As(V)]	6.2 ± 0.1 [with DMA(V)]
DMA_{red}	3.3 ± 0.1 [with MMA(V)]	7.9 ± 0.2 [7.6 ± 0.3]	5.5 ± 0.1 [5.5 ± 0.2]	12.8 ± 0.5 [12.7 ± 0.1]	7.1 ± 0.1 [7.2 ± 0.1]
MMA_{red}	3.0 ± 0.1 —	4.3 ± 0.1 [4.4 ± 0.0]	4.4 ± 0.1 —	7.7 ± 0.1 —	7.7 ± 0.1 —
AsB	4.8 ± 0.1	—	—	—	—
AsC	10.6 ± 0.1	—	—	—	—
TMA^+	12.5 ± 0.1	—	—	—	—
Sug.-OH	6.8 ± 0.2	—	—	—	—
Unknown A	—	—	—	— [4.4 ± 0.2]	— [4.3 ± 0.1]
Unknown B	—	—	—	— [9.2 ± 0.2]	— [8.1 ± 0.1]

contained As(III) or AsB, As(V), DMA(V) and MMA(V). The recovery of arsenic was 98 ± 5% and the species distribution (380 ± 40 μg l^{-1} As(V), 60 ± 2 μg l^{-1} DMA(V), 20 ± 3 μg l^{-1} AsB and 12 ± 4 μg l^{-1} MMA(V)) was similar to values given in the literature using anion exchange chromatography.[8] AsB was identified and quantified by cation exchange chromatography and the other species by anion exchange chromatography.

Anionic standard compounds (As(III), As(V), DMA(V), MMA(V), DMA_{red}, MMA_{red}) at concentrations below 50 μg l^{-1} were well separated using the anion exchange column with conditions AI1 and AI2 (Table 1, Figs 1 and 2). Greater concentrations of one or more compounds led to peak overlapping. This confirms the results published by Gailer and co-workers,[10,11] who studied the separation of anionic arsenic species on the PRP X100 column in dependence of pH and phosphate concentration of the eluent. The main difference between carbonate and phosphate buffer was reversal of the elution order of DMA_{red} and As(V). The cation exchange column separated the cationic arsenic standards, but failed to separate MMA(V), MMA_{red} and DMA_{red}. (Table 1, Fig. 3). Good separation of As(III) from the rest of the anionic standards was achieved using the SEC column under the separation conditions SEC1 and SEC2. The other anionic species (As(V), DMA(V), MMA(V), MMA_{red}) did not co-elute but had only small differences in retention time, except DMA_{red}, which was also well separated using the phosphate buffer (Table 1, Figs 4 and 5).

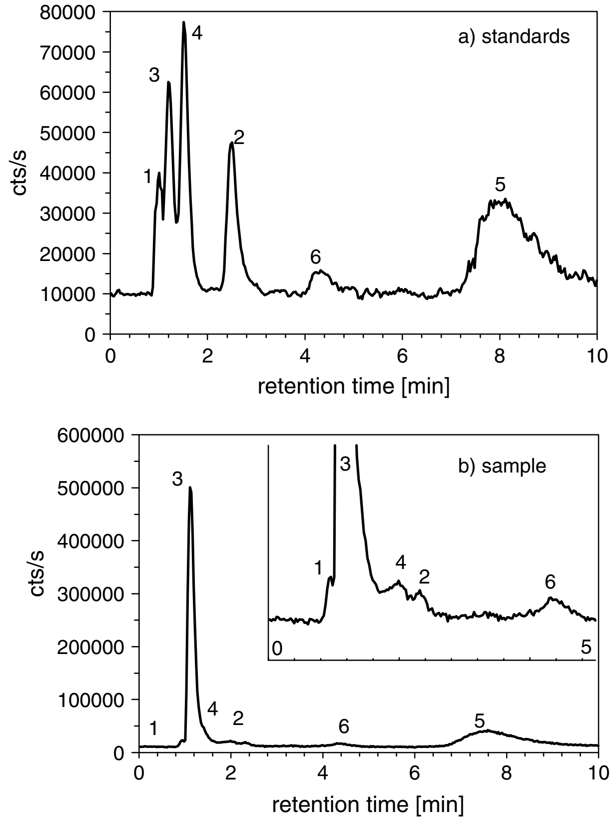

Figure 1. Separation with anion exchange chromatography, 30 mM phosphate buffer pH 6 (AI 1), of (a) arsenic species (1: As(III); 2: As(V); 3: DMA(V); 4: MMA(V); 5: DMA_{red}; 6: MMA_{red}; each *ca* 100 μg l^{-1}) and (b) wool extract.

Arsenic species in water extracts of wool

Separation of wool extracts showed that DMA(V) and DMA_{red} represent 70 to 80% of the arsenic species (Table 2). Trivalent and pentavalent inorganic arsenic accounts for 6 to 20% of total arsenic found in wool extract, and MMA(V)/MMA_{red} contribute 3 to 7% to the total arsenic concentration (Table 2). No cationic species, such as AsB, AsC or TMA^+, were identified in the wool extract (Table 1, Fig. 3b). Comparison of the retention times of the arsenic species in wool extract and standard solutions, as well as spiking experiments, showed

Table 2. Arsenic species concentration in wool (as arsenic) determined by external calibration (EC) and standard addition (SA). Different wool extracts used for each separation condition ($n = 2$)

		As concentration in wool (mg kg^{-1})								
Conditions	Standard	As(III)	As(V)	DMA(V)	DMA_{red}	MMA(V)	Unknown A	Unknown B	Sum	Total extractable
AI 1	EC	0.22 ± 0.02	0.15 ± 0.04	3.3 ± 0.6	1.4 ± 0.24	0.21 ± 0.02	—	—	5.3 ± 0.81	7.6 ± 0.43
	SA	0.23 ± 0.02	0.12 ± 0.02	3.0 ± 0.5	1.5 ± 0.25	0.21 ± 0.05	—	—	5.0 ± 0.78	
AI 2	EC	0.20 ± 0.02	0.25 ± 0.08	1.7 ± 0.8	1.3 ± 0.06	0.13 ± 0.03	—	—	3.5 ± 0.78	5.1 ± 0.37
	SA	0.25 ± 0.02	0.23 ± 0.03	1.3 ± 0.6	1.2 ± 0.02	0.14 ± 0.04	—	—	3.1 ± 0.63	
CAT[a]	EC	3.3 ± 0.15	0.19 ± 0.02	2.5 ± 0.2			—	—	6.1 ± 0.34	7.5 ± 0.22
	SA	3.2 ± 0.02	0.19 ± 0.04	2.3 ± 0.2			—	—	5.7 ± 0.11	
SEC 1	EC	0.08 ± 0.01	0.15 ± 0.02	2.9 ± 0.1	0.34 ± 0.02	0.08 ± 0.01	0.12 ± 0.01	0.07 ± 0.04	3.6 ± 0.24	5.9 ± 0.10
	SA	0.09 ± 0.02	0.13 ± 0.01	3.1 ± 0.1	0.35 ± 0.01	0.09 ± 0.01	—[b]	—[b]	4.0 ± 0.02	

[a] As(III), DMA_{red} and MMA(V) co-elute.
[b] No standards available.

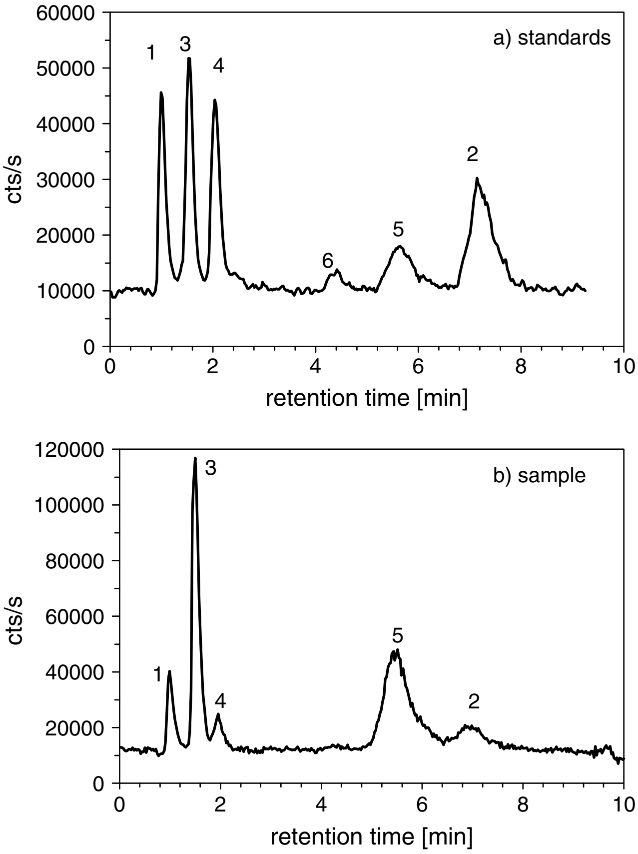

Figure 2. Separation with anion exchange chromatography, 20 mM carbonate buffer pH 8 (AI 2), of (a) arsenic species (1: As(III); 2: As(V); 3: DMA(V); 4: MMA(V); 5: DMA_{red}; 6: MMA_{red}; each *ca* 100 μg l^{-1}) and (b) wool extract.

that the wool matrix does not influence the retention times of the known arsenic species. The spiking experiments also served to confirm the identity of the species. Separation with the SEC column showed two unknown arsenic species with elution times of 4.4 min (A) and 9.2 min (SEC 2: 8.1 min: B) that did not co-elute with any standard (Figs 4b and 5b). One of the unknown species seems to be an arsenic compound with greater molecular mass, because it elutes faster than the other species. The retention times of these species using the cation and anion exchange columns are unknown; they either co-elute with other species or absorb to the columns.

Additional verification of the identity of the methylated arsenic species was carried out using anion exchange chromatography with ESI-MS detection. The Hamilton column was used with carbonate buffer. The presence of DMA(V) and MMA(V) was confirmed by their respective molecular mass peaks at m/z 139 (DMA(V) + H) (Fig. 6a–e) and m/z 141 (MMA(V) + H) (not shown) and spiking experiments. DMA_{red} did not give a molecular peak at the expected mass of DMA(III) ($(CH_3)_2AsOH + H$) of m/z 123, but showed, when present in high concentration, a small peak at m/z 139 corresponding to DMA(V) ($(CH_3)_2AsO(OH) + H$). If post-column hydride generation is used at pH 6 then this species generates a volatile arsine.[7] The conversion of DMA_{red} at the API interface was confirmed by using flow injection of pure DMA_{red}, where a signal at m/z 139 was also found. The problems encountered by using solely ESI-MS for the identification of species in a crude extract of biological material can clearly be seen at the m/z 139 trace (Fig. 6a). The trace of m/z 139 shows, in addition to the signal from DMA(V), another strong signal from a non-arsenic-containing compound at a retention time of 22 min. Spiking the extract with DMA(V) (Fig. 6a) showed that the signal at 3.9 min increased, but not the signal at 22 min (peak D). Using ICP-MS for detection, it can be seen that no arsenic-containing compound elutes at 22 min. Comparison of mass spectra of these two peaks shows clear differences (Fig. 6b–e). The signal at 22 min (Fig. 6d and e) contained m/z 185, 115 and 139 as major masses, whereas the signal at 3.7 min (Fig. 6b and c) showed no clear fragments: this could be attributed to DMA(V), since the fragmentor voltage was not high enough to fragment DMA(V) efficiently. Since many different organic species elute at this time, the mass spectrum is not clear. Spiking the extract with DMA(V) led to a strong

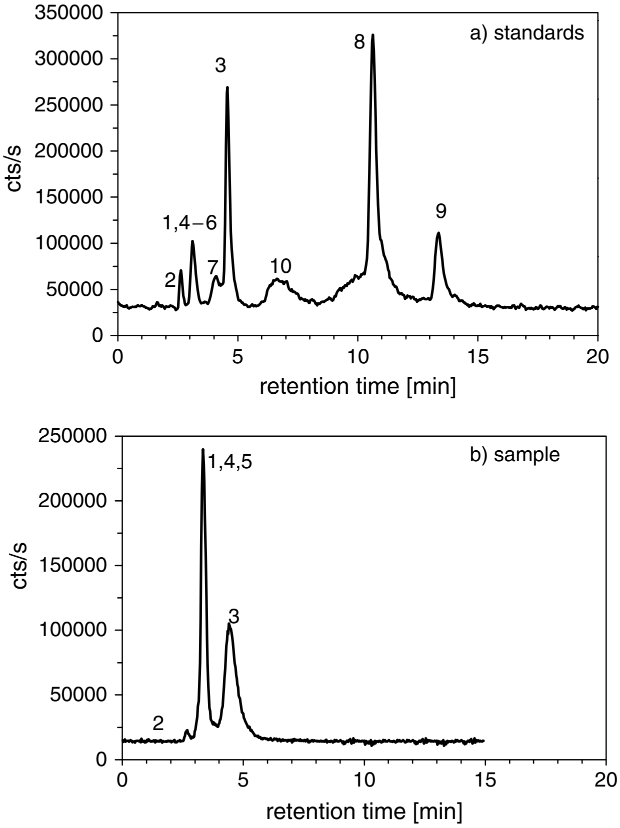

Figure 3. Separation with cation exchange chromatography, 20 mM pyridine buffer pH 3 (CAT), of (a) arsenic species (1: As(III); 2: As(V); 3: DMA(V); 4: MMA(V); 5: DMA_{red}; 6: MMA_{red}; each *ca* 100 μg l^{-1}) and (b) wool extract.

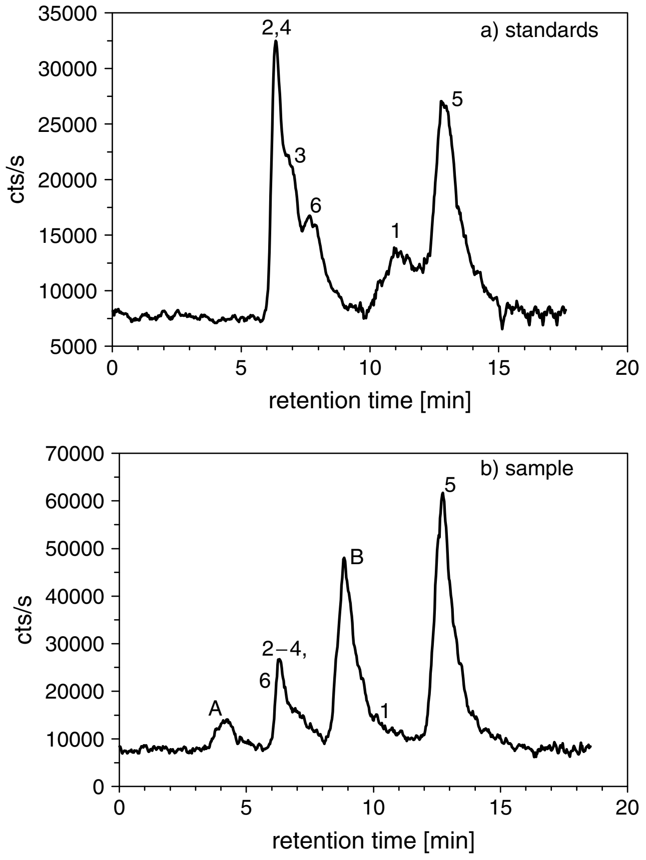

Figure 4. Separation with SEC, 30 mM phosphate buffer pH 6 (SEC 1), of (a) arsenic species (1: As(III); 2: As(V); 3: DMA(V); 4: MMA(V); 5: DMA_{red}; 6: MMA_{red}; each *ca* 100 μg l^{-1}) and (b) wool extract.

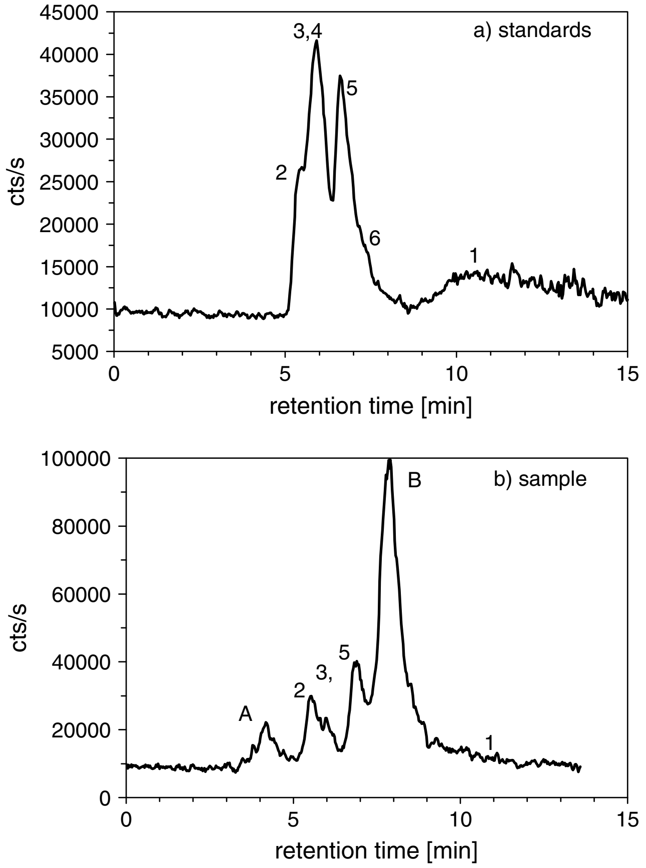

Figure 5. Separation with SEC, 20 mM carbonate buffer pH 8 (SEC 2), of (a) arsenic species (1: As(III); 2: As(V); 3: DMA(V); 4: MMA(V); 5: DMA_{red}; 6: MMA_{red}; each *ca* 100 μg l^{-1}) and (b) wool extract.

increase in the signal of m/z 139. Confirmation of DMA(V) was achieved by spiking and comparing signal intensities, in addition to comparison of retention times. Clear identification of the arsenic species in crude biological samples is only possible with a combination of ESI-MS and ICP-MS, spiking and consideration of fragmentation patterns from the ESI-MS.

The total extraction efficiency of arsenic from wool was about $80 \pm 9\%$. Complete recovery of extracted arsenic species was not possible after chromatographic separation, as determined by both external calibration and calibration via standard addition. Recovery rates were calculated by comparing the total extractable arsenic with the sum of arsenic species quantified from the chromatograms. For external calibration of the chromatograms, DMA(V) solutions of 10 to 100 μg l^{-1} were used. The mean recovery rate of total arsenic using cation exchange chromatography was 82%, with SEC it was 68% and the worst recovery, of about 45%, was found using anion exchange chromatography (Table 3). For standard addition, As(III), As(V), DMA_{red}, DMA(V) and MMA(V) were used. Standard addition did not improve recovery of the known species compared with external calibration (Table 2). Reduced recovery, therefore, is not due to a depression of arsenic intensity caused by co-elution of other compounds with arsenic species or differences in the nebulization process that may have interfered with the external calibration. There are obviously water-extractable arsenic species in wool that do not elute from the columns. On the other hand, human hair reference material GBW09 101, with a very similar matrix to wool, showed a chromatographic recovery of about 100%, despite the fact that the extraction procedure was the same as that used for wool.[7] It should be mentioned here that the chromatographic recovery from urine samples of the same sheep also varied between 4 and 100%, which might indicate that the metabolism of arsenosugars produces species that are not easy to elute (H. Hansen, personal communication).

To characterize these non-eluting compounds further, their stability during storage and against CuCl/HCl treatment was tested (Tables 3 and 4). Storage of the extract resulted in an increased chromatographic recovery (Table 3), mainly due to an increase in the concentrations of DMA(V) and MMA(V)

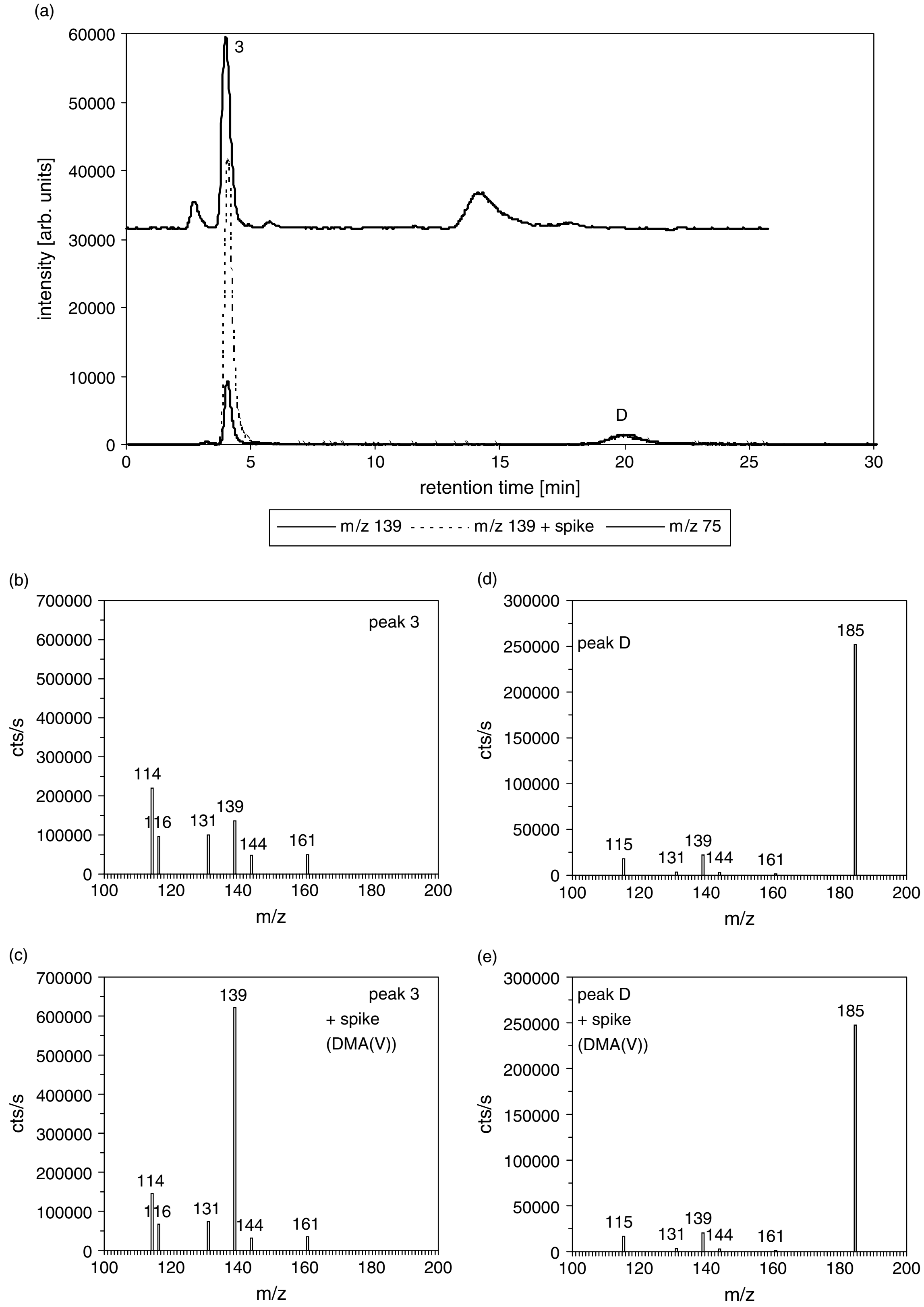

Figure 6. (a) Comparison of arsenic trace (*m/z* 75) measured by ICP-MS and trace *m/z* 139 (non-spiked and spiked with DMA(V) measured by ESI-MS, PRP X100, 20 mM carbonate, pH 8, flow rate 0.5 ml min^{-1}; peak 3: DMA(V); peak D: unknown organic compound. (b) Mass spectra of peak 3 without and (c) with spike of DMA(V). (d) Mass spectra of peak D without and (e) with spike of DMA(V).

(Table 5). The ability of CuCl/HCl to destroy arsenic–protein complexes[11] was also tested. The wool extract was mixed with 0.2 M CuCl/0.2 M HCl solution at a ratio of 1:10 and heated for 5 h at 37 °C. This improved the total recovery of arsenic by about 20% (Table 3). During this process, DMA_{red} was completely converted to DMA(V). The amount of As(III) increased by about 85%, As(V) increased by about 82%, MMA(V) increased 77% and DMA(total) increased by about 31% (Table 4), taking into account the results of the anion exchange and SEC columns. The results of the cation exchange column are not considered, because the separation efficiency of this column was not good enough to distinguish between most of the species. Although the recovery rates improved for anion exchange and SEC columns, only about 70% of the arsenic eluted from the anion exchange column and 83% from the SEC column, whereas recovery of the cation exchange column was around 90%. Since no new species were found with the cation exchange column, either co-elution of these species occurs or the species are acid-labile and decompose at pH 3. The latter is more likely, because separation of the extract using the anion exchange column with a phosphate buffer at pH 3 gave a recovery of 80% and no new species were found (data not shown). The conclusion of these experiments is that some of these non-elutable arsenic species are obviously protein-bound DMA(V) and MMA(V), since the use of CuCl/HCl is improving the chromatographic recovery of these species. The sensitivity to storage duration and the pH of the eluent also suggest the occurrence of protein-bound arsenicals in the extract.

Table 3. Recovery of extractable arsenic species from wool with different columns and separation conditions. Influence of CuCl/HCl treatment tested with two extracts from the same wool. Influence of storage time on recovery tested with two extracts from the same wool

	Recovery (%)				
	CAT	AI 1	AI 2	SEC 1	SEC 2
Untreated ($n = 15$)[a]	82 ± 19	42 ± 19	46 ± 17	67 ± 17	69 ± 16
Untreated ($n = 2$)	77 ± 1	30 ± 2	52 ± 2	50 ± 2	
With CuCl/HCl ($n = 2$)	90 ± 2	68 ± 3	75 ± 3	83 ± 3	
Storage at 4 °C for 12 days ($n = 2$)			105 ± 10		

[a] The large standard deviation is due to the use of wool from ten different sheep from North Ronaldsay.

Table 4. Comparison of arsenic species concentration in wool (as arsenic) for samples untreated or treated with CuCl/HCl ($n = 2$)

		As concentration in wool (mg kg^{-1})						
		As(III)	As(V)	DMA(V)	DMA_{red}	MMA(V)	Unknown A	Unknown B
AI 1		0.05 ± 0.005	0.05 ± 0.004	0.33 ± 0.02	0.33 ± 0.02	0.04 ± 0.001		
	+ CuCl/HCl	0.11 ± 0.02	0.1 ± 0.02	0.88 ± 0.04		0.15 ± 0.007		
AI 2		0.01 ± 0.002	0.02 ± 0.004	0.87 ± 0.04	0.13 ± 0.05	0.01 ± 0.0004		
	+ CuCl/HCl	0.01 ± 0.001	0.02 ± 0.002	1.2 ± 0.08		0.03 ± 0.001		
CAT		0.18 ± 0.006	0.17 ± 0.003	0.12 ± 0.002	0.33 ± 0.01	—[a]		
	+ CuCl/HCl	0.20 ± 0.002	0.24 ± 0.006	1.5 ± 0.04		0.19 ± 0.006		
SEC 1		0.09 ± 0.03	0.09 ± 0.005	0.2 ± 0.02	1.0 ± 0.04	0.15 ± 0.004	0.05 ± 0.004	0.08 ± 0.02
	+ CuCl/HCl	0.1 ± 0.01	0.1 ± 0.09	1.5 ± 0.14		0.12 ± 0.008	0.04 ± 0.008	0.12 ± 0.01

[a] Co-elutes with DMA_{red}.

Table 5. Comparison of arsenic species concentration in wool (as arsenic) for samples M1 (separation directly after extraction) and M2 (after 12 days, storage at 4 °C), using separation condition AI 2

	As concentration in wool (mg kg^{-1})				
	As(III)	As(V)	DMA(V)	DMA_{red}	MMA(V)
M1	0.07 ± 0.01	0.12 ± 0.01	0.04 ± 0.01	0.30 ± 0.03	0.01 ± 0.001
M2		0.28 ± 0.03	0.69 ± 0.13	0.17 ± 0.14	0.16 ± 0.02

For the unequivocal identification of arsenic species in biological samples, columns with different separation mechanisms and different detectors must be used. For aqueous wool extracts, the most effective separation is possible using an anion exchange column with phosphate or carbonate buffer. However, the most efficient separation does not always mean a 100% recovery of the species applied to the column. Separation efficiency and recovery need to be tested individually and optimized to the best possible compromise. Thus, real samples must be used for the optimization, in order to include the influence of the sample matrix, which cannot easily be predicted using pure standard solutions. It is highly recommended that mass balance of the eluting compounds and the total element concentration in the extract be conducted to identify the contribution of non-eluting compounds. Since our aim was to analyse species on-line, the compatibility of the separation technique with the detector is also a limiting factor. For the speciation of arsenic in wool the most promising way seems to be separation of the extract with and without CuCl/HCl treatment using an anion exchange column. This might be true for all matrices that contain arsenic species in a complexed form, such as arsenic–protein complexes.

Dimethylated arsenic species were the dominant form of arsenic, and monomethylated arsenic and inorganic arsenic were found in comparatively lower concentrations in the wool extract of sheep feeding on seaweed. Some water-soluble arsenic species formed in wool are acid labile and not stable long-term.

There can be different kinds of 'hidden' arsenic in biological matrices, depending on the extraction, separation and detection methods. Species where the detection method is the determining factor are detectable by using another detection method. For species that are not extractable, a change of the extraction medium sometimes helps (e.g. different polarity of the extractant). The most difficult problem is to find a method to measure species that do not elute from columns, since a change of the column is not necessarily a remedy.

Acknowledgements

We would like to thank the Sheep Court of North Ronaldsay and Kevin Woodbridge in particular for permission. Special thanks to Professor W. R. Cullen for the donation of MMA(V), AsB, AsC, Sug.-OH and TMA$^+$.

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