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Investigation of arsenic speciation in oyster test reference material by multidimensional HPLC-ICP-MS and electrospray tandem mass spectrometry (ES-MS-MS)

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Multidimensional (size-exclusion–anion-exchange–cation-exchange) liquid chromatography with ICP-MS detection was developed to produce a map of water-soluble species in an oyster test reference material. The presence of arsenobetaine, trimethyl(2-carboxyethyl)arsonium inner salt, arsenocholine, dimethylarsonic acid, tetramethylammonium ion, As(v) and two arsenosugars was demonstrated by ES-MS-MS. A previously unreported compound was isolated and identified by ES-MS-MS as 5-dimethylarsinoyl-β-ribofuranose. Anion-exchange chromatography was optimized to produce a chromatographically pure peak of arsenobetaine (accounting for *ca.* 64% of all water-soluble As present) that was used to quantify this compound.

Introduction

The fairly high amounts of arsenic found in marine organisms have stimulated studies of the chemical forms of this element aimed at the evaluation of the toxicological implications of consuming seafood and at the elucidation of the cycling of arsenic in the marine ecosystem. Arsenobetaine has been identified as the most abundant arsenic species in marine fauna, but a number of minor arsenic species accounting for up to 50% of total arsenic have been identified, especially in shellfish and molluscs.[1,2] They include simple methylated species, such as tetramethylarsonium and trimethylarsine oxide, monomethylarsonic and dimethylarsinic acids, arsenocholine, arsinoyl ribofuranosides and, recently, a 'new' arsenobetaine, trimethyl(2-carboxyethyl)arsonium inner salt.

The combination of high-performance liquid chromatography (HPLC) with on-line detection by inductively coupled plasma atomic emission spectrometry (ICP-AES)[3] or mass spectrometry (ICP-MS) has become an established technique for arsenic speciation. Single mechanism chromatographic techniques, such as anion-exchange (AE), cation-exchange (CE),[4] size-exclusion[3] and ion-pair reversed-phase (RP)[1,2] methods, were initially used to complete the characterization of a sample in terms of arsenic speciation. It soon turned out that the complexity of arsenic speciation required the parallel use of two (AE, CE)[5] or three (AE, CE, RP)[6] complementary separation mechanisms. These studies allowed the detection of 10 arsenic compounds in shellfish but unidentified peaks were still present.

The use of HPLC-ICP-MS suffers from several limitations, including a risk of misidentification of species based on the retention time matching with standards and the impossibility of identifying species for which standards are unavailable. Indeed, the large number of arseno compounds 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 unavoidable regardless of this mechanism. The literature on arsenic speciation gives the impression of being extremely abundant but its critical evaluation indicates, for example, that the anion-exchange HPLC peak referred to as arsenobetaine is likely to contain arsenosugar B, the 'new arsenobetaine', often As(III) and probably other cationic species such as arsenocholine. Hence there is a need for the optimization of multi-dimensional chromatography[7,8] for the separation and for molecule-specific techniques such as electrospray tandem MS (ES-MS-MS) for detection.

Pioneering studies on the use of ES-MS-MS for speciation analysis of arsenic go back to 1996,[9] but it is only in the last year that there has been substantial increase in applications of this technique to arsenic speciation studies.[7,8,10–14] All but one publication concerned algae samples and the most abundant class of compounds, arsenosugars, because of the apparent greater ease of analysing algae than molluscs.[9] Indeed, concentrations of arsenic in algae (100 μg g^{-1} level) are typically 10-fold higher than those in molluscs (10 μg g^{-1}), the matrix of animal samples is more complex than that of algae and the dominance of arsenobetaine in marine fauna drives the sum of the minor arsenic species close to the 1 μg g^{-1} level, *i.e.*, close to the limits of detection of the identification techniques available.

Characterizing and quantifying arsenic species in marine fauna is important for a better understanding of the arsenic metabolism and evaluation of the risk associated with the consumption of seafood. This concerns particularly shellfish because of the complexity of the As speciation. The quality of analytical results can be improved by the availability of certified reference materials (CRMs). Arsenic species have been characterized in some CRMs but only a few materials dedicated to speciation are available.[15,16]

The objectives of this research were (i) to investigate in detail speciation of arsenic in an oyster test reference material by multi-dimensional (employing consecutive orthogonal separation mechanisms) HPLC-ICP-MS and ES-MS-MS and (ii) to optimize the conditions of single mechanism HPLC-ICP-MS leading to a chromatographically pure arsenobetaine peak and thus allowing the quantification of this species.

Experimental

Apparatus

Low-pressure LC was carried out by means of a Minipuls 3 peristaltic pump (Gilson, Villiers-le Bel, France) and a Hewlett-

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Packard (Waldbronn, Germany) Series 1100 pump 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, Cotati, CA, USA). All the connections were made of PEEK tubing (0.17 mm id). Fractions for off-line analyses were collected using a Model FC-2 automatic fraction collector (Dynamax, France). A Model LP3 lyophilizer (Jouan, France) was used for freeze-drying of sample extracts and eluates.

The ICP-MS instrument was an ELAN 6000 (Perkin-Elmer SCIEX, Thornhill, ON, Canada). The sample introduction system used 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 also served for draining the spray chamber.

The electrospray MS instrument used was a Perkin-Elmer SCIEX API 300 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 (Danbury, CT, USA) model 1210 ultrasonic cleaner. The supernatant was separated by centrifugation using a Hettich (Tuttlingen, Germany) Universal 16 centrifuge. The rotary evaporator used for the elimination of methanol was a Heidolph (Germany) VV Mikro. A Hima CS120GX centrifuge (Hitachi, Tokyo, Japan) was used for removal of particles upon the dissolution of freeze-dried residues.

Chromatographic materials and reagents

A 700 × 16 mm id column (Pharmacia, Uppsala, Sweden) was filled with Sephadex G-15 gel (Pharmacia) according to the manufacturer's protocol for low-pressure size-exclusion LC. For semi-preparative anion-exchange LC an identical column was filled with DEAE Sephadex A-25. The reversed-phase (Inertsil ODS-2), cation-exchange and anion-exchange HPLC columns (Supelcosil SCX and SAX) were obtained from Supelco (St. Quentin Fallavier, France). A Superdex Peptide 10HR column (Pharmacia) was used for size-exclusion LC-ICP-MS analyses.

Analytical reagent grade chemicals were used throughout unless stated otherwise. 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 size-exclusion chromatography a 1% (v/v) solution of acetic acid 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. Dilute buffer solutions (pH 8.85 ± 0.05) were prepared by the dilution of this solution with water. A 25 mM phosphate buffer solution (pH 6.0) was prepared by mixing 57.5 ml of 0.2 M $NH_4H_2PO_4$ with 5.0 ml of 0.2 M $(NH_4)_2HPO_4$ solutions and diluting with water to 0.5 l. More dilute solutions were prepared by dilution with water. The buffers were de-gassed by sonication for 20 min before starting chromatography.

Standards and sample

Stock standard solutions at a concentration of 1.00 mg ml^{-1} (as arsenic) were prepared by dissolving the respective compound in water. Arsenic(III) and arsenic(V) standard solutions were prepared from disodium hydrogenarsenate and sodium arsenite (Sigma-Aldrich), in water. The four dimethylarsinoylriboside derivatives 3-[5′-deoxy-5′-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropanesulfonic acid (sugar A), 3-[5′-deoxy-5′-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropylene glycol (sugar B), 3-[5′-deoxy-5′-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropyl hydrogensulfate (sugar C) and 3-[5′-deoxy-5′-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropyl 2,3-hydroxypropylphosphate (sugar D) were kindly donated by Professor J. Edmonds (De Montfort University, Leicester, UK). Arsenobetaine was a gift from Professor 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). The purity of standards was verified by CE-HPLC-ICP-MS and AE-HPLC-ICP-MS. With the exception of arsenosugar D that contained some (*ca.* 10%) arsenosugar B, all the other standards had purities exceeding 98%. A secondary stock standard solution 1 μg ml^{-1} of each of the compounds was prepared for HPLC-ICP-MS analyses. Working standard solutions were prepared on the day of analysis by appropriate dilution of the stock standard solutions with water. The stock standard solutions were kept in a refrigerator at 4 °C in the dark.

Oysters were collected from Venice Lagoon in the frame of the MULSPOT project, financed by the SM&T programme (SMT4-CT98-2232) and coordinated by Dr. R. Morabito (ENEA, Rome, Italy). They were transported to the EU Joint Research Centre in Ispra (Italy) within one day. The oysters were stored at −15 °C. They were de-shelled and the tissue was minced, homogenized (Ultra-Turrax) and freeze dried. The dried oyster was then ground and sieved and the fraction of < 125 μm was taken for analysis.

Procedures

Extraction of arsenic species from oyster sample. Three types of extractants, water, methanol–water (1 + 1 v/v) and methanol, were investigated. Extractions were carried out on 0.25 g of material with two consecutive portions of extractant (15 ml) for 1 h in an ultrasonic bath. The supernatants were combined, reduced in volume using a rotary evaporator (at room temperature) and then rediluted in 10 ml of water. Extracts from three sample intakes (a total of 0.77 g dry weight) were combined, freeze-dried and dissolved in 3 ml of water.

Extract clean-up by size-exclusion chromatography (SEC). The size-exclusion column was conditioned by washing with water and flushing the column with the prepared eluent (1% v/v acetic acid). A 3 ml sample of the extract solution was ultracentrifuged (50 000 rpm at 4 °C for 30 min) and filtered though a 0.45 μm filter. The sample was eluted isocratically with acetic acid solution at a flow rate of 0.9 ml min^{-1}. Fractions were collected every 2 min (1.8 ml) for 3.5 h. Arsenic was determined in a 100 μl aliquot of each fraction (diluted 1 + 7) to reconstitute the elution profile [Fig. 1(a)]. Fractions 34–40 (peak A) and 41–46 (peak B) were pooled and freeze-dried. The residues were dissolved in 2 ml of water producing two samples, referred to as samples A and B.

Purification of arsenic compounds by anion-exchange chromatography. The solutions of samples A and B were introduced on to an anion-exchange column. The eluent (ammonium carbonate buffer) flow rate was 0.9 ml min^{-1}. The program was as follows: 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; 10–13.5 h, 10 mM buffer, fractions collected every 5 min; and 13.5–25.3 h, 50 mM buffer, fractions collected every 5 min. An aliquot of each fraction was analysed 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 fractions comprising the apex of each of the peaks A_2, A_3, B_2, B_3 and B_4 (*cf.*, Fig. 1) was analysed by CE-HPLC-ICP-MS, RP-HPLC-ICP-MS and SE-HPLC-ICP-MS. The operating conditions for each of the chromatographic separation mechanisms are summarized in Table 1. The rest of the fractions were freeze-dried, dissolved in 40 μl of 0.1 M formic acid in 40% methanol and analysed by ES-MS-MS.

Fractionation of the void from anion-exchange chromatography by cation-exchange chromatography. Fractions eluted in the void from the AE column (referred to as A_1 and B_1) were lyophilized and dissolved in 120 μl of water. An aliquot of 100 μl was injected onto a cation-exchange column. Arsenic was determined in fractions collected every 15 s to reconstruct the elution profile. Fractions corresponding to the peaks were pooled, lyophilized, dissolved in 40 μl of 0.1 M formic acid in 40% methanol and analysed by ES-MS-MS.

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. Typical examples are a nebulizer gas flow rate of 1.05 l min^{-1}, ICP rf power of 1100 W and a lens voltage of 8 V. 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 reconstituted by

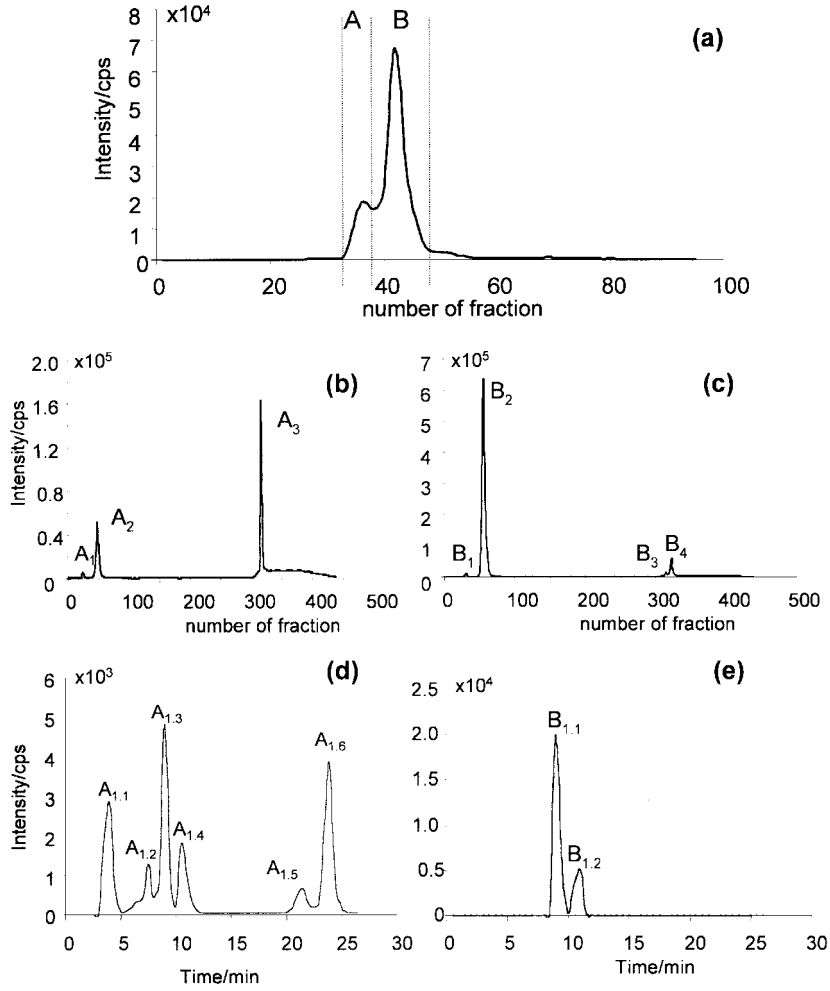

Fig. 1 Fractionation of arsenic species in oyster extract by three-dimensional liquid chromatography (monitored by ICP-MS). (a) size-exclusion LC: A, arseno sugar fraction; B, low molecular mass fraction. (b) Anion-exchange LC of the arseno sugar fraction (A). (c) Anion-exchange LC of the low molecular mass fraction (B). (d) Cation-exchange LC of the void (A_1) of the anion-exchange chromatogram shown in (b). (e) Cation-exchange LC of the void (B_1) of the anion-exchange chromatogram shown in (c).

Table 1 HPLC-ICP-MS separation conditions

	Separation mechanism		
	Cation exchange	Reversed phase	Size exclusion
Column	Supelcosil LC-SCX	Intersil ODS-2	Superdex Peptide 10HR
Dimensions	250 × 4.6 mm id × 5 μm	250 × 4.6 mm id × 5 μm	300 × 10 mm id × 13 μm
Eluent	Pyridine–formate (pH 3)	Malonic acid	Acetic acid
Elution program (buffer concentration)	Isocratic (20 mM)	Isocratic (4.0 mM)	Isocratic (1.0%)
Flow rate/ml min^{-1}	1.0	0.75	0.75
Operating pressure/bar	90–100	110	20
Injection volume/μl	100	100	100
Run time/min	14.85	14.85	35.58

plotting the intensity of this signal *vs.* 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 Turbochrom4 software (Perkin-Elmer). All signal quantifications were done in the peak area mode.

ESI-MS-MS conditions. A sample solution for analysis was prepared from a lyophilized chromatographic fraction by its dissolution in water and addition of an appropriate amount of methanol (30%) and formic acid (0.6%). 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 as follows: 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 a collision energy of 35 V and the product ions were scanned in the range of interest within 7.17 s. The dwell time was varied to give an appropriate run duration. The multiplier voltage was 2400 V. The conditions were slightly readjusted daily for the maximum sensitivity.

Determination of arsenobetaine and DMA. Quantification of the species was carried out by anion-exchange HPLC using the method of standard additions at two concentration levels, approximately once and twice the original concentration. Elution (1 ml min^{-1}) was carried out with phosphate buffer (pH 6) at 2 mM for 5 min following by a gradient from 2 to 25 mM within 20 min. Turbochrom software was employed to integrate the peak of interest and the areas *versus* standard additions were plotted to obtain the concentration of arsenobetaine and DMA in the extract. Standard addition best fit lines with r^2 values of $\geqslant 0.99$ were allowed.

Results and discussion

Extraction of arsenic species from oyster tissue sample

Different extractants specified in the Procedure were employed to compare the effects of the extractants on the species extracted and the total arsenic extracted. The total concentration of arsenic in the sample was 15 mg kg^{-1}. The amount of arsenic extracted was independent of the extractant and amounted to 8 ± 1 mg kg^{-1}. The residue after three consecutive extractions still contained 7 ± 1 mg kg^{-1} of arsenic. The extracts, analyzed by AE-HPLC-ICP-MS, showed no difference in the chromatographic profiles as a function of the extractant used.

Speciation of arsenic in oyster tissue extract

Organoarsenic species were purified by three-dimensional chromatography. In the first step size-exclusion chromatography was used to enrich the arsenosugar fraction and separate it from high molecular mass water-soluble proteins and from the sample matrix containing smaller organoarsenic species. The two SE chromatographic fractions containing arsenic (arsenosugar fraction and low molecular mass fraction) were further fractionated by anion-exchange LC. The third chromatographic fractionation step was applied to the void of the anion-exchange column and cation-exchange chromatography was carried out. The As-specific chromatograms obtained at the different stages of the fractionation scheme are shown in Fig. 1.

Investigation of size-exclusion fractions by anion-exchange LC. The SEC-ICP-MS chromatogram [Fig. 1(a)] indicates the presence of a front shoulder, apparently containing anionic arsenosugar(s), on the major peak. Anion-exchange LC [Fig. 1(b)] allows the separation of the three basic fractions: void, medium-retained species and well-retained species, for both of the peaks observed in the size-exclusion chromatogram. The ratio of strongly retained (anionic) species to the others in the shoulder peak SEC fraction (A) is a factor of 50 higher than in the major peak (B). The chromatographic purity of the peaks corresponding to strongly retained compounds (A_3, B_3 and B_4) and to weakly retained species (A_2 and B_2) was further investigated using different mechanisms of HPLC-ICP-MS. The compounds eluted were tentatively identified by retention time matching with standards and, unambiguously, by ES-MS-MS.

Characterization of the strongly retained compounds in AE-LC. The chromatographic purity of the late eluted peaks [peaks A_3, B_3 and B_4 in Fig. 1(b) and (c)] was verified by CE-HPLC-, RP-HPLC- and HR-SE-HPLC-ICP-MS. The chromatograms are shown in Fig. 2. Fig. 2(a) indicates that fraction A_3 is chromatographically pure. The CE-HPLC-, RP-HPLC- and SE-HPLC-ICP-MS chromatograms indicate the presence of Arsenosugar D as the major ($> 98\%$) species in this fraction. The residual arsenic probably corresponds to As(v). The presence of arsenosugar D was confirmed by ES-MS-MS (spectra not shown). The signal of As(v) in ES-MS-MS of this fraction is too small to allow the positive identification of this species.

The low molecular mass SEC fraction (B) indicates the presence of at least two strongly anionic compounds, eluting as peaks B_3 and B_4. The verification of the chromatographic purity of peak B_3 confirms the presence of arsenosugar D (the major species, *ca.* 80%) and As(v) (*ca.* 10%) by standard matching in the CE-HPLC-, RP-HPLC- and SE-HPLC-ICP-MS chromatograms. A third compound (*ca.* 10%) is present at a retention time that cannot be matched by any of the standards available. Note that arsenosugar D, despite being the major peak, accounts only for 10% of arsenosugar D eluted as fraction A_3. Peak B_4 seems to be chromatographically pure and to contain DMA, as demonstrated by the three HPLC separation mechanisms in Fig. 2(c). This finding was confirmed by ES-MS-MS, which also indicated the presence of As(v) in this fraction (mass spectra not shown).

Characterization of the weakly retained compounds in AE-LC (fractions A_2 and B_2). The verification of the chromatographic purity of peak A_2 by CE-HPLC-, RP-HPLC- and HR-SE-HPLC-ICP-MS (Fig. 3) demonstrates that at least four compounds (two major and two minor) are present. The same number of compounds can be found in fraction B_2, but at different concentration ratios. Arsenobetaine is the major species found in fraction B_2 but only a minor one in fraction A_2. The presence of this species in both fractions is further confirmed by ES-MS-MS (spectra not shown). Arsenosugar B is a major species in fraction A_2 and a minor species in fraction B_2. Its identity is further confirmed in fraction A_2 by ES-MS-MS (spectra not shown). The retention times of the other two compounds cannot be matched with any of standards available. However, ES-MS-MS (Fig. 4) allows us to identify (in both fractions A_2 and B_2) the presence of a species at *m/z* 193 of which the molecular mass and the CID fragmentation pattern correspond to an arsenobetaine-like compound, trimethyl(2-carboxyethyl)arsonium inner salt, recently reported by Francesconi *et al.*[14] However, another species is present in fraction A_2. Note that the intensity of the ES-MS signal of arsenobetaine in fraction A_2 is double that in fraction B_2, despite its 10-fold lower concentration. This indicates a strong matrix suppression effect in the case of fraction B_2.

Characterization of the void of the anion-exchange chromatography by CE-HPLC-ICP-MS and CE-HPLC-ES-MS-MS (fractions A_1 and B_1). Cation exchange is the natural orthogonal mechanism for fractionation of the void of anion-exchange LC. CE-HPLC-ICP-MS chromatograms of the fractions containing compounds unretained by AE-LC are shown in Fig. 1(d) and (e). They demonstrate the presence of two different species in fraction B_1, and of at least (some As elutes in the void) six different species in fraction A_1. The two species present in fraction B_1 are also found in fraction A_1 and elute at retention times characteristic of arsenocholine ($A_{1.3}$ and $B_{1.1}$) and tetramethylarsonium ion ($A_{1.4}$ and $B_{1.2}$). Their identity was unambiguously confirmed by ES-MS-MS (Fig. 5). ES-MS-MS was also applied to the identification of minor peaks ($A_{1.1}$, $A_{1.2}$, $A_{1.5}$, and $A_{1.6}$) in the CE-HPLC-ICP-MS chromatogram that did not match retention times of any of the available standards. No interpretable MS and MS-MS spectra could be obtained, except the major peak $A_{1.6}$ (Fig. 6). The fragments observed

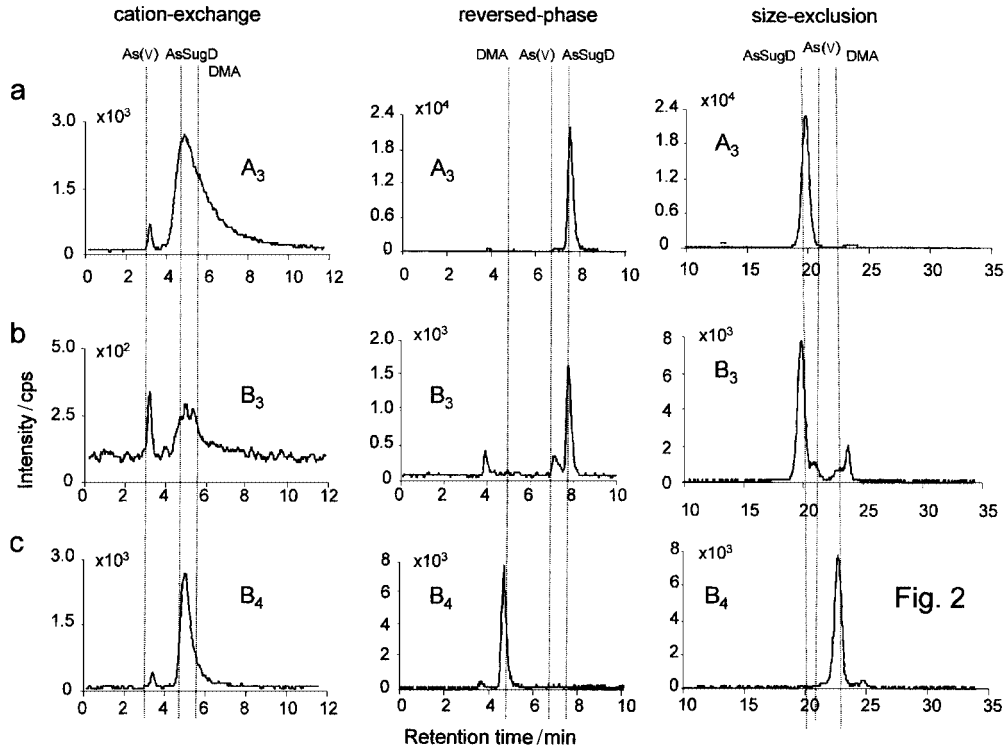

Fig. 2 Verification of the chromatographic purity of the late-eluting fractions in anion-exchange chromatography [*cf.* Fig. 1(b) and (c)]. (a) Peak A_3; (b) peak B_3; (c) peak B_4. Left panels: CE-HPLC-ICP-MS. Middle panels: RP-HPLC-ICP-MS. Right panels: SE-HPLC-ICP-MS. Dotted lines indicates retention (elution) times of the corresponding standards.

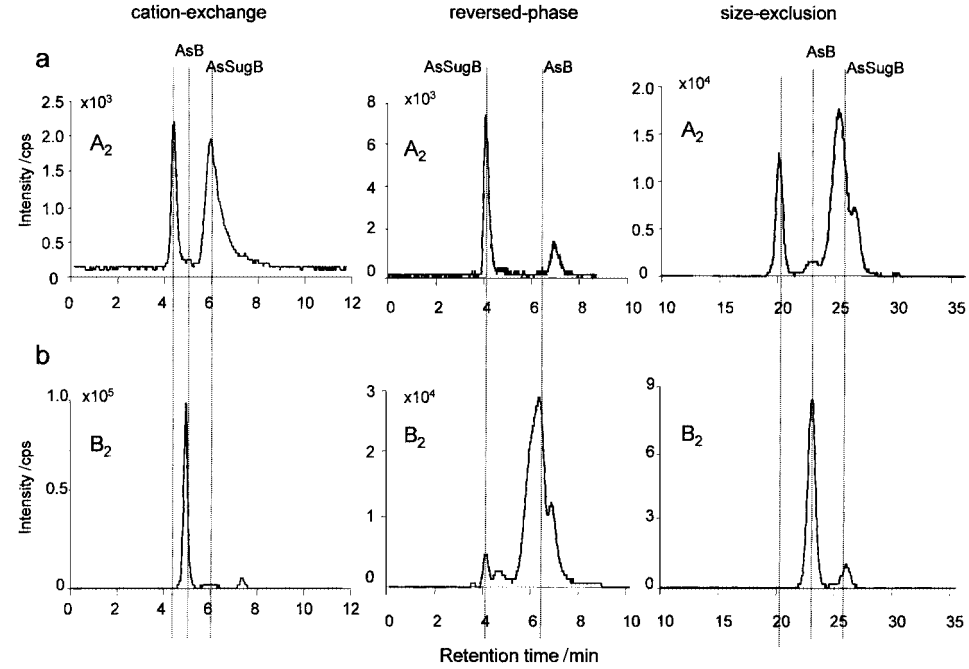

Fig. 3 Verification of the chromatographic purity of fractions in anion-exchange chromatography [*cf.* Fig. 1(b) and (c)]. (a) Peak A_2; (b) peak B_2. Left panels: CE-HPLC-ICP-MS. Middle panels: RP-HPLC-ICP-MS. Right panels: SE HPLC-ICP-MS. Dotted lines indicate retention (elution) times of the corresponding standards.

allow the attribution of a tentative structure shown in Fig. 6(b). This compound, 5-dimethylarsinoyl-β-ribofuranose, has, to our knowledge, never been reported in a natural product so far.

In summary, the method has allowed the identification of the presence of 13 different species. Nine of these species [eight previously reported, *i.e.*, As(v), arsenobetaine, trimethyl(2-carboxyethyl)arsonium inner salt, arsenocholine, dimethylarsinic acid, tetramethylammonium ion and arsenosugars B and D, and one previously unreported, *i.e.*, 5-dimethylarsinoyl-β-ribofuranose] could be identified by ES-MS-MS without the need for retention time standards. This makes, to our knowledge, this study the most comprehensive ever reported for oyster. ES-MS-MS data also indicate that none of the unidentified species has previously been reported in the literature.

Determination of organoarsenic species in oyster tissue extract

The quantification of arsenic species is possible provided that separation conditions allowing the baseline separation of the species present are found. Anion exchange is the most appropriate mechanism, but the choice of packing has a tremendous effect on the separation. A study of nine different AE-HPLC columns showed that the most efficient separation was achieved by elution with a gradient of phosphate buffer at pH 6.0[17] from a Supelco SAX column. The other columns did not allow the separation of arsenobetaine from either As(III), arsenosugar B, the 'new arsenobetaine' or other cationic species. A typical chromatogram obtained under optimized conditions is shown in Fig. 7. It shows nine fairly well resolved peaks. The identities of three peaks, 4, 5 and 6 (the peaks are not numbered on the chromatogram), were confirmed by ES-MS-MS to be arsenobetaine, trimethyl(2-carboxyethyl)arsonium inner salt and DMA, respectively. No ES-MS signal could be obtained either for arsenic species eluted prior to arsenobetaine, because of the co-elution of matrix components, or for the late eluting species, arsenosugar D and As(v), because of the signal suppression by the buffer. The identification of the species was based on the above described knowledge of the occurrence of the particular species and on spiking experiments.

The concentrations of arsenic species identified in the oyster tissue sample are summarized in Table 2. The mean result for arsenobetaine has a low RSD and shows that the method is reproducible and that the tissue is homogeneous. The lower concentration of DMA in the sample makes standard additions much more difficult, resulting in a high RSD. Arsenic(v) is at the limit of quantification of the method (*ca.* 0.01 μg g^{-1}) and

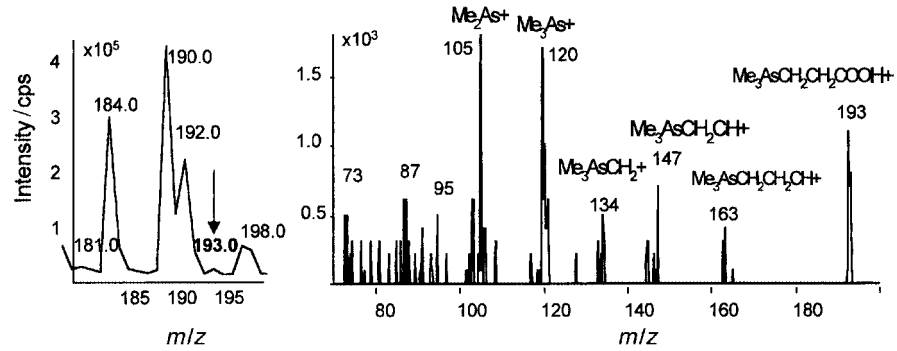

Fig. 4 Confirmation of the identity of trimethyl(2-carboxyethyl)arsonium inner salt *(m/z* 193) found in the weakly retained fraction (A_2) in anion-exchange chromatography [*cf.* Fig. 1(b) and (c)] by ES-MS-MS.

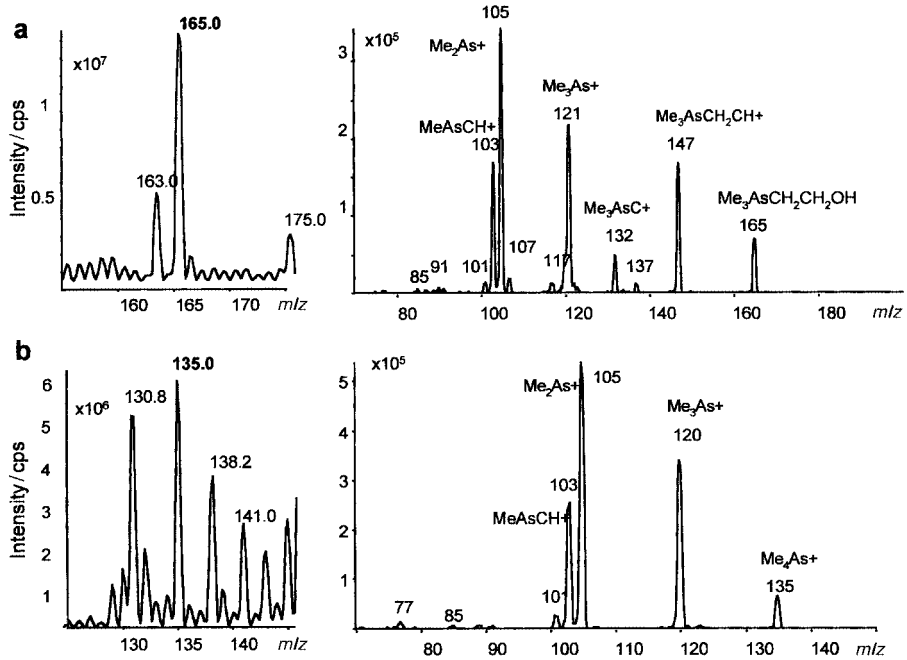

Fig. 5 Confirmation of the identity of peaks in CE-HPLC-ICP-MS by ES-MS-MS. Examples concern $B_{1.1}$ and $B_{1.2}$ peaks. (a) CID mass spectrum of *m/z* 165 ion (arsenocholine); (b) CID mass spectrum of *m/z* 135 ion (tetramethylarsonium). The ES-MS *m/z* ranges of interest are given in the left panels.

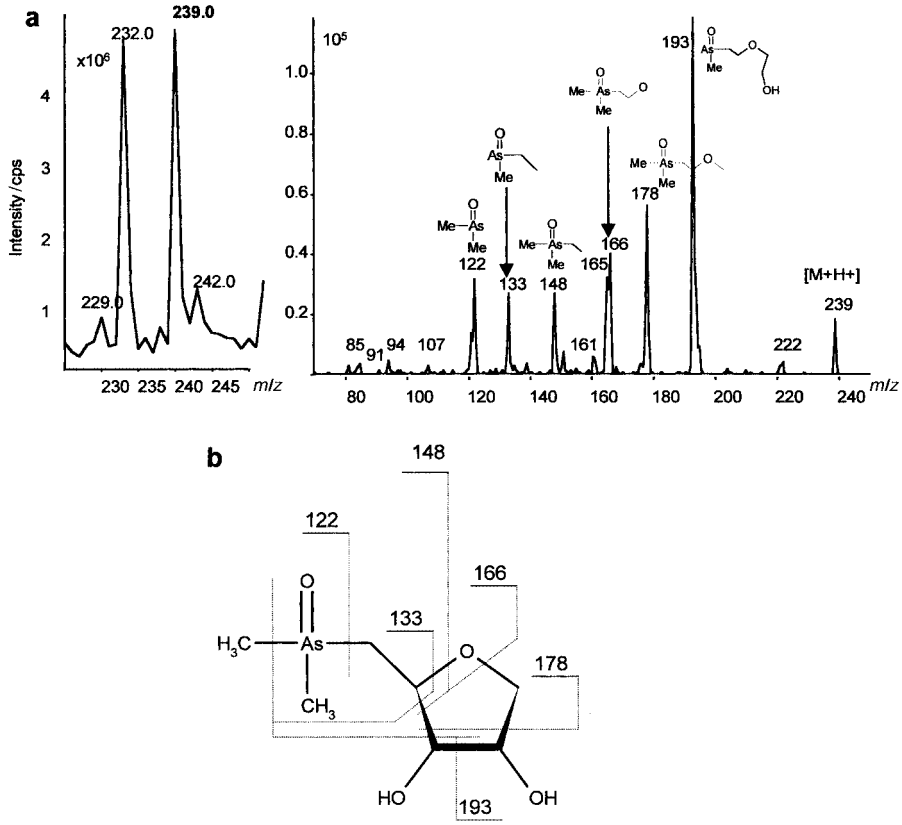

Fig. 6 Identification of peak A1.6 [*cf.* Fig. 1(d)] in CE-HPLC-ICP-MS by ES-MS-MS. (a) CID mass spectrum of the *m/z* 239 ion (the *m/z* range of interest in ES-MS is given in the left panel); (b) tentative fragmentation pattern.

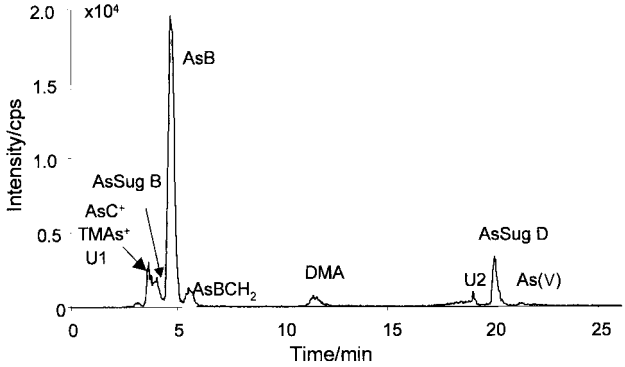

Fig. 7 Anion-exchange HPLC-ICP-MS chromatogram of the raw sample extract under optimized conditions.

Table 2 Quantification of arsenic species in the oyster sample studied

Species	Concentration of As/mg kg^{-1}	Concentration of species/mg kg^{-1} of species	Contribution to the total arsenic in the sample (%)
Unknown 1	0.31		5.3
AsC	0.06	0.123	1
$TMAs^+$	0.02	0.03	0.3
AsSug B	0.39	1.7	6.6
AsB	3.56	8.46 ± 0.34[a]	63.9
AsB-CH_2	0.22	0.58	3.8
DMA	0.28	0.52 ± 0.16[a]	4.6
Unknown 2	0.29		4.9
AsSug D	0.56	3.61	9.6
Total	5.69		100

[a] Five independent (including sample preparation) analyses.

was not quantified. Quantification of the other species can only be approximate. There were no standards available to us for trimethyl(2-carboxyethyl)arsonium inner salt and for the new ribofuranose compound. Species such as arsenocholine and tetramethylarsonium were not fully separated by anion exchange and their contents were estimated from the cation-exchange chromatograms of the void (the whole peak) of the preparative anion-exchange chromatography.

Conclusions

Three-dimensional HPLC-ICP-MS allows the mapping of minor (total concentration at the low μg g^{-1} level) arsenic species in animal tissues. The occurrence of 13 different arsenic species was shown in an oyster extract, eight of which (accounting for > 90% of the water-soluble arsenic) could be unambiguously identified by ES-MS-MS. This study is the first to demonstrate the successful use of ES-MS-MS to the characterization of arsenic species in animal tissues at such low levels based on a sample size of < 1 g. A new arsenic species could be isolated and tentatively identified on the basis of the CID fragmentation pattern as 5-dimethylarsinoylribofuranose. The knowledge of the number of species present is a prerequisite for the successful optimization of single mechanism HPLC-ICP-MS to eliminate the insufficient peak purity as the source of quantification error.

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