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Evidence of the Presence of Dimethylated, Trimethylated and 'Refractory' Arsenic Compounds in Estuarine Salt-marsh Halophytes

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Five species of halophytes were sampled in the salt marshes of the Tagus estuary, dried, ground and digested. They were further extracted with ethanol and the extracts passed through weak and strong cationic ion-exchange resins, purified through TLC and submitted to pyrolysis mass spectrometry and HPLC–ICP/MS. Arsenic content and hydride-forming arsenic species were verified, in each step, by GF–AA and HG–QFAA. A high content of arsenic was found in the samples of halophytes studied, both di- and tri-methylated arsenic compounds being present. A considerable fraction of this arsenic content seems to be refractory to hydride generation. Moreover, the arsenic fraction found seems to have the same ion-exchange behaviour as the refractory fractions formerly studied in estuarine water.

A partial characterization of these structures by pyrolysis–GC–MS suggests the presence of arsenobetaine and arsenocholine compounds. Furthermore, HPLC–ICP/MS data seem to confirm the presence of these compounds. In addition, the latter hyphenated technique strongly suggests the presence of a number of other organoarsenicals including tetramethylarsonium (TMAs), trimethylarsine oxide (TMAO), cacodylate (DMA) and possibly an arsenosugar-type compound. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

Arsenic is a powerful poison and suspected carcinogen, its toxic potential being strongly dependent on its chemical speciation and biogeochemical processing by ecosystems.

Marine biota seem to be more efficient in the processing of arsenic than terrestrial organisms, despite recent findings in the freshwater and terrestrial environments.[1,2] In fact, the quaternary arsenic compound trimethyl(carboxymethyl)arsonium zwitterion [arsenobetaine, $(CH_3)_3As^+CH_2COOO^-$)] has been found to be virtually ubiquitous in marine animals, particularly in those contributing to human diet.[3] These include crustacea (reptantia, Decapoda), Holo-

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thuroidea, Echinoderma, Mollusca (Bivalvia, Gastropoda, Cephalopoda) and Fish (Elasmobranchii, Teleostei).[4]

Arsenocholine $[(CH_3)_3As^+CH_2CH_2OH]$, a probable precursor of arsenobetaine, and trimethylarsine oxide $[(CH_3)_3AsO]$, one of its metabolites, have both been found in marine and freshwater organisms.[5–8] Its acetylated analogue, acetylarsenocholine[6,9] and tetramethylarsonium, $(CH_3)_4As^+$,[10,11] have also been identified in marine organisms.

However, the generalized mechanism through which arsenobetaine accumulates in the tissues of marine animals is far from being understood. Indeed, the biosynthesis of arsenobetaine from arsenate, in seawater, does not seem to be supported by previous empirical evidence. Preliminary assays with marked [^{74}As]arsenate assimilated from water by fish and crustacea gave puzzling results.[3] Also, the oral administration of inorganic arsenic to fish did not lead to the accumulation of arsenobetaine in their tissues.[3,12,13]

Several authors therefore suggest that the food chain, rather than seawater, may be the source of organic arsenic in marine animals.[11,14] The presence of heavier arsenic compounds occurring as algal metabolites and identified as di- and trimethylated arsenoribosides[15,16] further suggested a mechanism of accumulation through the food chain.[14,17–19] The identification of dimethylarsinylriboside derivatives (arsenosugars) in shellfish[20–22] and the suggestion of a theoretical mechanism for the conversion of trimethylarsenoribosides into arsenocholine[4,23] seem to reinforce this idea.

This hypothesis, however also suffers from some difficulties. In particular, the feeding of Fish, Crustacea, Bivalvia, Copepoda and bacteria with algal arsenoribosides does not seem to produce arsenobetaine.[13,16,24,25]

The identification of dimethyloxarsylethanol as a degradation product, in sediments, of the arsenoribosides present in *Ecklonia radiata* tissues suggested a new pathway. Neverthless, most biochemical steps have never been clarified or confirmed.[4,14] Moreover, the authors who examined this matter on an enlarged base of evidence concluded that there is no obvious relationship between the concentration of arsenobetaine in the tissues of marine animals and their trophic position in the food web.[27]

The question of the origin of arsenobetaine present in marine animals therefore remains the subject of active controversy in marine environmental biogeochemistry. This controversy is most relevant, given the toxic properties of the inorganic and simple methylated arsenic species and the non-toxic properties of arsenobetaine.

The detection of trimethylarsenic forms[4,28] and the quantification and partial identification in estuarine waters of two new arsenic fractions refractory to hydride generation, at least some of which are trimethylated,[28–31] appears, in this context, as a new avenue.

A complete positive identification of the chemical structure of these refractory arsenical fractions has not yet been achieved, apparently due to the low levels in which they occur (< ppb) combined with the interference effects of the complex estuarine water matrix.[32,33] Nevertheless a mass-fragmentometry determination performed on an extract of these waters suggests the presence of an arsenocholine moiety.[4,33] Recent analytical determinations using HG–QFAA, HPLC and DCI–MS/MS further suggest the presence of halogen-bonded arsenobetaines in those fractions.[33]

The biochemical theory of osmoregulation[34–36] may provide a key for the investigation of another possible mechanism for the production and accumulation of arsenobetaine in the marine environment.

In fact it has been found that, apparently in response to osmotic stress, most halophytes, but also some algae and halophilic bacteria, readily accumulate a number of imino-acids and also methylated quaternary nitrogen and methylated sulphur compounds. These include proline, pipecolic acid, glycine betaine, homobetaine, stachydrine, sorbitol, mannitol and dimethylsulphonium-3-propanoic acid.[35–41] Choline, the precursor of glycine betaine, is apparently involved in salt resistance of halophilic bacteria.[32,43]

This osmoregulation mechanism seems to be present in the metabolism of many halophytes, and in some circumstances of other plants.[44,45] Some of these compounds are nitrogen or sulphur analogues of the structures that have been proposed for the refractory arsenicals.[33] This suggests that these may be biosynthesized, in similar conditions, by the same mechanisms.

As a first step to clarify this issue, we therefore decided to investigate the presence of this type of compound in the tissues of estuarine halophytes, particularly those present in salt-marsh formation of the Tagus estuary.

MATERIALS AND METHODS

Collection of samples and general processing

Samples of the halophytes *Artrochnemum perenne* (sample 965), *Artrochnemum fruticosum* (sample 966), *Spartina maritima* (sample 967), *Halimione portulacoides* (sample 968) and *Halimione* spp. (sample 969) were collected at the Pancas salt-marsh study site within the Tagus Nature Reserve, and kept cold till further processing.

In the laboratory the samples were cut into small pieces and dried at 40 °C. Dry weights were determined on sub-samples after being dried at 100 °C to constant weight.

The remaining portions were processed according to procedures in use at the Estação Agronómica Nacional (EAN), Portugal, for vegetable material; in particular they were ground in a blade mill until they turned to powder. The resulting samples, for the selected five species, varied between 20 and 100 g (dry wt).

These samples were extracted with ethanol and one of the extracts (966) was passed, mainly for purification purposes, through thin-layer chromatography (TLC-I).

As arsenic compounds hardly eluted in the system used, the arsenic-containing sections of the TLC plate were scraped, extracted with methanol, evaporated to dryness and recovered with Milli-Q water (L_1/966). The remaining plant extracts (965, 967, 968 and 969) were not subjected, in this phase, to TLC, and were instead evaporated to dryness and recovered with Milli-Q water (L_1/965, 967, 968 and 969).

The five fractions resulting from this first step (L_1) were diluted to a small volume (10–15 cm^3) and separately passed through a weak cationic ion-exchange column.

The five water eluates of this column (L_3) were passed separately through a strong cationic ion-exchange column. The eluates obtained on this second column were then pooled according to the eluent (L_6, $L_7+L_8+L_9$ and L_{10}), concentrated and purified again through TLC (TLC-II).

The arsenic peaks detected in this last TLC application were finally concentrated to three small-volume pairs of samples referred as extract (1,2) from L_{7+8+9} extract (3,4) from L_6 and extract (5,6) from L_{10}. The pair (3,4) was further submitted to pyrolysis mass spectrometry and HPLC–ICP/MS.

The total arsenic content of each fraction obtained was verified, all through the process, through graphite furnace atomic absorption spectrophotometry (GFAA) or hydride generation–quartz furnace atomic absorption spectrophotometry (HG–QFAA) of digested samples. Inductively coupled plasma emission spectrometry (ICP–AES) was also used for the first digest of the plant material.

The content of hydride-forming arsenic species was determined in some undigested fractions by HG–QFAA.

The general scheme is depicted in Fig. 1.

Digestion procedures

Acid digestion

Duplicate portions (1 g) of each sample of material were digested with 5 cm^3 of H_2SO_4 (dil.1:1) according to the *US Pharmacopeia* method: the plant powder was gently heated in a sand bath and portions of H_2O_2, 30% (v/v), were added; as the process proceeded the temperature was increased moderately (not exceeding 120 °C), until a clear supernatant liquid was obtained.

Total (dry-ashing) digestion

Total digestions were also obtained according to the dry-ashing process of Uthe *et al.*[46] An adequate volume (2.5 cm^3) of ashing aid (a slurry of 7 g of MgO + 10.5 g of $Mg(NO_3)_2 \cdot H_2O$, in 100 cm^3 of Milli-Q water) was added to the samples (1 g) and the mixture dried overnight at 80 °C. The evaporates were then heated in a muffle furnace (1 h/200 °C + 1 h/300 °C + 12 h or overnight at 460 °C). The dry residues were recovered with 2 M Suprapur HCl and made up to 50 cm^3 with Milli-Q water. Blanks of Milli-Q water are run in the same way and cacodylate, arsenocholine or tetramethylarsonium was used as internal standard to determine the yield of the digestions.

Solvent extraction of plant tissue

The plant powder samples were extracted according to the Larher and Hamelin[34] procedure: 5 g of each sample was introduced into an Erlenmeyer flask to which 50 cm^3 of ethanol (80° GL) was added. The flasks were agitated in a flask-shaker Gallenkamp for about 5 h.

The mixture was then filtered through pre-cleaned Whatman 41 filters and the extract evaporated to dryness in a rotary evaporator, at

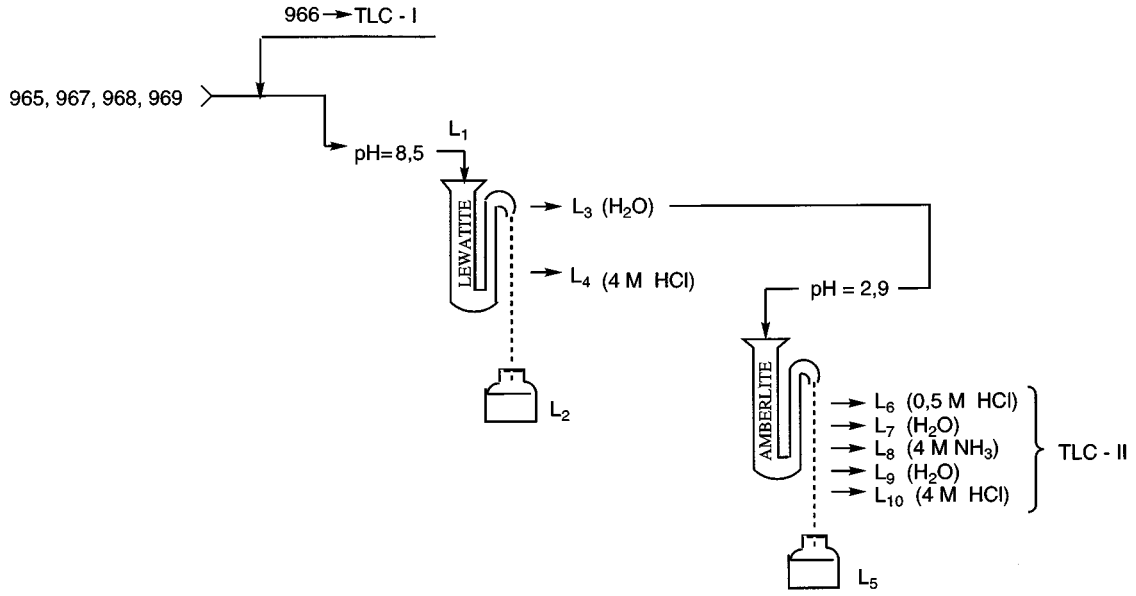

Figure 1 Scheme of ion-exchange purification; 965, 966, 967, 968 and 969, original plant extracts; L_1, water fractions recovered from the dried plant extracts; L_2 and L_5, leakages; L_3 and L_4, eluates of the weak cationic ion-exchange column; L_6 to L_{10}, eluates of the strong cationic ion-exchange column.

37 °C. The residue was recovered with 4 cm^3 of double-distilled Milli-Q water.

Concentration and purification procedures

Concentration

Most fractions were concentrated by evaporation on a thermal plate or sand bath, in a hood, or in rotary evaporators, at a low temperature, as indicated. Small volumes were, in general, concentrated in an Altech concentrator under argon or nitrogen. For the concentration of the final samples for MS analysis, Pasteur pipettes were used with the same gases.

Ion-exchange chromatography

The purification of the plant tissue extracts was attempted by following a simplified version of former procedures that proved to be adequate for the purification of the arsenic compounds contained in marine animal muscle.[5,47] As already mentioned, this procedure includes a sequence of weak and strong cationic ion-exchange resin columns.

The weak cationic ion-exchange column was a glass column 150 mm × 13 mm (i.d.), packed with Lewatite CNP 80, H^+ form (standard grade). It was equilibrated by successive elution with 3% hydrochloric acid, Milli-Q water, 3% sodium hydroxide and Milli-Q water.[4] After correction of the pH to 8–8.5, the extract was applied to the column and this was eluted with Milli-Q water followed by 5% hydrochloric acid and sometimes also 4 M HCl.

The strong cationic ion-exchange, with the same dimensions as the weak cationic ion-exchange column, was packed with the resin Amberlite IR-120, Na^+ form (analytical grade). This column was equilibrated with Milli-Q water, 5% hydrochloric acid and Milli-Q water.

After the application of the extract (at pH about 3), the column was eluted with 0.5 M HCl, Milli-Q water, 4 M ammonia, Milli-Q water and 4 M HCl (Suprapur).

Blanks of Milli-Q water were run in the same way and their arsenic content verified by GF–AAS.

Thin-layer chromatography (TLC)

As mentioned, one plant tissue extract (966) was concentrated and applied to cellulose (Merck aluminium foil, F254, 0.2 mm, 20 cm × 20 cm) TLC plates and eluted with chloroform/methanol/water/ammonia (65:35:4:1, by vol.) in a Camag chromatographic vat. A total of 2900 μl was applied on three plates. These were conditioned as indicated previously.[4] A mixture of

synthetic standards [arsenate (As^{5+}), monomethylarsonate (MMA), dimethylarsinate (DMA), arsenocholine (AsC) and arsenobetaine (AB)] was also applied and developed by spraying with a solution of iodine on chloroform (0.5 g/100 cm^3).[4,5]

The whole set of eluates obtained from the strong cationic ion-exchange resin column (L_6, $L_7+L_8+L_9$ and L_{10}) was pooled, concentrated and applied on an identical thin-layer (TLC) plate conditioned in the same way. This time chloroform/methanol/water/acetic acid (65:50:15:1, by vol) was used as eluent.

In each experiment the cellulose layer was scraped in sections, poured into Pyrex cups, extracted with methanol and filtered through Whatman 541 filters. The arsenic content of the resulting fractions (T_i) was determined by AA as indicated below.

Arsenic detection and quantification

Atomic absorption spectrophotometry

Arsenic hydride-forming species were detected and quantified, as before, by HG–QF AA in a Perkin-Elmer 5000 AA spectrophotometer equipped with deuterium background correction, EDL source and lamp; the wavelength was 193.7 nm; the reactor vessel, was 25 cm^3 in volume and the glass surfaces were passivated with silanizing agents.[4]

Total arsenic content was determined either by the application of the same analytical method on digested samples, either by use of GFAA or by ICP–AES. GFAA determinations were performed in a Perkin-Elmer 2380 AA spectrophotometer equipped with an HG-500 graphite furnace; L'vov platforms, pyrolytic tubes and matrix correction with $Ni(NO_3)_2$ were used. The heating program was the following: drying at 120 °C, 50 s (including 15 s ramp); charring at 500 °C, 10 s (inc. 5 s ramp); gas interruption 500 °C, 5 s; atomizing at 2300 °C, 11 s (inc. 3 s ramp); ashing at 2650 °C, 6 s (inc. 2 s ramp).

Inductively coupled plasma emission spectrometry

ICP–AES analyses were performed on acid digests of the plant samples in a sequential ICP Jobin Yvon JY 24. The wavelength was $\lambda = 193.699$ nm and argon was used as the carrier gas. Simple calibration curves and the standard additions method have both been used in these determinations.

Identification of arsenic compounds by mass spectrometry

Pyrolysis–GC–MS

Pyrolysis–gas chromatography–mass spectrometry determinations were performed on a ZAB-HF VG mass spectrometer. The samples were applied in general on the platinum rod of the pyrolyser unit. A Curie-Point pyrolyser was used on the gas chromatograph (HRGC 5160 Mega Series, Carlo Erba) equipped either with a CP-SIL8-CB column or with a CP-SIL19-CB, 50 m × 0.25 mm (i.d.), film thickness 0.20 μm, WCOT fused silica GC column.

The pyrolysis attained 510 °C in 5 s. The GC column of the ZAB-HF was pre-heated according to the following programs: 10 min at 40 °C; ramp of 10 °C/min till 100 °C or 10 min at 40 °C; ramp of 15 °C/min till 250 °C. The mass range and the electron energy were *m/z* 45–400 and 70 eV respectively. Samples of 25 μl were injected as received, or after additional dilution in methanol.

HPLC–ICP/MS

The dry residues of the final extracts 3 and 4 (from L_6) were redissolved in 100 μl methanol and 500 μl water.

For the HPLC separation a Waters 6000 A HPLC pump and a U6K injector valve were used in conjunction with a cation-exchange column and a mobile phase. The chromatographic system was interfaced with the ICP–MS instrument by 20 cm of 1/16 in (1.6 mm) polytetrafluroethylene, Teflon®, capillary tubing (0.5 mm i.d.) that connected the HPLC column outlet to the inlet hole of the cross-flow nebulizer of the ICP–MS instrument.

The separations were carried out by a cation-exchange chromatographic column (Ionosphere-c, 100 mm × 3 mm i.d.) as the stationary phase with 2 mM or 20 mM pyridinium formate and 3% (v/v) methanol in water at pH 2.7 as the mobile phase.[48]

RESULTS

In Table 1 we present the results obtained for the total arsenic content of the first extracts (acid and dry-ashing digests) and other ion-exchange fractions obtained from samples 965, 966, 967, 968 and 969, reported to a normalized dry weight of

Table 1. Total arsenic content of first extracts and ion-exchange fractions of samples 965, 966, 967, 968 and 969 (5 g)

	Total arsenic content (ng)				
Fraction	Sample 965	966	967	968	969
First extract					
Acid digest	N.A.	17850	N.A.	N.A.	44050
Dry-ashing digest	2322	10328	111488	1268	N.A.
Weak cationic					
H_2O eluate (L_3)	N.A.	N.A.	1603	1133	N.A.
HCl eluate (L_4)	521	N.A.	106	193	1000
Strong cationic					
Leakage (L_5)	N.A.	N.A.	N.A.	N.A.	328
0.5 M HCl eluate (L_6)	1131	465	N.A.	302	566
H_2O eluate (L_7)	N.A.	880	N.A.	N.A.	N.A.
4 M NJ_4 eluate (L_8)	488	1097	584	47	3074 (44 DMA)
H_2O eluate (L)	N.A.	502	N.A.	N.A.	N.A.
4 M HCl eluate (L_{10})	N.A.	236	N.A.	N.A.	726

5 g. All the ion-exchange blanks were below detection level.

Fractions that have not been analysed by GF–AAS or HG–QFAA, and therefore have no assigned value for arsenic content, are indicated by NA in Table 1. The presence of 44 ng of DMA is also acknowledged for the 4 M NH_4 eluate of sample 969 (L_8/969) in this table.

In Table 2 we present the results obtained for arsenic speciation in the strong cationic ion-exchange fractions that resulted from the processing of sample 966.

As mentioned before in the first application of the TLC system (extract of sample 966, TLC-I) the arsenic compounds present hardly moved from the injection base line, 72% of the arsenic being present in the first section T_0 and 21% in the second section T_1.

In Table 3 (and Fig. 2) we present the results found for total arsenic and arsenic speciation of the pooled fractions collected in the second TLC application (TLC-II). The behaviour in this case is distinct from that of TLC-I, with a dominant peak of arsenic in section T_2 and a secondary peak in section T_5.

Figures 3–6 depict mass spectra, obtained from the pair of samples (3,4/L_6) by pyrolysis–GC–MS, that have been compared with the corresponding standards, whereas Figs 7 and 8 depict the chromatograms obtained with the HPLC–ICP/MS system on the same extracts 3 and 4. On the other hand Fig. 9 depicts the chromatogram obtained with the same HPLC–ICP/MS system on a mixture of standards [arsenate (As^{5+}), monomethylarsonate (MMA), cacodylate (DMA), arsenobetaine (AB), trimethylarsine oxide (TMAO), arsenocholine (AsC) and tetramethylarsonium (TMAs)].

Table 2. Arsenic specification[a] (ng) of final ion-exchange fractions obtained from the processing of sample 966

Fraction	As_{total}	As^{5+}	MMA	DMA	TMA	$As_{ref.}$
0.5 M HCl eluate	465	17	N.D.[b]	N.D.	N.D.	448
4 M NH_4 eluate	1097	N.D.	N.D.	35	480	582
4 M HCl eluate	236	N.D.	N.D.	N.D.	236	N.D.

[a]As^{5+}, arsenate; MMA, monomethylarsenic; DMA, dimethylarsenic; TMA, trimethylarsenic; $As_{ref.}$, refractory arsenic. [b]N.D., below detection level.

Table 3. Total arsenic and speciation of TLCII collected fractions (ng)

Fraction	As_{total}	As^{5+}	MMA	DMA	TMA	$As_{ref.}$
T_0 injection	124	N.A.[a]	N.A.	N.A.	N.A.	
T_1	405	N.A.	N.A.	N.A.	N.A.	
T_2	762	N.A.	N.A.	N.A.	N.A.	
T_3	745	39	18	73	30	585
T_4	339	N.A.	N.A.	N.A.	N.A.	
T_5	395	35	21	142	N.D.	197
T_6	219	N.A.	N.A.	N.A.	N.A.	
T_7	321	52	26	157	N.D.	86
Solvent front						

[a]N.A., not analysed; N.D., below detection level.

DISCUSSION

The levels obtained for total arsenic content of halophyte tissue digests by ICP–AES and by HG–QFAA in the Tagus salt marshes appear as relatively high, when compared with the values given in the literature for the arsenic content of marsh plants (0.45–12 μg/g), in other estuaries.[49–51] This is particularly the case for *Artrochnemum* sp. and *Halimione* sp. shoots and leaves. The high arsenic content of *Spartina maritima* material (967) may be explained by the

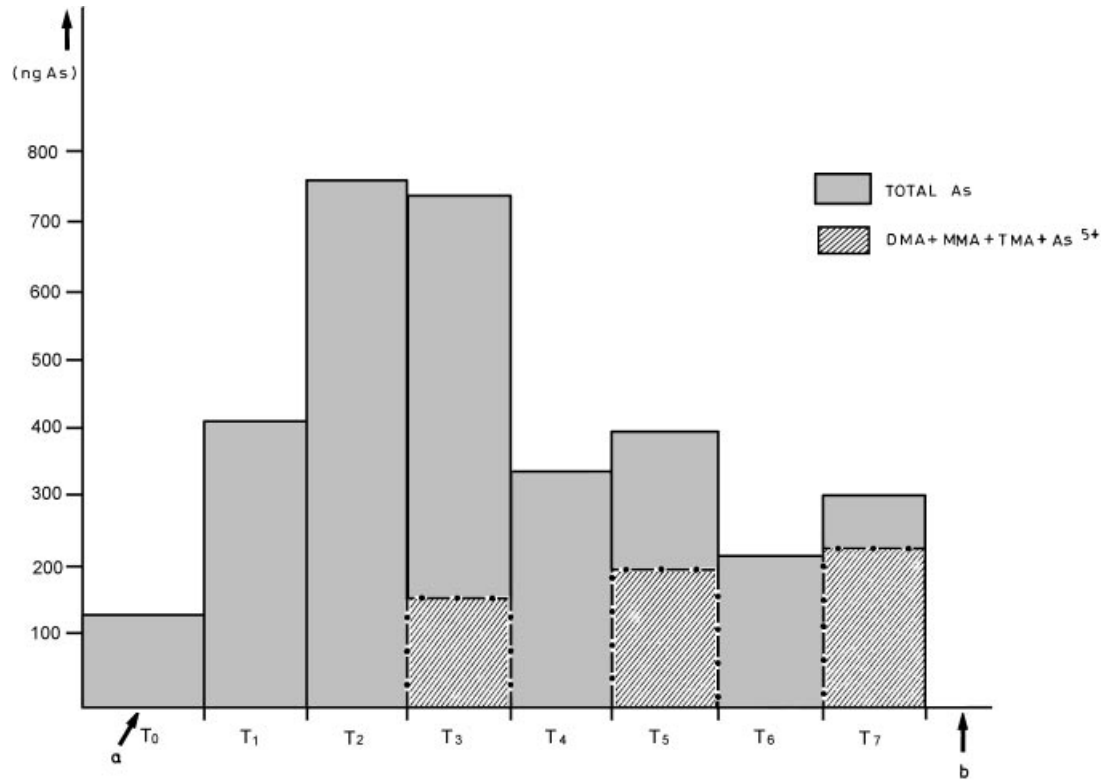

Figure 2 TLC-II chromatogram of L_{7+8+9}: (a) injection line; (b) solvent front; T_0 to T_7 sections sequentially scraped along the TLC; the grey bars represent total arsenic contents for sections T_0 to T_7; for sections T_3, T_5 and T_7 in addition to total arsenic, the sums of inorganic arsenic ($As^{5+} + As^{3+}$), MMA, DMA and TMAO have been determined and are represented by the hatched bars.

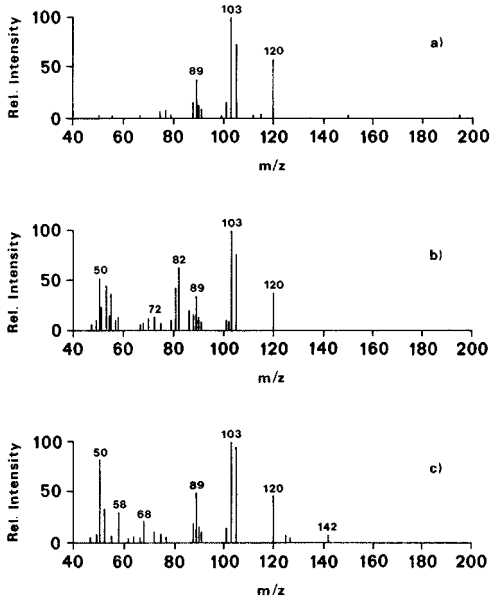

Figure 3 Pyrolysis–GC–MS spectra: (a) scan 36 of arsenobetaine; (b) scan 44 of extract 3; (c) scan 38 of extract 4.

fact that this sample included the root biomass and has not been specifically cleaned of root iron plaque.[49,50]

Comparing these values with those obtained for the subsequent extracts (Table 1) it seems clear that most of the arsenic present in the samples richer in arsenic (966, 967, 969) was lost, which may be due to the poor yield of the

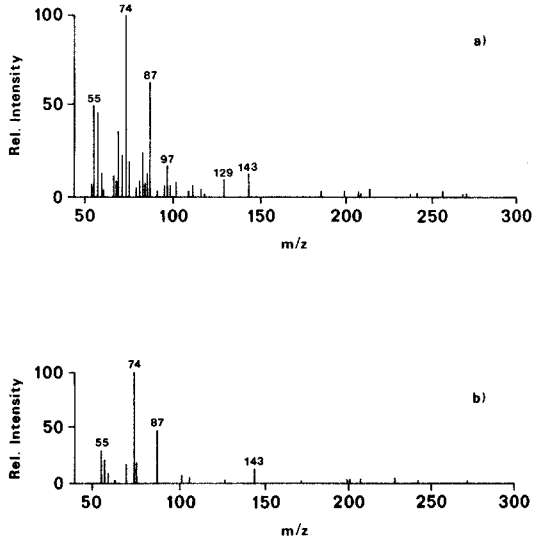

Figure 4 Pyrolysis–GC–MS spectra: (a) scan 349 of extract 4; (b) scan 326 of arsenobetaine.

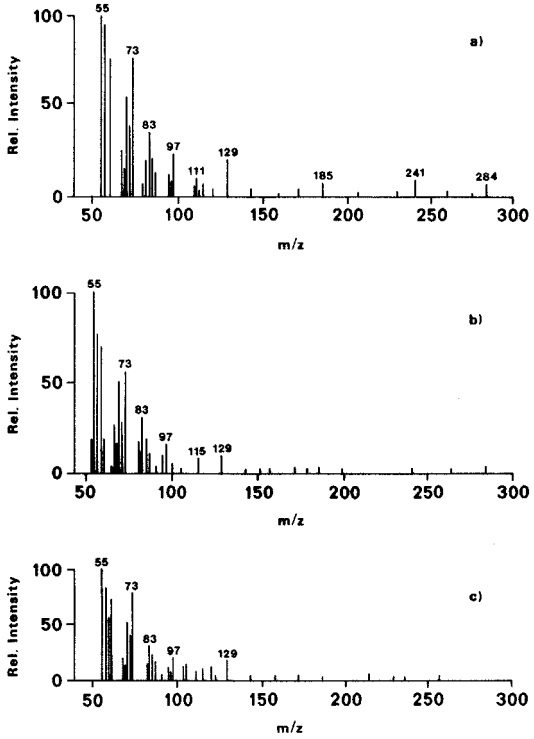

Figure 5 Pyrolysis–GC–MS spectra: (a) scan 371 of extract 4; (b) scan 373 of extract 3; (c) scan 343 of arsenobetaine.

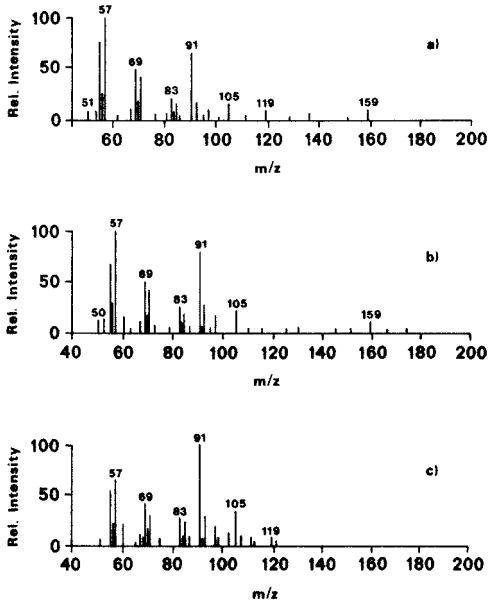

Figure 6 Pyrolysis–GC–MS spectra: scans 274 of (a) extract 4; (b) extract 3; (c) arsenocholine.

solvent extraction procedure.

An important conclusion, that seems beyond reasonable doubt, is that, besides dimethylated arsenic forms, there are trimethylated arsenic forms present in the tissues of the halophytes under study. These latter forms are at the origin of the species detected by HG–QFAA as TMA in the extracts and fractions obtained from them (Tables 2 and 3). This is certainly an interesting finding, given the previous detection of TMA forms in the waters of the same estuary.[4,28]

Although preliminary, the data collected suggest some interspecific difference in the processing of arsenic by these plants. In fact while TMA forms dominate in the strong cationic ion-exchange fractions (L_8 and L_{10}) of sample 966 (*Artrochnemum fruticosum*) (Table 2), DMA forms have been observed to occur

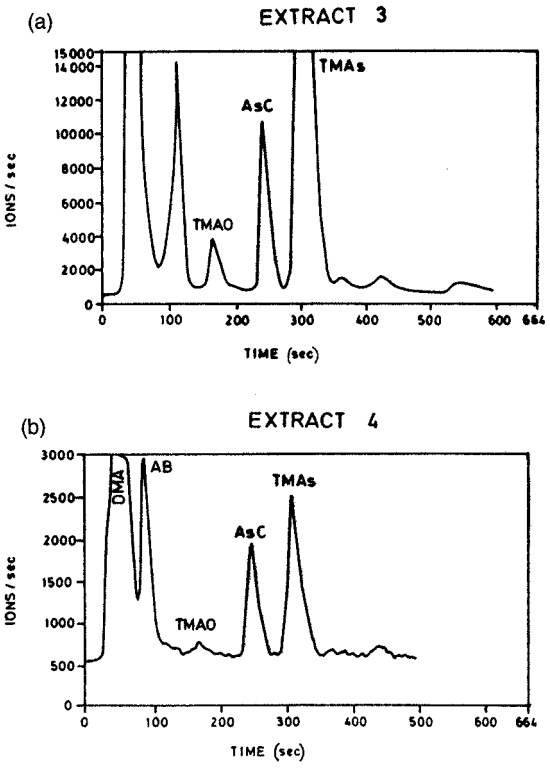

Figure 7 HPLC–ICP/MS chromatograms (20 mM pyridine mobile phase) of: (a) extract 3, showing As^{5+} + monomethylarsonate + dimethylarsinate, trimethylarsine oxide (TMAO), arsenocholine (AsC), tetramethylarsonium (TMAs) and some unknown arsenic compounds eluting after TMAs; (b) extract 4, presenting As^{5+} + monomethylarsonate + dimethylarsinate, arsenobetaine (AB), trimethylarsine oxide (TMAO), arsenocholine (AsC) and tetramethylarsonium (TMAs) peaks.

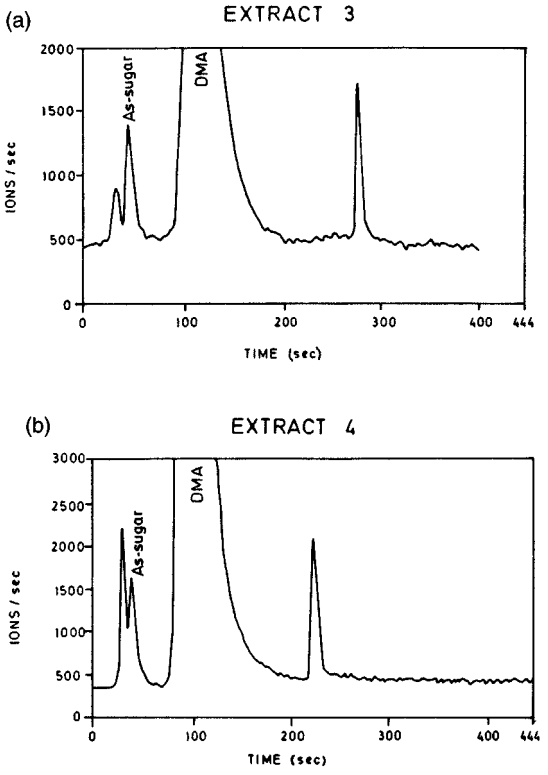

Figure 8 HPLC–ICP/MS chromatograms (2 mM pyridine mobile phase) of: (a) extract 3, presenting an As-sugar, dimethylarsinate (DMA) and an unknown arsenic compound eluting at 280 s; (b) extract 4 presenting an incompletely separated arsenosugar, dimethylarsinate (DMA) and an unknown arsenic compound eluting at 220 s.

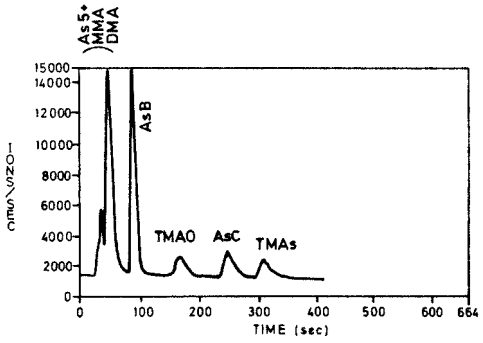

Figure 9 HPLC–ICP/MS chromatogram (20 mM pyridine mobile phase) of a mixture of standards showing peaks corresponding to arsenate (As^{5+}), monomethylarsonate (MMA), dimethylarsinate (DMA), arsenobetaine (AB), trimethylarsine oxide (TMAO), arsenocholine (AsC) and tetramethylarsonium (TMAs).

exclusively in fractions (L_8) of sample 969 (*Halimione* spp.) (Table 1).

Most of the arsenic present in these fractions proved to be 'refractory' to hydride generation, as follows from close analysis of Tables 2 and 3. The particular ion-exchange behaviour of the arsenic present in these fractions, being eluted by practically all the eluents used, is consistent with what has been previously observed for the 'refractory' arsenic fraction in estuarine water.[28,31,32]

A question that remains open is the chemical nature of these constituents of plant tissue. The comparison of pyrolysis–GC–MS mass spectra of extracts 3 and 4 and the mass spectra of the synthetic standards of arsenobetaine and arsenocholine, using the same technique, enables us to make some suggestions, however.

As an example, it may be observed that the mass spectra of both scan 44 of extract 3 and scan 38 of extract 4 are closely similar to the mass spectrum we have obtained of scan 36 of the standard of arsenobetaine (Fig. 3). The latter is a representative spectrum of arsenobetaine which is in agreement with literature results.[5]

It is noteworthy that other similarities may be found in further scans of both extracts, suggesting the presence of an arsenobetaine moiety. For example, the mass spectrum of scan 349 of extract 4 presents similarities with the mass spectrum of scan 326 of arsenobetaine (Fig. 4) and the mass spectra of both scan 371 of extract 4 and scan 373 of extract 3 are similar to the mass spectrum of scan 343 of arsenobetaine (Fig. 5). In addition, it appears that the presence of arsenocholine may not be excluded. In fact, the mass spectra of scans 274 of both extract 3 and extract 4 are identical to the mass spectrum obtained in the same scan of arsenocholine (Fig. 6).

It seems therefore reasonable to suggest that arsenobetaine and arsenocholine moieties are present in both extracts besides other interfering compounds.

On the grounds of what is known about the chemical structure of dimethylated and trimethylated arsenosugars, the possibility that we also are dealing with this type of compound cannot be entirely excluded. In fact the EI–MS mass spectra obtained on synthetic DMA arsenoribosides by Liu[52] also presents a number of peaks ($m/z = 89$, 103, 105, 120) that could possibly correspond to the moieties observed in these pyrolysis–GC–MS applications.

The non-selective ion-exchange behaviour exhibited by these extracts, and mentioned above, does not seem much in favour of an arsenobetaine or arsenocholine moiety. According to the literature these should elute, in fact, in the 4 M NH_4 and 4 M HCl fractions respectively of the strong cationic ion-exchange column, which is not the case.[4,5,47] Nevertheless, the results obtained for the same plant extracts with the HPLC-ICP/MS system confirm the presence of AsC in both extracts 3 and 4 (Figs 7 and 9) and of AsB in extract 4 (Figs 7b and 9). These results are in agreement with the previous suggestion of the presence of arsenobetaine and arsenocholine that follows from pyrolysis–GC–MS spectra.

In addition the HPLC–ICP/MS results suggest the presence, in these extracts, of tetramethylarsonium (TMAs) and trimethylarsine oxide (TMAO) (Fig. 7a and b) as well as dimethylarsinate (cacodylate) (DMA) and, apparently, an arsenosugar (Fig. 8a and b).[22,48] The HPLC–ICP/MS chromatogram further indicates the presence of a number of unidentified arsenic compounds eluting after TMAs (Fig. 7a) and after DMA at 280 s and 220 s (Fig. 8a and b, respectively).

These results, although a definite identification of the chemical structure of these compounds has not been possible so far, are interesting in the context of the arsenobetaine-origin controversy. Apparently they constitute the first detection of arsenobetaine, arsenocholine and tetramethylarsonium in marine plants, namely estuarine Angiosperms.

Nevertheless one cannot entirely discard the possibility that some degradation might have occurred during the manipulation of the extracts under study. Their distinct behaviour in the two TLC systems would be in favour of such a hypothesis, since neither arsenobetaine nor arsenocholine is likely to have, in the TLC-I system, the R_f observed in the present case.[4] However, the end-products of such a possible degradation process seem to be those that we have determined.

The fact that we are dealing with a number of organoarsenic compounds may help to explain the non-selective ion-exchange behaviour exhibited by the 'refractory' fraction which appears in almost all the eluates.

If arsenobetaine and arsenobetaine-like compounds are present in the tissues of estuarine halophytes, which seems quite probable on the available evidence, it would be reasonable to assume, as postulated, that these compounds are

the result of a metabolic biosynthesis by plants. This biosynthesis might occur through the same mechanisms and in response to the same stimulus that apparently explains the accumulation of the normal betaines and other compatible solutes—the need to respond to osmotic stress.

The presence of the tetramethylarsonium ion and the possible presence of arsenosugars may need, however, to be explained in a different way. If these compounds are present in halophyte tissues they are likely to pass, by mineralization or exudation, to the surrounding estuarine waters. This would be convergent to a certain extent, with the results of the previous mass fragmentometry determination, based on which the presence of arsenocholine has been postulated for the first time, in the Tagus estuarine waters.[4] It would also be in agreement with the more recent DCI–MS/MS determinations that suggest the presence of halo arsenobetaines, most of them trimethylated, in the same waters.[33]

These observations are therefore consistent with the hypothesis that osmoregulation mechanisms, typical of halophyte behaviour, may be involved in the estuarine biogeochemical cycle of arsenic, particularly in the process that leads to accumulation of arsenobetaine in marine animals.

CONCLUSIONS

Relatively high concentrations of arsenic were found in five species of halophytes in Tagus estuarine salt marshes. Di- and tri-methylated arsenic species are present and a considerable fraction of this arsenic content seems to be refractory to hydride generation. The arsenic fraction found in halophyte extracts seems to have the same ion-exchange behaviour as the 'refractory' arsenic fractions studied previously in estuarine water.

A partial characterization of these structures by pyrolysis–MS suggests the presence of arsenobetaine and arsenocholine moieties.

The results obtained in the same extracts by HPLC–ICP/MS confirm the presence of both these compounds. They further indicate the presence, in these extracts, of tetramethylarsonium (TMAs), trimethylarsine oxide (TMAO) and cacodylate (DMA), and suggest the presence of one arsenosugar, in addition to several unidentified arsenic-containing compounds.

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