Anal Bioanal Chem (2002) 372 : 457–466
DOI 10.1007/s00216-001-1182-x

SPECIAL ISSUE PAPER

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Multidimensional liquid chromatography with parallel ICP MS and electrospray MS/MS detection as a tool for the characterization of arsenic species in algae

Received: 3 July 2001 / Revised: 1 October 2001 / Accepted: 13 October 2001 / Published online: 21 December 2001

Abstract An analytical strategy was developed for the characterization of arsenic species in a *Laminaria* algae. The approach was based on multidimensional liquid chromatography (LC) including sample extract cleanup by size-exclusion LC, separation of arsenic species by anion-exchange LC, verification of the chromatographic purity of arsenic-containing fractions, and their further purification, if necessary, by reversed-phase (RP) HPLC. The complementarity of ICP MS, used as the chromatographic detector, and ES MS/MS, employed for the identification of the peaks observed, was demonstrated. The species found were: arsenosugar A 11.7±0.5 μg g^{-1}, As^{V} 10.9± 2.1 μg g^{-1}, arsenosugar B 2.22±0.07 μg g^{-1}, arsenosugar D 1.5±1.2 μg g^{-1}, a newly detected arsenosugar 1.13± 0.07 μg g^{-1}, arsenosugar C 0.61±0.04 μg g^{-1}, DMA 0.42± 0.02 μg g^{-1} and these accounted for >99% of the arsenic present. The identities of all the species, except the newly detected compound, were doubly checked by matching the retention times of chromatographically pure (after the 3rd LC dimension) species with standards and by ES MS/MS.

Keywords Speciation · Arsenic · Algae · Liquid chromatography

Introduction

The complex metabolism pathways and bioaccumulation of arsenic have resulted in the occurrence of more than 25 different species of this element in a broad concentration range (up to hundreds of μg g^{-1}) in marine plants and animals [1, 2]. In addition to simple inorganic anions of As^{III} and As^{V} and methylarsinic acids, these include arsenobetaine, which is the major organoarsenic compound in marine fauna, and arsinoyl-ribosides (arsenosugars) which are products of As metabolism in algae and have been found in some bivalves [1, 2].

Essential differences in toxicities of the arsenic species have spurred the development of analytical methods for their specific determination, especially in countries with a considerable consumption of seafood [3]. High performance liquid chromatography combined with inductively coupled plasma (ICP) MS detection (HPLC-ICP MS) has been the primary analytical technique used to study speciation, that is the determination of one or more individual chemical species, of arsenic in biological materials [3, 4, 5]. Limitations of this technique have, however, been rapidly appearing with the increasing number of studies of natural samples. They include a risk of co-elution of arsenic species, the impossibility of identifying species for which standards are unavailable, and a risk of misidentification of species based on the retention time matching with standards.

Indeed, the large number of species with similar physicochemical properties present in a marine sample and the insufficient separation efficiency of chromatographic techniques make the co-elution of some species in a single separation mechanism practically unavoidable, regardless of this mechanism. This problem can be partly alleviated by careful optimization of the separation conditions [5, 6] or by running the same sample with different principle separation techniques, in parallel [7] or in sequence [8, 9], was also reported.

In terms of identification of arsenic species, the pioneering works used NMR and X-ray crystallography which required processing kilograms of samples [10, 11]. Alternatives, on the analytical laboratory scale, appeared in the late 80s with the wider availability of mass spectrometry, employing desorption [12], fast atom bombardment (FAB) [13] and electrospray (ES) ionization [14, 15,

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16]. However, although mass spectra were abundantly published, successful attempts of identification of arsenic species in biological matrices were scarce. The studies lacked a comprehensive approach and were limited to individual target compounds. The use of FAB MS enabled Pergantis et al. to identify one arsinoyl-riboside in a Sargassam extract; the positive ES MS identification of another riboside could not be confirmed by HPLC-ICP MS [13]. The coupling of HPLC with electrospray tandem mass spectrometry (ES MS/MS) allowed the detection of dimethylarsinic acid (DMA), arsenobetaine, a cationic arsenosugar in oyster [14], and three arsenosugars in kelp [15]. In our former work, two arsenosugars in *Hizikia* and *Laminaria* algae were detected by ES MS/MS after two-dimensional HPLC [8, 9]. The same sugars were detected independently in *Laminaria* and one more in *Hizikia* algae by LC-ES MS using variable fragmentor voltages to allowing controlled fragmentation of analyte species [17].

The common denominator of the above works was the awareness of the authors that only a few of the arseno-compounds present in a sample could actually be identified. The insufficient sensitivity of ES MS/MS problematic matrix suppression effects, and the impossibility of unambiguous attribution of peaks in mass spectra to arseno-compounds meant that only a few major species could be targeted. Variable fragmentor voltage [17] or the parallel use of ICP MS [8, 9] was necessary to obtain assurance of arsenic presence in the molecular ion. An elegant demonstration of ES MS for the identification of an unknown organoarsenic compound was recently reported [18]. The major limitation of ES MS is the insufficient purity of the arseno-species introduced into the ion source.

The aim of this study was to develop an analytical strategy able to provide a complete map of arsenic speciation (species identity and their relative mass contribution to the total element concentration) in a *Laminaria* algae on the analytical laboratory scale, by exploiting high performance analytical tools such as multidimensional HPLC, ICP MS for sensitive detection of arsenic in LC effluents, and electrospray MS/MS for the identification of the species detected.

Experimental section

Apparatus

Low-pressure LC was carried out by means of a model Minipuls 3 peristaltic pump (Gilson, France) and a Hewlett Packard series 1100 pump (Hewlett-Packard, Waldbronn, Germany) was used as the sample delivery system for HPLC separations. For the latter, injections were made using a Model 7725 injection valve with a 100 μL injection loop (Rheodyne, CA, USA). All the HPLC connections were made of PEEK tubing (internal diameter (i.d. 0.17 mm) whereas 1 mm i.d. tubing was used in semi-preparative setups. Fractions for off-line analyses were collected using a Model FC-2 automatic fraction collector (Dynamax, France). A Model LP3 lyophilizer (Jouan, Saint-Herblain, France) was used for freeze-drying of sample extracts and eluates.

The ICP MS instrument used in this work was an ELAN 6000 (PE-SCIEX, Thornhill, ON, Canada). The sample introduction system included a Ryton™ spray chamber fitted with a cross flow nebulizer. For the determination of As in chromatographic fractions, samples were fed by means of a Minipuls 3 peristaltic pump that was also used to drain the spray chamber.

The ES MS instrument used was an API 300 (PE SCIEX, Thornhill, ON, Canada) pneumatically-assisted electrospray (ionspray) triple-quadrupole mass spectrometer. Samples were introduced using a syringe pump (Harvard Apparatus, South Natick, MA, USA).

Ultrasonic extraction was performed using a Branson 1210 ultrasonic cleaner (Branson, Danbury, CT, USA). The supernatant was separated by centrifugation using model Hettich Universal 16 centrifuge (Hettich, Tuttlingen, Germany). A Model Hima CS120GX centrifuge (Hitachi, Tokyo, Japan) was used to remove particles on the dissolution of freeze-dried residues.

Chromatographic material and reagents

A 700×16 mm column (Pharmacia, Uppsala, Sweden) was filled with G-15 Sephadex Gel (Pharmacia) according to the manufacturer's protocol. For semi-preparative anion-exchange LC an identical column was filled with DEAE Sephadex A-25. RP HPLC was carried out using an Inertsil ODS-2 4.6×250 mm×5 μm column (Supelco, St. Quentin Fallavier, France).

Analytical reagent grade chemicals were used throughout unless otherwise stated. Methanol (Sigma-Aldrich, St. Quentin Fallavier, France) was of LC grade. Water was purified to 18.2 MΩ cm resistance using a Milli-Q water purification system (Millipore, Bedford, MA, USA). For SE chromatography a 1% (v/v) solution of acetic acid solution was prepared. A 50 mM ammonium carbonate buffer (pH 8.90) was prepared by the dissolution of 3.9275 g of an equimolar mixture of $(NH_4)_2CO_3$ and NH_4HCO_3 in 1 L of water. The dilute buffer solutions (2.5, 5, and 10 mM; pH 8.85± 0.05) were prepared by the dilution of this solution with water. The 4 mM malonic acid solution was prepared by dissolving 0.2082 g of malonic acid in 500 mL water. The mobile phases were degassed by sonication for 20 min before chromatography.

Standards and samples

Standard stock solutions (1.00 mg mL^{-1}) were prepared by dissolving the respective compound in water. Arsenic (III) and arsenic (V) standards were prepared using sodium arsenate and sodium arsenite (Sigma Aldrich, St. Quentin Fallavier, France). The four dimethylarsinoyl-riboside derivatives: 3-[5'-deoxy-5'-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropanesulfonic acid (referred to later as sugar A), 3-[5'-deoxy-5'-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropylene glycol (sugar B), 3-[5'-deoxy-5'-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropyl hydrogen sulfate (sugar C), and 3-[5'-deoxy-5'-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropyl 2,3-hydroxypropyl phosphate (sugar D), were kindly donated by Prof J. Edmonds (De Montfort University, Leicester, UK). Arsenobetaine was a gift from Prof W. Cullen (UBC, Vancouver, Canada). Standard solutions of the other arsenic compounds were a gift from Dr Erik Larsen (Danish Veterinary and Food Administration, Søborg, Denmark). Secondary stock solutions (1 μg mL^{-1}) of the compounds were prepared for HPLC-ICP MS analyses. Working solutions were prepared on the day of analysis by the appropriate dilution of the stock solutions with water. The stock solutions were kept in the refrigerator at 4 °C in the dark.

The *Laminaria* macroalgae collected at the coast of Iceland were available on the French food market as a food product ground into powder. The commercial product is soaked in cold water for 10 min before preparation for consumption in soups or salads. According to the analysis carried out by an accredited laboratory (DGCCRF, Talence, France) it contained 30 μg g^{-1} of arsenic (by GF AAS), 60% of which was found to be able to generate hydrides under standard conditions [8].

Procedures

Extraction of arsenic species from algal samples

Arsenic species were extracted with a methanol/water mixture (1:1, v/v) according to Shibata and Morita [19] and Lai et al. [20]. A sample of 1 g of an algae powder was weighed into a 40 mL centrifuge tube and diluted with 20 mL of methanol/water (1:1, v/v). The tube was sonicated for 3 h and centrifuged for 20 min at 2500 rpm. After centrifugation the extract was removed using a Pasteur pipette and placed in a round-bottom flask. The extraction procedure was repeated once with methanol/water mixture (9:1, v/v). The residue was then washed with 10 mL of methanol/water (9:1, v/v) and the tube was then sonicated for 20 min and centrifuged. The washing procedure was repeated three times and the extracts and washings were combined and solvent was removed under reduced pressure at 40 °C using a rotary evaporator. The residue after evaporation to dryness was dissolved in 10 mL of water prior to analyses.

Extract cleanup by size-exclusion chromatography

The SE column was conditioned by washing with H_2O and flushing the column with eluent (1% (v/v) acetic acid). A 5 mL sample of the extract solution was ultracentrifuged (50 000 rpm at 4 °C for 30 min) and filtered through a 0.45 μm filter. A 4 mL extract sample was eluted isocratically with the acetic acid solution at a flow rate of 0.9 mL min^{-1}. Fractions (1.8 mL) were collected every 2 min for 2.5 h. Arsenic was determined in a 100 μL aliquot of each fraction to reconstitute the elution profile. Fractions 32–50 were pooled and freeze-dried. The residue was re-dissolved in 4 mL water and ultracentrifuged.

Purification of arsenic compounds by anion-exchange chromatography

An aliquot of 3.5 mL of the solution prepared as above was eluted from the anion-exchange column with the carbonate buffer at 0.9 mL min^{-1}. The following program was used: 0–3 h (2 mM buffer, fractions collected every 2 min), 3–7 h (5 mM buffer, fractions collected every 2 min), 7–10 h (5 mM buffer, fractions collected every 4 min), 10–15 h (10 mM buffer, fractions collected every 5 min), 15–25 h (50 mM buffer, fractions collected every 5 min). An aliquot of each fraction was analyzed by ICP MS to reconstruct the elution profile.

Verification of the chromatographic purity by reversed-phase and cation-exchange HPLC

An aliquot (100 μL) of each of the fractions comprising the apex of each of the peaks: N°40, 48, 93, 247, 284, 315, 326, 371, and 394 was injected onto the analytical RP HPLC. The elution was carried out isocratically with 4 mM malonic acid. In addition, a 100 μL aliquot of fractions 40 and 48 was injected onto a cation-exchange column and isocratically eluted with 20 mM pyridine-formate buffer (pH 3.0) at 1 mL min^{-1}. The rest of the fractions were freeze-dried individually for ES MS/MS analyses.

Purification of arsenic compounds by reversed-phase chromatography

Fractions 39 and 41were combined and freeze-dried (the same was done with fractions 47 and 49). The residue was re-dissolved in 100 μL of water and eluted from the RP HPLC column with 4 mM malonic acid at 0.75 mL min^{-1}. Fractions were collected every 30 s. An aliquot was analyzed by ICP MS to reconstitute the elution profile. The fraction corresponding to the peak (one for fraction 40 and two for fraction 48) apex was combined with the adjacent fractions and freeze-dried for ES MS analyses.

ICP MS determination of As in eluates and reconstitution of elution profiles

The ICP MS measurement conditions (nebulizer gas flow, RF power and lens voltage) were optimized daily using a standard built-in software procedure. Typically, a nebulizer gas flow of 1.05 L min^{-1}, ICP RF power of 1100 W, and a lens voltage of 8 V were used. The chromatographic off-line elution profiles were obtained by introducing an aliquot of the eluate into an ICP until a steady state signal was established for 3–5 s. The chromatogram was reconstructed with Microsoft Excel by plotting the intensity of this signal versus the fraction number (corresponding to the elution time or volume). For the on-line acquisition of elution profiles the dwell time was 60 ms and the number of replicates allowing the continuous data acquisition for the duration of the chromatogram was applied. Typically, 1000 replicates were applied to give a scan duration of 1323 s. Chromatographic signals were processed using the Turbochrom4™ software (Perkin–Elmer). All signal quantifications were done in the peak area mode.

Electrospray MS/MS conditions

A sample solution was prepared from a lyophilized chromatographic fraction by dissolution in water and addition of an appropriate amount of methanol (30%) and formic acid (0.6%) to obtain the highest sensitivity. The optimization of the ES MS conditions was done using a 1 μg mL^{-1} solution (10 μg mL^{-1} for arsenosugars) of each individual standard in the above mixture. The typical operating conditions in the MS mode were: orifice voltage 20 V, ionspray voltage 4100 V, scan range 70–500 u within 8.6 s, dwell time 10 ms, and step size 1 u. For the MS/MS experiments the $M+H^+$ ion was fragmented by setting the collision energy to 35 V and the product ions were scanned in the range of interest within 7.17 s. The dwell time was varied to give a required run duration. The multiplier voltage was 2400 V. These were slightly readjusted daily for the maximum sensitivity.

Results and discussion

Purification of organoarsenic compounds from algae extracts by multidimensional LC

Choice of chromatographic separation mechanisms

The separation protocol to be developed should: (i) assure the isolation of each As species present in the sample in chromatographically pure form (one species in each fraction), and (ii) achieve the maximum simplicity of the matrix in which the As species would have been isolated. This is important in view of ES MS since As is monoisotopic and the attribution of peaks in an MS spectrum to As compounds may not be straightforward. The achievement of the second objective implied the choice of a ready-to-be-removed (volatile) mobile phase buffer and an efficient elimination of non-As-containing matrix components by sequentially applying chromatography with orthogonal separation (different principle) mechanisms.

The most efficient separations of As species in marine biota were achieved by taking advantage of their different charge (anion-exchange) or hydrophobicity (RP HPLC) [4]. The combination of these mechanisms was therefore judged the most suitable for our purpose and was investigated in detail. For two-dimensional chromatography it is convenient that the first separation employed can handle a

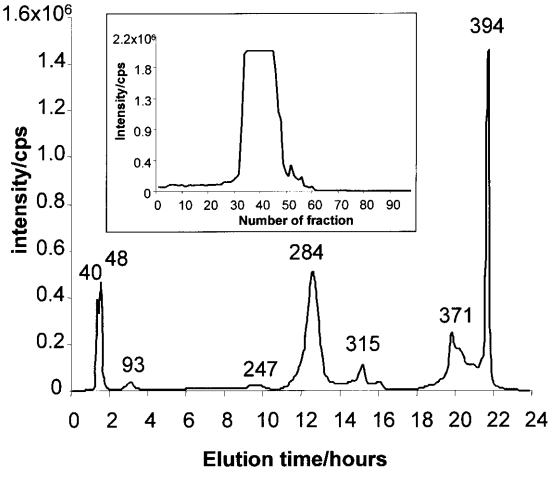


Fig. 1 Anion-exchange chromatographic separation of organoarsenic species in an algae extract (*Laminaria*). The numbers denoting peaks correspond to numbers of collected fractions. The *inset* shows the elution of organoarsenic species during the cleanup step from a size-exclusion column

larger quantity of sample to provide sufficient quantities of analyte, in individual fractions, for further chromatography. The sequence anion-exchange (AE)-RP HPLC was chosen since, in comparison with reversed-phase columns, anion-exchange supports are cheaper, easier to pack in any dimensions, and LC can be run with milliliter quantities with relatively low pressures.

An algae matrix contains high molecular mass biopolymers (polysaccharides, proteins) and other compounds that may be adsorbed on the chromatographic stationary phase or co-eluted with arsenic compounds. In order to avoid the degradation of chromatographic resolution, reduction of the column lifetime, and interference with ES MS when the co-eluted species were pre-concentrated by freeze-drying , a sample cleanup step using gel-permeation chromatography was introduced prior to fractionation of arsenic compounds by AE.

Extract cleanup by size-exclusion chromatography

The inset in Fig. 1 shows the elution of As species from a gel-permeation column. A stationary phase with a small exclusion limit (ca. 15 kDa) was chosen because of the small size of As compounds. Arsenic elutes as a broad peak apparently containing all the As species present. Note that the elevated background preceding the peak is not due to the elution of As since 99±2% of arsenic introduced on the column is found in the major peak. The investigation of the chromatographic purity of the individual fractions within this peak by RP HPLC-ICP MS (results not shown) demonstrates that some fractionation of arsenic species occurred but none of the fractions could be considered as chromatographically pure. Therefore the fractions 32–50 were pooled and freeze-dried for further investigation.

Fractionation of organoarsenic compounds by anion-exchange chromatography

An ammonium carbonate buffer, which allowed the baseline separation of anionic As species and was compatible with an electrospray ion source [15, 17, 21], was chosen for preparative anion-exchange separations. A chromatogram obtained (Fig. 1) shows three major peaks and a number of small ones. The peaks close to the void (cationic species) and the late eluting As^{V} are split. No visible salt crystals were obtained after freeze-drying of all the fractions eluted with the carbonate buffer except those collected in or close to the void (fractions 40–48). The chromatographic purity of the eight fractions containing the apices of peaks, marked by numbers of the fraction collected in Fig. 1, was investigated below by RP HPLC-ICP MS.

Verification of the chromatographic purity of AE peaks by RP HPLC

RP HPLC has been frequently used for the separation of As species in marine organisms [4]. In all these separations, however, an ion (either cation- or both)-pairing reagent was used in up to 25 mM. This creates a considerable problem in view of ES MS, especially upon preconcentration of fractions eluted from RP HPLC. It was therefore necessary to investigate the separation of As species by RP HPLC with a buffer that would not interfere with ES MS analyses.

Malonic acid, which shows slight cation-pairing properties, is a convenient mobile phase component since it does not significantly interfere with ES MS measurements and can be eliminated to a significant degree by freeze-drying. The separation efficiency is still reasonably good for many species. The arsenosugars were well separated from each other as demonstrated by the retention times marked for the appropriate standards in Fig. 2. However, without the ion-paring reagent arsenosugar B co-eluted with As^{III}. Since these species also co-eluted in the void of anion-exchange chromatography, the chromatographic purity of the void in AE LC should ideally be examined by cation-exchange HPLC.

Fig. 2 shows RP HPLC-ICP MS chromatograms of fractions corresponding to peak apices in Fig. 1. The chromatogram for fraction 371 was not shown since it was identical to that of fraction 394 (bottom panel in Fig. 2). The identification of As species in five fractions with numbers 93–394 seemed to be straightforward. They all contained one major peak for which the retention time matched the retention time of a standard. The small peak in Fraction 315 corresponded to the beginning of elution of arsenosugar C; the peak from arsenosugar A had not disappeared completely.

The chromatogram of fraction 394 (and 371) shows two peaks that both increase proportionally when the fraction is spiked with an As^{V} standard. It is therefore likely that they both correspond to As^{V}. This hypothesis is cor-

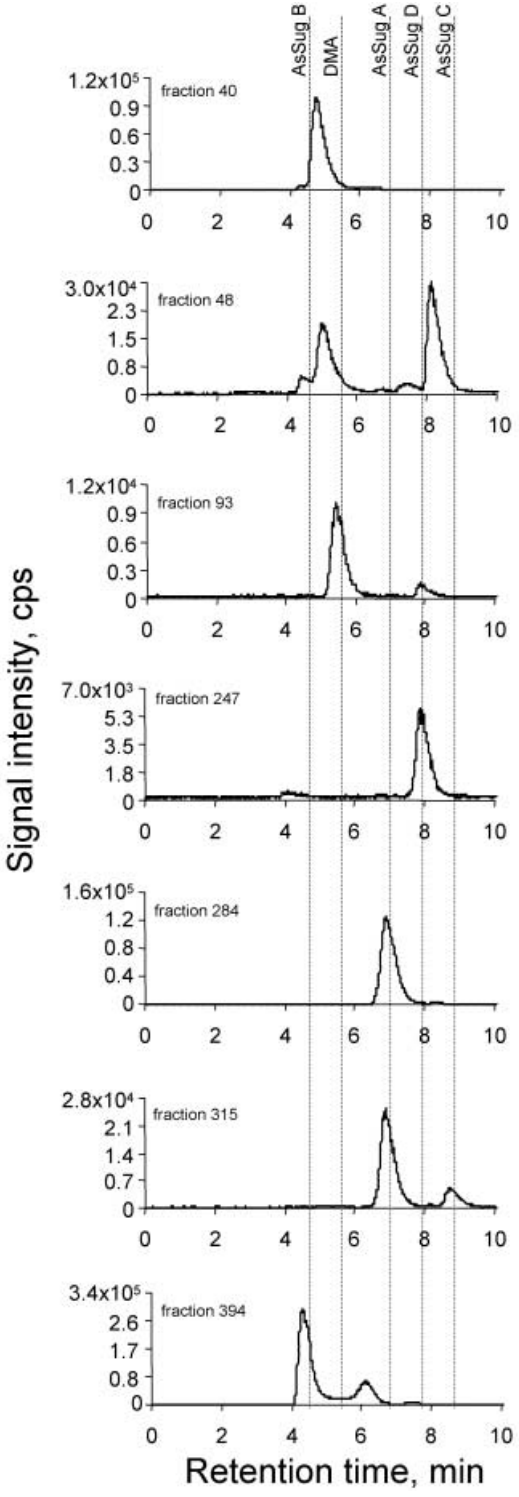


Fig. 2 Verification of the chromatographic purity of arsenic species eluted from an anion-exchange column with carbonate buffer (cf. Fig. 1) by RP HPLC-ICP MS. *Dotted lines* mark the retention times of the standards of arsenic species

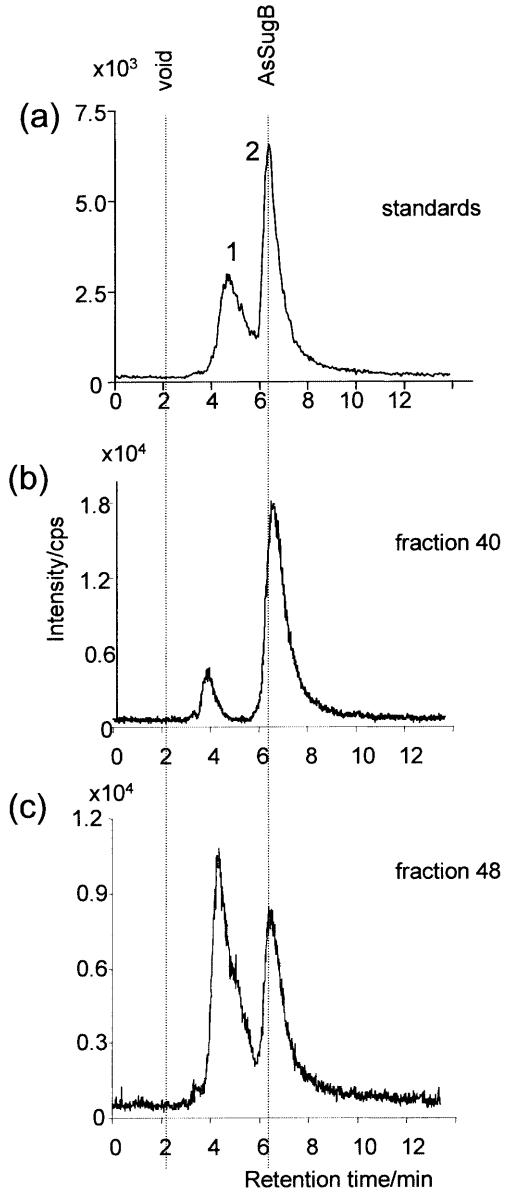
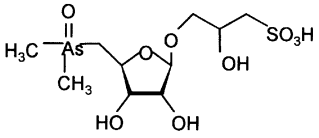
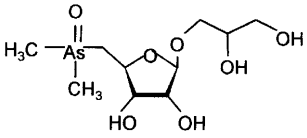
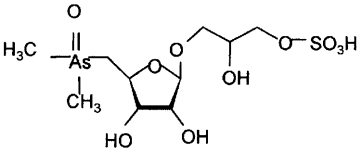
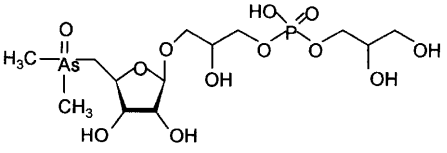
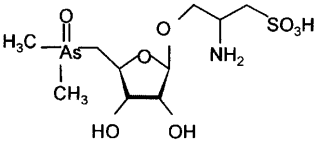
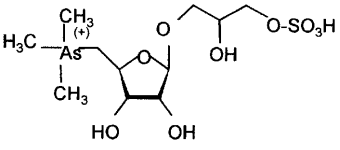
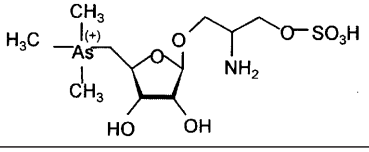


Fig. 3 Verification of the chromatographic purity of arsenic species eluted in or close to the void of an anion-exchange column by cation-exchange HPLC-ICP MS. (**a**) standards of a mixture of: 1 – arsenosugar D and 2 – arsenosugar B (degradation product of arsenosugar D), (**b**) chromatogram of fraction 40 in Fig. 1, (**c**) chromatogram of fraction 48 in Fig. 1

roborated by the fact that arsenic from this fraction eluted as a single peak from anion-exchange and size-exclusion columns at the retention time of As^V standards (results not shown). This observation highlights a major source of error in peak identification by retention time matching. Without the preceding AE chromatography and a spiking experiment this chromatogram would have been interpreted as showing two unknown species.

Some problems may occur with identification of species that elute from the anion-exchange column in or close to the void (fractions 40 and 48). Arsenic from fraction 40 (void in AE) was slightly retained on the reversed-phase column at the retention time corresponding to that of arsenosugar B (Fig. 2). A verification of the purity of this peak by cation-exchange HPLC (Fig. 3) confirmed that the majority of the species was indeed arsenosugar B, but

Table 1 Mass spectrometric data for arsenic species encountered in biological matrices

Compound	Mass $[M+H]^+$	Fragments	Formula
Arsenous acid (arsenite)	126		$OH\text{-}As(OH)_2$
Arsenic acid (arsenate)	142 [143]	143, 141, 125, 109, 91	$O{=}As(OH)_3$
MMA	140 [141]	141, 123, 109, 91	$CH_3AsO(OH)_2$
DMA	138 [139]	139, 121, 109, 91, 89, 75	$(CH_3)_2AsO(OH)$
TMAO	136 [137]	137, 122, 117, 107, 91, 77	$(CH_3)_3AsO$
$TMAs^+$	135	135, 120, 105, 91	$(CH_3)_4As^+$
AsB	178 [179]	179, 161, 137, 120, 105, 91	$(CH_3)_3AsCH_2COOH$
AsC	165	165, 147, 132, 121, 105, 91	$(CH_3)_3AsCH_2CH_2OH$
DMAsEt	166 [167]	167, 149, 139, 123, 107, 93	$(CH_3)_2AsO(CH_2)_2OH$
DMAsAc	180 [181]	181, 163, 153, 139, 121, 107, 91, 75	$(CH_3)_2AsOCH_2COOH$
Arsenosugar A	392 [393]	393, 375, 237, 195, 97	
Arsenosugar B	328 [329]	329, 237, 195, 97	
Arsenosugar C	408 [409]	409, 329, 237, 195, 97	
Arsenosugar D	482 [483]	483, 391, 237,195, 97	
Arsenosugar E	390 [391]	standards not available	
Arsenosugar F	407 [408]	standards not available	
Arsenosugar G	406 [407]	standards not available	

10% of As nevertheless eluted in the void of CE column. Regarding fraction 48, the second peak in RP HPLC-ICP MS corresponded to arsenosugar B but another compound that eluted at a retention time which could not be matched by any of the available standards was also observed. This compound was only slightly retained by cation-exchange chromatography (Fig. 3) that all together indicated a lack of positive charge and weak polarity.

Note that when RP HPLC-ICP MS was applied to the fractions isolated by AE chromatography using the phosphate buffer (results not shown), practically none of the peaks could be matched with the standards because the retention times of analytes in the sample were affected by the salt-rich matrix [22]. However, positive identification was obtained by spiking the experiments–confirming the results shown in Fig. 2.

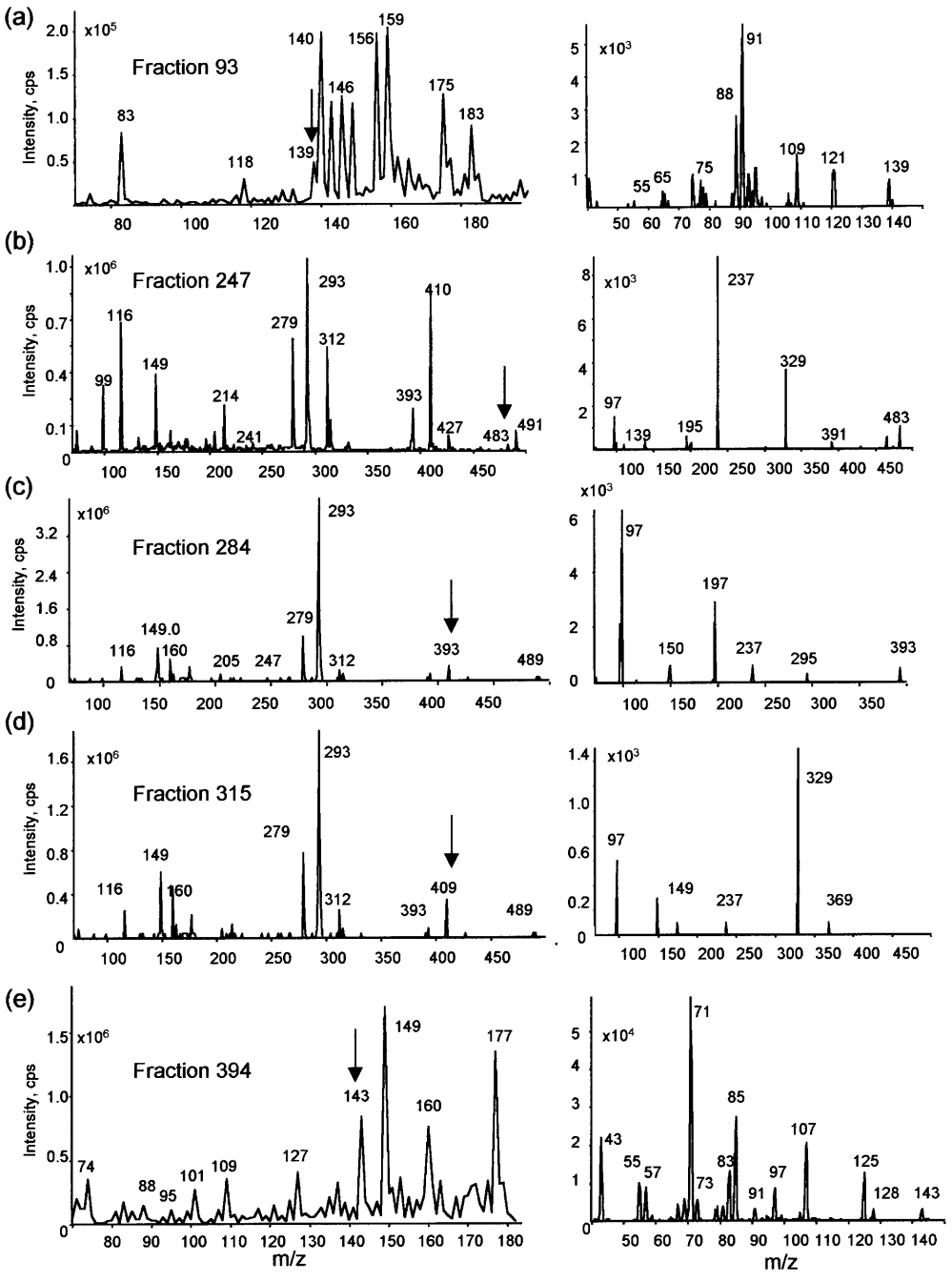


Fig. 4 Identification of signals from chromatographically pure fractions obtained by AE chromatography (Fig. 1) by electrospray MS/MS (arrows indicate the position of the molecular ion fragmented). Left panels: ES MS spectra. Right panels: CID MS spectra. (**a**) Fraction 93, (**b**) fraction 247, (**c**) fraction 284, (**d**) fraction 315, (**e**) fraction 394. See Table 1 for matching the fragments observed with those from standards

Confirmation of the peak identification by ES MS/MS

The identity of organoarsenic compounds in AE-RP HPLC-ICP MS was also investigated by electrospray MS/MS. In the first place the reference spectra of the standards available were acquired. The data (m/z of the protonated molecular ion and m/z values of collision induced dissociation (CID) MS fragments) are shown in Table 1. The data are consistent with literature values where available. For several species, notably As^{V}, no data in the positive ion mode could be found in the literature for comparison. However, in the negative mode MS/MS spectra for As^{V} reported by Florencio et al. [23], the fragments were similar (–2 u due to different ionization modes) to those we observed.

ES MS/MS of AE HPLC fractions

Electrospray MS/MS spectra of the five fractions numbered in Fig. 2 from 93–394 are shown in Fig. 4. Owing to the chromatographic purity (demonstrated above) of the investigated fractions, the positive identification of one compound in each fraction is sufficient. This is an important consideration in view of arsenic being monoisotopic and the virtual impossibility of demonstrating otherwise

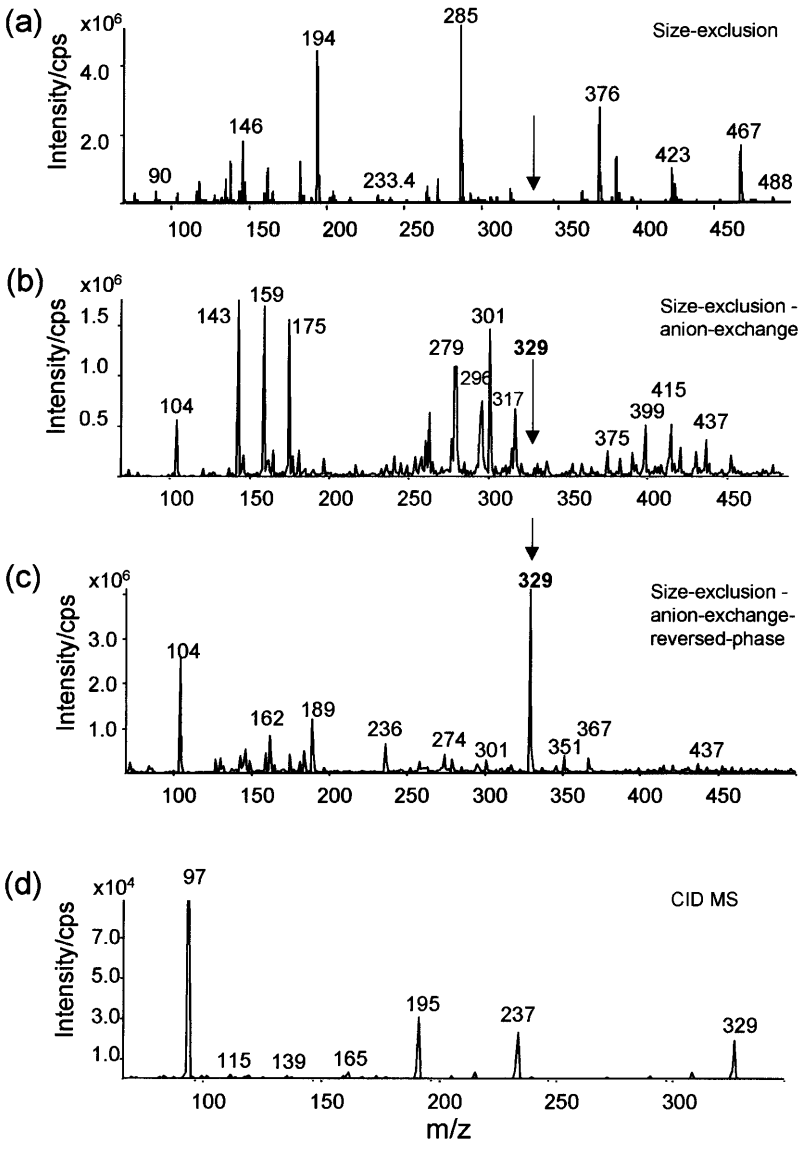


Fig. 5 Effect of the multidimensional chromatographic purification on the quality of electrospray mass spectra. (**a**) Extract purified by size-exclusion chromatography only, (**b**) extract purified by SE-AEC, (**c**) extract purified by SE-AEC-RP HPLC, (**d**) CID MS fragmentation pattern of the peak marked with an arrow (arsenosugar B)

that no other peak in the MS spectrum can be attributed to an arsenic species.

The ES MS/MS data unambiguously confirm the identification of DMA, arsenosugars D, A and C, and As^V in the corresponding fractions. Note that in fraction 315 (Fig. 4) peaks of both arsenosugar C and A can be seen.

ES MS/MS analysis of the AE HPLC void

The first two fractions considered (40 and 48) gave ES MS spectra of very poor quality probably because of the presence of all the cationic (salt) matter from the sample (a considerable amount of powder remained after freeze drying). Therefore, these two fractions were further purified by RP HPLC as described in the Experimental section. The effect of the multidimensional chromatographic purification on the quality of electrospray mass spectra is best seen in Fig. 5 on the example of arsenosugar B. No signal was seen in the As-containing fraction after size-exclusion chromatography (neither in MS nor in MS/MS mode) (Fig. 5a), probably as a result of suppression of ionization by the co-eluting matrix. Anion-exchange chromatography simplified the matrix (suppression of all the intense peaks in the SE fraction) (Fig. 5b). A peak at m/z 329 became visible (a product ion mass spectrum of acceptable quality could be obtained in the CID MS mode) but the choice of the ion to be fragmented required knowledge of the compound's existence. A third chromatographic dimension (RP HPLC) cleaned up the matrix efficiently and sugar B become by far the major sample constituent (Fig. 5c). Indeed, the fragmentation pattern unambiguously confirmed its identity (Fig. 5d).

The arsenosugar 329 peak was also readily seen in the purified fraction 48. In addition to it a peak with m/z 407, fragment peaks at 164, 178, 213, and 236 in the CID MS mode were observed. The latter might be an analogue of arsenosugar C (referred to as arsenosugar G in Table 1) in

Table 2 Summary of analytical methods and findings for the characterization of organoarsenic compounds in marine samples and related food

Sample investigated	Purification technique	Species found	Identity confirmation	Ref.
Oyster	CE	Arsenosugar B, DMA, arsenobetaine	ES MS/MS	[14]
Sargassam	SE-AE	Arsenosugar C	nano ES MS/MS, FAB MS/MS	[13, 21]
Kelp	AE	Arsenosugars B, D, A	ES MS/MS	[15]
Hizikia	SE-AE	Arsenosugars C, D	ES MS/MS	[8]
Fucus	AE	Arsenosugars A, C, D	ES MS	[17]
Fucus	CE	Arsenosugar B	ES MS	[21]
	AE	Arsenosugars A, C, D		
Laminaria	SE-AE	Arsenosugars A, D	ES MS/MS	[9]
Laminaria	AE	Arsenosugars A, D	ES MS	[17]
Laminaria	SE-AE	Arsenosugars A, C, D, As^V, DMA	ES MS/MS	This work
	SE-AE-RP	Arsenosugars B and G		

which the =O group was replaced by a -CH_3 group, and the side chain -OH group by -NH_2. Note that arsenosugar C analogues in which either of the replacements took place have previously been reported in the literature [10, 24]. This evidence can be considered as only circumstantial as no standard for this compound was available.

Organoarsenic compounds in Laminaria algae

The literature on organoarsenic species in this particular algae is scarce. Shibata et al. [25] found arsenosugar A to be the dominant organoarsenic species, which confirms our findings. In our previous work on the same algae we could see arsenosugars A and D, but the quality of mass spectra was poor because of the insufficient matrix removal [9]. This observation was independently made by Pedersen and Francesconi [17]. A small peak eluted near the solvent front which could not be unambiguously assigned because of the limited separation efficiency in this region [17]. Arsenosugars C and B have not been previously reported in *Laminariales*, despite their being referred to as arsenic constituents in other brown algae.

An important aspect of arsenic mapping in algae is the quantification of individual species. The total arsenic concentration found by ICP MS, after microwave-assisted sample decomposition using HNO_3 and H_2O_2, was 28.1± 1.0 μg g^{-1}, in good agreement with the 30 μg g^{-1} value (precision not given) obtained from an accredited laboratory (DGCCRF) by GF AAS. The analysis of the extract obtained according to the Experimental section gave a value of 28.8±2.4 μg g^{-1} of water-methanol soluble arsenic which accounts for 100% of the arsenic present in the sample. The concentrations (given as arsenic) of the individual organoarsenic compounds were calculated on the basis of two independent anion-exchange chromatograms (and CE HPLC-ICP MS for the compounds eluted in the void from the anion-exchange column). They were: arsenosugar A 11.7±0.5 μg g^{-1}, As^V 10.9±2.1 μg g^{-1}, arsenosugar B 2.22±0.07 μg g^{-1}, arsenosugar D 1.5±1.2 μg g^{-1}, arsenosugar G 1.13±0.07 μg g^{-1}, arsenosugar C 0.61± 0.04 μg g^{-1}, and DMA 0.42±0.02 μg g^{-1}. Note that according to the value obtained by HG AAS in the DGCCRF laboratory the "mineral arsenic" should account for 18 μg g^{-1} whereas the sum of As^V and DMA in our measurement accounted for only 11.3 μg g^{-1}. The reason for this discrepancy remains for the moment unknown.

The analytical strategy developed above is, to the best of our knowledge, the first based on ICP MS and ES MS/MS that allows the establishment of a complete map of arsenic species in a natural sample. As demonstrated in Table 2, the study seems to be the most comprehensive of the published research on the characterization of marine samples and relevant food carried out by LC with dual ICP and ES MS/MS detection.

Conclusions

The common ambiguities regarding the purity of peaks in HPLC-ICP MS of arsenic species can be alleviated by multidimensional LC-ICP MS which is demonstrated here to be a convenient and versatile technique for mapping arsenic compounds in biological matrices. The combination of size-exclusion with carefully optimized anion-exchange separation mechanisms allows the isolation of the majority of naturally present arsenic compounds in microgram quantities in chromatographically pure form, but a third dimension by reversed-phase (or cation-exchange) HPLC is required to resolve the cationic species. The buffers proposed in this study can be completely removed by freeze-drying which, in combination with the elimination of sample matrix components assured by the chromatographic multidimensionality, offers the possibility of obtaining high-quality mass spectra by ES MS/MS for identification purposes. Consequently, the separation scheme developed provides a convenient way for gaining commercially unavailable organoarsenic standards, e.g., arsenosugars, from readily available algae. The elimination of the matrix and the buffer allows high pre-concentration factors for organoarsenic species to be obtained by lyophilization. This makes the developed analytical protocol promising in studies of arsenic metabolism in terrestrial

organisms in which this element is present in concentrations orders of magnitude lower than in marine biota.

Acknowledgements S. McSheehy acknowledges a Marie Curie fellowship from the European Commission (Grant N° SMT-4CT-982232). We thank Ms. Ela Preś-Skrzydlewska, a Socrates student from the Warsaw University of Technology for help with the preliminary experiments.

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