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Evaluation of microwave and ultrasound extraction procedures for arsenic speciation in bivalve mollusks by liquid chromatography–inductively coupled plasma-mass spectrometry

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ABSTRACT

Liquid chromatography–inductively coupled plasma-mass spectrometry (LC–ICP-MS) was used for arsenic speciation analysis in tissues of bivalve mollusks (*Anomalocardia brasiliana* sp. and *Macoma constricta* sp.). Microwave and ultrasound radiation, combined with different extraction conditions (solvent, sample amount, time, and temperature), were evaluated for As-species extraction from the mollusks' tissues. Accuracy, extraction efficiency, and the stability of As species were evaluated by analyzing certified reference materials (DORM-2, dogfish muscle; BCR-627, tuna fish tissue; and SRM 1566b, oyster tissue) and analyte recovery tests. The best conditions were found to be microwave-assisted extraction using 200 mg of samples and water at 80 °C for 6 min. The agreement of As-species concentration in samples ranged from 97% to 102%. Arsenobetaine (AsB) was the main species present in bivalve mollusk tissues, while monomethylarsonic acid (MMA) and arsenate (As(V)) were below the limit of quantification (0.001 and 0.003 μg g^{-1}, respectively). Two unidentified As species also were detected and quantified. The sum of the As-species concentration was in agreement (90 to 104%), with the total As content determined by ICP-MS after sample digestion.

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1. Introduction

Marine organisms, such as bivalve mollusks, are widely consumed by humans. The consumption of bivalve mollusks is common in all coastal regions of Brazil due to the abundance of natural resources of the aquatic ecosystem. Todos os Santos Bay (Bahia, Brazil) is an important fishing community, the main activity of which is collecting mollusks in the nearby mangrove [1]. However, bivalve mollusks are among the biggest accumulators of contaminants from the environment, and their consumption is associated with human intoxication [2].

One of the main elements that mollusks can bioaccumulate is arsenic, mainly organic arsenic. Inorganic arsenic (i-As) can be bioconverted to methylated species such as monomethylarsonic acid (MMA) or arsenobetaine (AsB) [3]. The biotransformation rate depends on the intake of As and the transformation mechanisms in animals. The correlation between the total element concentration and the metabolized fraction is not completely known [1,3].

The quantification of total As concentration in seafood already is performed routinely. However, the toxic and biological effects of As cannot be associated only with total arsenic concentration. The toxicities of As species differ mainly in terms of their oxidation states and whether they are present in inorganic or organic forms. For example, inorganic As is considered carcinogenic [4], while organic As – such as AsB, arsenocoline (AsC), and several arsenosugars – are considered nontoxic.

The predominant As species in bivalve mollusks is AsB, the concentration of which ranges from 1 to 100 μg g^{-1} [5]. Minor species – such as MMA, dimethylarsenic acid (DMA), and tetramethylarsonium ion (TMA) – also have been found [6,7]. Therefore, the total concentration of As species in seafood should be determined in order to effectively evaluate the toxicity of the element and potential effects on humans.

Several studies have focused on As speciation analysis [6–11]. Usually, a combination of analytical techniques is necessary to achieve sufficient selectivity and sensitivity for speciation analysis. Liquid chromatography (LC) with inductively coupled plasma mass spectrometry (ICP-MS) has been used most often. This technique allows good separation of As species, in addition to the selective detection of species present in low concentrations.

The inclusion of a hydride generation system after the chromatographic column can improve the limit of detection (LOD) and can reduce interferences [8,12–14]. However, arsenic speciation analysis is still challenging because the integrity of the As species must be

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maintained during all steps of the analysis, and sample storage and sample preparation are most critical steps. Established methods to extract arsenic species from solid samples are still not available, and certified reference materials (CRMs) with certified mass fractions values of As species are scarce. So, the accuracy of the method is not easily accessed [8,15]. The difficulty of i-As speciation analysis in seafood has been demonstrated recently [8,15]. The authors of those studies highlighted that there are strong discrepancies among the results found by different laboratories [15].

As stated before, sample preparation is critical in speciation analysis, mainly for solid matrices. Diluted acids, water, methanol, and a methanol–water mixture are the most common solvents used for As-species extraction from biological samples [15–18]. To accelerate the As-species extraction, mechanical stirring and Soxhlet extraction are usually employed [19]. More recently, ultrasound (US) extraction [20–23], microwave-assisted extraction (MAE) [11,24–30], supercritical fluid extraction, and pressurized liquid extraction [31,32] have been evaluated in order to reduce the extraction time and volume of extractant. The extraction efficiency depends on the sample matrix, species to be extracted, type of solvent, extraction time, and temperature. However, in general, there is no universal procedure for species extraction in speciation analysis and it is necessary to investigate the extraction conditions for each sample matrix.

Most of the As species present in biological tissues are soluble in water and can be extracted with water alone or with a mixture of water and methanol. Orthophosphoric acid and trifluoroacetic acid have been used to improve extraction efficiency. Extraction of As species from fish tissue also has been carried out by means of enzymes [22,33].

In the present study, the application of MAE and US combined with different solvents were investigated for As-species extraction from edible tissues of bivalve mollusks. Arsenic species were separated using anion-exchange chromatography and detected using ICP-MS. The method was applied for As speciation analysis in samples of bivalve mollusks collected in an area that is highly impacted by human activities.

2. Experimental

2.1. Instrumentation

For As detection, an inductively coupled plasma-mass spectrometer (Elan DRC II, PerkinElmer Sciex, Thornhill, Canada) equipped with a concentric nebulizer (Meinhard Associates, Santa Ana, CA, USA), a cyclonic spray chamber (Glass Expansion, Inc., West Melbourne, Victoria, Australia), and a quartz torch with a quartz injector tube (2 mm i.d.) was used. The nebulizer gas flow rate, ion lens voltage, and torch alignment were adjusted following the manufacturers' instructions, using conventional nebulization. Single-ion monitoring for m/z 75 was used to collect the As signal. The LC system consisted of a quaternary pump (Series 200, PerkinElmer) equipped with a Rheodyne six-port injector valve (200 μL sample loop) and an anion-exchange separation column (Hamilton, PRP-X100, 250 cm length and 4.1 mm i.d.). The column outlet was connected to the pneumatic nebulizer of the ICP-MS instrument through PEEK-tubing. All separations were conducted at room temperature. The operation conditions of the LC–ICP-MS system are summarized in Table 1.

Total arsenic was determined using a simultaneous, inductively coupled plasma optical emission spectrometer (Spectro CIROS CCD, Spectro Analytical Instruments, Kleve, Germany) with axially viewed plasma. The selected wavelength for As and operational parameters are listed in Table 1. Instrumental conditions used were those recommended by the instrument manufacturer.

Argon with a purity of 99.996% (White Martins—Praxair, São Paulo, SP, Brazil) was used as plasma, auxiliary, and nebulization gas.

A Multiwave 3000 microwave oven (Anton Paar, Graz, Austria) was used for sample digestion and As-species extraction.

Table 1
Instrumental conditions for total As determination by ICP OES and for As-species determination by LC–ICP-MS.

Total As determination by ICP OES	
RF power	1500 W
Plasma gas flow rate	14 L min^{-1}
Auxiliary gas flow rate	1.0 L min^{-1}
Nebulizer gas flow rate	0.70 L min^{-1}
Spray chamber	Double pass, Scott type
Nebulizer	Cross-flow
Plasma observation view	Axial
Wavelength	189.042 nm
ICP-MS	
RF power	1400 W
Plasma gas flow rate	15 L min^{-1}
Auxiliary gas flow rate	1.2 L min^{-1}
Nebulizer gas flow rate	1.10 L min^{-1}
Sampler and skimmer cones	Pt
m/z monitored	75
Dwell time	500 ms
As speciation by LC–ICP-MS	
Sample volume	200 μL
Mobile phase flow rate	1.25 mL min^{-1}
Mobile phase A (20 mmol L^{-1})	$(NH_4)_2HPO_4$, pH 6.0
Mobile phase B (20 mmol L^{-1})	$(NH_4)_2CO_3$, pH 8.5
LC program	0–1.4 min: 30% A 1.4–2.7 min: 100% B 2.7–15 min 30% A

Extractions of arsenic species using US were performed by means of a 20 kHz (130 W, nominal power) ultrasonic processor manufactured by Sonics and Materials, Inc. (Model VCX 130PB, Newton, CT, USA).

A centrifuge (3k30, Sigma, Osterode am Harz, Germany) was used for the extract centrifugation.

2.2. Reagents and solutions

Water was distilled and subsequently purified using a Milli-Q system (Millipore Corp., Billerica, MA, USA) in order to obtain resistivity of 18.2 MΩ cm. Nitric acid (Merck, Darmstadt, Germany) was purified by sub-boiling distillation (Milestone system, Model Duopur, Sorisole, Italy). Ammonium phosphate (Merck) and ammonium carbonate (Merck) solutions, both used as mobile phases, were prepared in water and then filtered through a 0.45 μm membrane filter (Millipore) before use. The pH of the mobile phase and sample was adjusted with 1.0 mol L^{-1} ammonium hydroxide (Merck) or 1.0 mol L^{-1} nitric acid.

Stock solutions containing 1000 mg L^{-1} As in the form of dimethylarsonic acid ($C_2H_6AsO_2H$, Sigma Aldrich, St. Louis, Missouri, USA), arsenite (As(III) — $NaAsO_2$, Merck), arsenate (As(V) — $Na_2HAsO_4 \cdot 7H_2O$ from Riedel-de Haën, Seelze, Germany), and monomethylarsonic acid (CH_5AsO_3, donated by Dr. J. Feldmann from the University of Aberdeen, Aberdeen, UK) were prepared in water. Stock solutions containing 1000 mg L^{-1} As of p-arsanilic acid (p-ASA, $C_6H_8AsNO_3$, Sigma) and arsenobetaine ($C_3H_6AsCH_2COOH$, donated by Dr. W. Goessler from Karl-Franzen University, Graz, Austria) were prepared in ammonium hydroxide (Merck). The total arsenic concentration in each stock solution was measured by ICP-MS. The standard solutions for total As determination were prepared by serial dilution of the SCP33MS (SCP Science, Baie d'Urfé, Canada) solution, which contained 10 mg L^{-1} As.

Solutions containing 10 mg L^{-1} As as As(III), As(V), AsB, DMA, MMA, and p-ASA were prepared in water and stored at 4 °C in a dark environment. These stock solutions were used to prepare standard solutions ranging from 0.10 to 10 μg L^{-1} As in water.

The CRM DORM-2 (dogfish muscle) from the National Research Council of Canada (NRCC, Ottawa, Ontario, Canada), BCR627 (tuna fish muscle) from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), and SRM 1566b (oyster tissue) from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) were analyzed to evaluate the accuracy of the method.

2.3. Samples and sample preparation

Samples of bivalve mollusks (*Anomalocardia brasiliana* sp. and *Macoma constricta* sp.) were collected in Todos os Santos Bay (Bahia, Brazil). After collection, the edible parts were removed from the shells with a plastic spoon, washed with ultrapure water, and transferred to polyethylene vials, which was sealed and kept at −20 °C until the analysis was conducted. Composite samples were prepared by pooling specimens of medium size.

Before analysis, the frozen samples were thawed, homogenized in a blender, lyophilized, and ground to obtain particle sizes lower than 100 μm in diameter. The lyophilized samples were placed in polyethylene flasks and kept at 4 °C in a dark environment. Speciation analysis of As was carried out in the lyophilized samples.

2.3.1. Microwave-assisted digestion

For total As determination, about 200 mg of samples was accurately weighed and transferred to quartz vessels of the microwave oven and then 6 mL of HNO_3 was added to the sample. The program was used according to the conditions recommended by the microwave oven manufacturer, which consists of (i) 10 min ramp to reach 1400 W, (ii) 1400 W for 15 min, and (iii) 0 W for 20 min (the cooling step). Pressure and temperature were set at 80 bar and 280 °C, respectively. After cooling, the digest was transferred to a polypropylene vessels, the volume was completed to 30 mL with water, and the solution was stored at 4 °C until the As determination by ICP OES. The conditions used for ICP OES are summarized in Table 1.

2.3.2. Microwave-assisted extraction

For As-species extraction assisted by microwave energy, approximately 200 mg of samples was accurately weighed and transferred to quartz vessels of the microwave oven, and 6 mL of water or a methanol–water solution (1:1, 2:1, and 3:1) was added to the samples. The following program was used: (i) 500 W for 5 min (ramp of 10 min), (ii) 1400 W for 6 min (ramp of 10 min), and (iii) 0 W for 20 min (cooling). The temperature was set at 80 °C. After cooling, the mixtures were transferred to polypropylene vessels, and the volumes were completed to 30 mL with water and then centrifuged at 935 *g* for 5 min. Subsequently, the supernatants were filtered through a 0.45 μm membrane syringe filter (Chromafil PTFE, Macherey-Nagel, Düren, Germany) and 200 μL of the filtered extract were injected into the chromatographic column used for As-species separation. The conditions used for As speciation analysis by LC–ICP-MS are summarized in Table 1.

2.3.3. Ultrasonic extraction

For As-species extraction assisted by US energy, about 200 mg of samples was accurately weighed and transferred to 50 mL polypropylene vessels, and 6 mL of water or a methanol–water solution (1:1, 2:1, and 3:1) was added to the sample. The mixtures were sonicated at 130 W for 6 min at an amplitude of 40%. Subsequently, the volumes of the mixtures were completed to 30 mL with water and then treated as described above (Section 2.3.2). The conditions used for As speciation analysis by LC–ICP-MS are summarized in Table 1.

The extraction efficiencies of MAE and US were evaluated by the analysis of certified reference material analyzing CRM oyster tissues and tuna fish. A mass balance of As was calculated by comparing the sum of the concentrations of all As species and the total As concentrations determined in the extracts as well as after sample decomposition.

2.3.4. Stability of As species submitted to microwave irradiation or sonication

The effects of microwave and ultrasound energy on the stability of the As species were studied using the CRM oyster tissue. Approximately 200 mg of CRM was accurately weighed and transferred to quartz vessels of the microwave oven or to 50 mL polypropylene flasks and spiked with As(III), As(V), AsB, DMA, MMA, and p-ASA in order to obtain final concentrations of 1.0 mg L^{-1} As. The speciation analysis of As in the spiked CRM was carried out as previously described.

2.3.5. Total As determination by ICP OES

Total As concentration in extracts and digested samples of bivalve mollusks and CRM were determined by ICP OES. Standard solutions ranging from 10 to 100 μg L^{-1}As were prepared in 5% (v/v) HNO_3. The instrumental conditions used for As measurements are cited in Table 1 and were adjusted to achieve the lowest limit of quantification (LOQ). The LOQ was calculated using the 10*s* criterion, where *s* was the standard deviation of 10 consecutive measurements of the sample blank.

2.3.6. Arsenic speciation analysis by LC–ICP-MS

Arsenic speciation analysis using LC–ICP-MS was carried out for CRM and bivalve mollusks. Quantification of each arsenic species was based on external calibration. Standard solutions were obtained from a multi-species solution prepared by the appropriate dilution of single-species stock solutions. The concentration range of standard solutions for each As species was 0.1 to 10 μg L^{-1} As and were prepared in water and used to obtain the calibration curves. The instrumental conditions set for the As measurements are summarized in Table 1 and were adjusted to achieve the lowest LOQ, calculated as cited above. The chromatographic peaks of As were processed using area integration.

Possible interference by Cl ($As^{40}Ar^{35}Cl^{+}$ with m/z 75) was evaluated for As in the mollusk extracts, which contained Cl^{-}. A 100 mg L^{-1} Cl^{-} solution was injected in the chromatographic column and the As signal (m/z 75) remained at the blank level. In previous studies using the same system [9], only a very small signal at m/z 75 was observed for 1000 mg L^{-1} Cl^{-}. Since the Cl^{-} concentration in the mollusk extracts was lower than 200 mg L^{-1} in the final solution, it was concluded that Cl^{-} did not interfere with As.

3. Results and discussion

3.1. Total As determination

The total As concentration found in the certified oyster tissue and tuna fish that were digested or extracted using MAE or US is shown in Table 2.

According to the t-student test at a 95% confidence level, recovery of total As was significantly better for MAE than US extraction. By considering only MAE, such significantly better As recovery was obtained by using pure water or a 1:1 methanol–water solution. These results accord with those found by other authors [34–38]. However, the efficiency of As-species extraction may vary from one laboratory to another. For example, in an interlaboratory study [15], the results obtained for i-As in a certified dogfish liver (DOLT-4) were spread.

Table 2
Total As recoveries from MAE and US extraction using different extractants. Results (in %) are the average and standard deviations of three replicates of each sample. Total As determinations are by ICP OES.

Extractants	SRM 1566b		BCR 627	
	MAE[a]	US[b]	MAE[a]	US[b]
Water	99 ± 2	91 ± 3	101 ± 5	93 ± 2
Methanol–water (1:1)	97 ± 4	89 ± 5	99 ± 7	86 ± 3
Methanol–water (2:1)	92 ± 2	83 ± 3	94 ± 4	81 ± 7
Methanol–water (3:1)	89 ± 2	77 ± 8	91 ± 2	72 ± 6

[a] MAE: microwave-assisted extraction.
[b] US: ultrasonic-assisted extraction.

Different conditions used for sample preparations as well as different techniques used for separation and detection were possible causes of diverging results. It also was affirmed that i-As determination in such a matrix is difficult [15].

In the present study, extraction of As species by using US energy was not efficient, which could be due to the conditions used or the sample matrix. According Table 2, different results are observed for the MAE, US, and extraction mediums. More accurate results (using a *t*-test at a 95% confidence level) were obtained by using MAE and water. Therefore, these conditions were chosen for further As-species extraction.

Analyte recovery in the spiked CRM submitted to MAE and extraction using 1:1 methanol–water was evaluated. Analyte recoveries in the range of 95% to 102% were achieved. Furthermore, the total As concentration found in the digested samples was similar to that found in the sample submitted to MAE (using a *t*-test at a 95% confidence level). In both cases, the total As concentration found agreed with the certified value.

3.2. Extraction of arsenic species from bivalve mollusks samples

Analyte extraction is critical in speciation analysis, mainly for solid biological tissues. Analyte losses, species interconversion, or incomplete species extraction are possible. Therefore, to achieve quantitative extraction without changing the species of the element, it is necessary to use mild extraction conditions. Even though several studies dealing with extraction procedures for arsenic species were reported [6,20–34], in the present study we investigated the use of MAE and US associated with water and mixtures of water and methanol for As-species extraction from edible tissues of mollusks. The volume of extractant and the sample mass were fixed at 6 mL and 200 mg, respectively. The extractant and the time of exposure to microwave radiation or sonication were then evaluated.

According to the results shown in Table 2 for total As concentration, better results were obtained when the samples were submitted to microwave radiation in water or a 1:1 water–methanol solution. These results agree with those found in previous research [10,25,30,36,38]. Although several extraction steps for 2 min at pre-defined maximum temperatures under microwave irradiation have been employed [6], in the present study only one step for 6 min at 80 °C was effective. In these conditions, the analyte extractions were quantitative (Table 3), without significant interconversion of As species. Therefore, for the subsequent tests, water (used as an extractant) and the microwave oven program described in Section 2.3.2 were used.

As mentioned before (Table 2), lower recovery of total As (using a *t*-test at a 95% confidence level) was observed when US was used for As-species extraction. Additional experiments were carried out by using a US probe to improve the extraction efficiency. However, As-species interconversion was observed. Due to this fact and the lower extraction efficiency achieved by means of sample sonication in a bath, US was not used further.

3.3. Arsenic speciation analysis

Arsenic species separation was conducted by using anion-exchange chromatography (PRP-X100 column). Ammonium phosphate (20 mmol L^{-1}) and ammonium carbonate (20 mmol L^{-1}) solutions were used as mobile phases. Initial experiments were carried out using these mobile phases in isocratic or gradient elution. The influence of the pH of the mobile phase was evaluated, and better As-species separation was obtained by using ammonium phosphate at pH 6.0 and ammonium carbonate at pH 9.0. Gradient elution was tested for both mobile phases, starting with 5% (water was used as a diluent) of a 20 mmol L^{-1} ammonium phosphate or a 20 mmol L^{-1} ammonium carbonate. The concentration of the mobile phases was increased to 100% in 25 min.

Tests were also carried out for isocratic mode elution. In this case, 6 mmol L^{-1} ammonium phosphate at pH 6.0 and 20 mmol L^{-1} ammonium carbonate at pH 9.0 were used. Good As-species separation was observed for standards by using gradient and isocratic elutions. However, the separation of As species in oyster tissue (NIST 1566b) was not satisfactory. In this case, good separation of As species in oyster tissue and standards were achieved by using ammonium phosphate and ammonium carbonate solutions at pH 6.0 and 9.0, respectively. To achieve better separation of As species, the elution was started using 6 mmol L^{-1} of the ammonium phosphate solution, and from 1.4 to 2.7 min the ammonium phosphate solution was changed to a 20 mmol L^{-1} ammonium carbonate solution. From 2.7 to 15 min, a 6 mmol L^{-1} ammonium phosphate solution was used again. The conditions employed for LC separation are summarized in Table 1.

By using these chromatographic conditions, the retention times of AsB, DMA, and As(V) were the same for standards and certified oyster tissue. However, As(III), MMA, and p-ASA were not detected, and an unidentified As species in oyster tissue was found. As observed by

Table 3
Results for total As and As species in CRM and samples of *Macoma constricta* sp. (MC) and *Anomalocardia brasiliana* sp. (AB). Results (in μg g^{-1}) are the average and standard deviations of three replicates of each sample.

CRM/ samples	As species (LC–ICP-MS)									Total As		
	AsB	As(III)	DMA	MMA	p-ASA	As(V)	UK_1	UK_2	UK_3	Σ As species (LC–ICP-MS)	MAE (ICP OES)	MW (ICP OES)
DORM-2[a]	16.8 ± 0.3	0.61 ± 0.04	0.841 ± 0.003	<0.001	<0.001	<0.003	–	–	–	18.25 ± 0.93	18.75 ± 0.66	18.1 ± 0.4
Certified value	16.4 ± 1.1	–	–	–	–	–	–	–	–	–	–	–
BCR-627[b]	3.80 ± 0.11	0.29 ± 0.04	0.147 ± 0.013	0.085 ± 0.005	<0.001	0.035 ± 0.001	–	–	–	4.35 ± 0.18	4.68 ± 0.03	4.56 ± 0.04
Certified Value	3.90 ± 0.22	–	0.150 ± 0.020	–	–	–	–	–	–	–	–	–
NIST 1566b[c]	5.40 ± 0.20	<0.001	0.40 ± 0.01	<0.001	<0.001	1.16 ± 0.01	0.33 ± 0.03	–	–	7.25 ± 0.25	6.94 ± 0.2	7.2 ± 0.3
MC	71.0 ± 4.0	16.0 ± 1.1	1.30 ± 0.05	<0.001	1.07 ± 0.01	<0.003	7.13 ± 0.05	3.62 ± 0.09	–	100 ± 4	99 ± 9	100 ± 2
MC	18.2 ± 0.7	3.4 ± 0.1	<0.001	<0.001	2.51 ± 0.02	<0.003	5.22 ± 0.02	1.80 ± 0.05	–	31.1 ± 0.7	30.0 ± 0.4	33.0 ± 3.0
AB	6.4 ± 0.4	1.70 ± 0.03	<0.001	1.70 ± 0.02	<0.001	<0.003	<0.001	<0.001	<0.001	9.8 ± 0.5	9.8 ± 0.7	10.9 ± 0.3
AB	11.1 ± 0.7	2.81 ± 0.02	<0.001	2.51 ± 0.06	<0.001	<0.003	<0.001	<0.001	<0.001	16.4 ± 0.7	16.0 ± 1.0	17.1 ± 0.8

[a] Certified value for total As in DORM-2: 18.0 ± 1.1 μg g^{-1}.
[b] Certified value for total As in BCR-627: 4.80 ± 0.3 μg g^{-1}.
[c] Certified value for total As in NIST 1566b: 7.65 ± 0.6 μg g^{-1}.

Nischwitz and Pergantis [39], several As species are present in the certified oyster tissue (NIST 1566b), including AsB, DMA, arsenocholine, tetramethylarsonium ion, and two arsenosugars. Probably the unidentified As species was an arsenosugar, which could not be identified in the present work. Note that As(III) and AsB elute practically at the void volume of the column, and As(III) eluted slightly earlier than AsB. This can occur because As(III) at pH 6.0 is present as an uncharged species ($pKa_1 = 9.3$) and AsB ($pKa = 2.18$) as a zwitterion.

3.4. Arsenic species determination in mollusks

The developed method was applied for As speciation analysis in bivalve mollusks sampled in Todos os Santos Bay (Bahia, Brazil). The chromatogram obtained from the *M. constricta* sp. sample extracted using MAE with water and *M. constricta* sp. sample spiked with As species at a level of 10 μg L^{-1} As are shown in Fig. 1. As can be observed in this figure, the retention times of As(III), AsB, DMA, and p-ASA in standard solutions and samples are the same. These results demonstrated that there were not matrix interferences. MMA and As(V) were not detected in the mollusk samples, whereas two undefined As species (UK_1 and UK_2) were detected.

A peak of As with retention time equal to that of p-ASA is observed in Fig. 1. Probably, this peak does not correspond to p-ASA, because this species is not usually present in mollusks. It is also important that As(III), the most toxic i-As species, was present in a relatively high concentration in all mollusk samples analyzed. Sloth and Julshamn [40] reported a relatively high i-As concentration in blue mussels (*Mytilus edulis* L.). However, As(III) is usually not detected in seafood, or its concentration is very low [41,42]. The relatively high concentration of total As (Table 3) found in one sample was also noteworthy.

Fig. 2 shows the chromatogram obtained for one sample of *A. brasiliana* sp. As(III), AsB, and MMA were detected in this sample, and only one unknown (UK_3) peak was observed. This peak also could correspond to an arsenosugar species. Arsenosugar species, such as dimethylarsinoylsugarglycol and dimethylarsinoylsugarphosphate, were identified in fish and mollusks by Nischwitz and Pergnatis [39] as well as by Schmeisser et al. [43].

The sensitivity for all As species is similar; the mean value of the slope of the calibration curves was 51.3 ± 6.8 counts per mg L^{-1} As. Therefore, the unknown As species were quantified against the calibration curve of As(III). The same strategy of quantification of unknown As species has been used by Slot et al. [44] and Geng et al. [45].

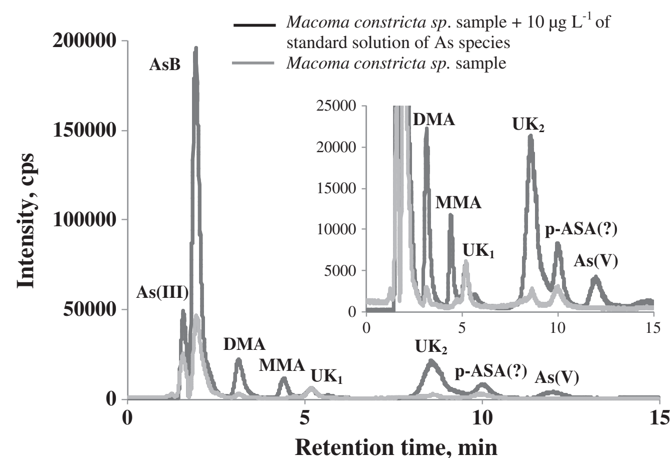

Fig. 1. LC–ICP-MS chromatograms of As species in *Macoma constricta* sp. after MAE with water extraction and *M. constricta* sp. spiked with a 10 μg L^{-1} standard solution of As species. Chromatographic conditions are reported in the Experimental section.

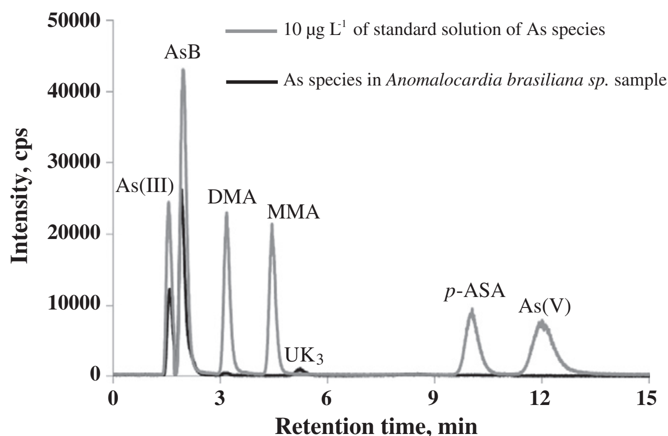

Fig. 2. LC–ICP-MS chromatograms of As species in *Anomalocardia brasiliana* sp. after MAE with water extraction and a 10 μg L^{-1} standard solution of As species. Chromatographic conditions are reported in the Experimental section.

The results for all analyzed sample tissues of bivalve mollusks and CRM are given in Table 3. As can be observed in this table, AsB was the main As species present in the samples. The concentrations of some As species were lower than their respective LOQs.

The extraction efficiency and As-species determination were evaluated by analyzing certified dogfish muscle (DORM-2), tuna fish tissue (BCR-627), and oyster tissue (SRM 1566b); the results of these evaluations are shown in Table 3. The total As in the extracts and the sum of the As-species concentrations were not different at a 95% confidence level. This indicates that MAE and water are appropriate for As-species extraction from bivalve mollusks.

The AsB concentration determined in the CRM was in agreement with the certified values. Similar AsB concentrations were found and reported by other authors [39,42,44]. However, a higher concentration of DMA than that reported [39] was found in oyster tissue (NIST 1566b) in the present study. Higher concentrations of AsB and DMA than those reported [39] also were found in the certified oyster tissue. The difference might be due to the species quantification methods. In Table 3, it can be observed that As(III) also was detected in DORM-2.

The precision of the method was evaluated by injecting 200 μL of a standard solution containing 2.5 ng As. The coefficients of variation for five consecutive measurements were lower than 10%. The LOQ was typically 0.003 μg g^{-1} for As(V) and 0.001 μg g^{-1}As for the other As species. The LOQs are of the same level as those reported for As species (Table 4). The linear correlation coefficient of the calibration curves was typically 0.999.

4. Conclusions

A method for As speciation analysis in bivalve mollusks was developed using LC–ICP-MS. The extraction of As species from the samples was quantitative by using microwave energy and water as an extractant. However, US-assisted extraction led to lower analyte recovery. It was demonstrated that it is possible to separate As species in standard solutions and sample solutions by using an anion-exchange column and ammonium phosphate as well as ammonium carbonate as mobile phases.

As expected, AsB was the main As species in bivalve mollusks. Unidentified As species were detected: two in *M. constricta* sp. and one in *A. brasiliana* sp. A peak with the same retention time as p-ASA was detected in one sample of *M. constricta* sp., probably correspondent to an unknown As species. In this sample, the As(III) concentration was higher than in the others.

Table 4
Limits of quantification for As-species determination by LC–ICP-MS (values in μg g^{-1}).

Sample	As(III)	AsB	MMA	DMA	p-ASA	As(V)	Ref.
Mollusks	0.001	0.001	0.001	0.001	0.001	0.003	This work
Seafood	0.020	0.020	0.020	0.020	–	0.020	[11]
Freeze-dried tissue of fish, chicken, rice, and soil	0.0011–0.0018	0.0021–0.0024	0.0016–0.0054	0.0017–0.0054	–	0.0016–0.0045	[21]
Mollusk, white fish, and cold water fish	0.021–0.042	0.043–0.086	0.058–0.116	0.045–0.090	–	0.078–0.156	[46]
Food	0.001	–	0.001	0.002	–	0.002	[47]
Marine periwinkle	0.250	0.250	0.250	0.250	–	0.250	[48]

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