APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2001; **15**: 658–666
DOI: 10.1002/aoc.204

Determination of arsenic species in oyster tissue by microwave-assisted extraction and liquid chromatography–atomic fluorescence detection

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A method for the determination of arsenic species in oyster tissue is established. The extraction of arsenic species is carried out by using low-power microwaves. Quantitative extraction is obtained at a power of 40 W, and in 5 min, using the extracting agent methanol/water (1 + 1). The measurements are carried out using liquid chromatography–UV irradiation–hydride generation–atomic fluorescence detection (LC–UV–HG–AFS). Three arsenic species were detected in oyster tissue: arsenobetaine (AsBet) (87%), a probable arsenosugar (AsS) (4.9%), and dimethylarsinate (DMA) (4.7%). No influence of the clean-up, the microwave field or the IR drying system on the stability of the arsenic compounds was observed. The extracts can be kept stable up to 3 days at 4 °C. The performance of the method is proved on fresh samples, as they are usually analysed in routine laboratories. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: arsenic; speciation; oyster tissue; hyphenated techniques; methylarsenic

Received 20 March 2000; accepted 6 February 2001

INTRODUCTION

Arsenic is widely distributed in the environment in organic and inorganic forms that differ in their toxicity.[1] Marine organisms, especially bivalves and algae, accumulate arsenic in their structure, which is metabolized as organic compounds of arsenic. Of these, arsenocholine (AsChol) and mainly arsenobetaine (AsBet) are the predominant metabolites, and their levels commonly range from 1 to 100 mg kg^{-1}.[2–5]

The most studied bivalves, in terms of toxicity and contamination, are mussels and oysters, since they are used in human food. Mussels are included in the common diet, and they are normally consumed after cooking. This practice causes the removal of the soluble arsenic species from that seafood. Oysters are usually consumed fresh; therefore, all the arsenic species present are introduced into the human organism.

Thus, it is necessary to study the distribution of arsenic species in both marine tissues. The use of water/methanol mixtures has been exhaustively described as the most common way to extract arsenic species from marine biological matrices.[6–11] Other extracting agents have given good results, such as methanol/chloroform mixtures,[12,13] or methanol/chloroform/water.[14] With all these methods, arsenic species are extracted simultaneously from the sample matrix with little sample manipulation. Traditionally, extraction has been carried out by successive steps, leading to high consumption of extracting agents. Shaking can be carried out either by mechanical stirring[7,9,14] or sonication;[6,10–13] the latter considerably reduces extraction time. However, common ultrasonic extraction processes involve three or four steps and about 1 h of sample treatment.

More recently, the use of microwave ovens has increased, especially for digestion of samples and further determination of total amounts of elements. A wide review has been published on this topic.[15] However, microwaves are not widely used to

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Contract/grant sponsor: DGICYT; Contract/grant number: PB 98-1259.

promote quick arsenic species extraction from a complex matrix. Low-power focused microwaves have been applied successfully for arsenic speciation in mussels,[8] but no use of microwaves for arsenic speciation in oyster tissues has been described.

Here we present an optimization of the microwave-assisted extraction and determination of arsenic species in oyster tissue. We used a similar procedure to those described elsewhere for extraction of arsenic species from mussel tissue.[8] However, the differences in matrix composition between the two marine organisms made it necessary to optimize the method for the oyster tissue, in order to ensure maximum extraction of the arsenic species. It is well known that in speciation analysis the particular matrix composition influences the extraction and the distribution of the species.

Measurements were performed using the previously developed coupled technique or liquid chromatography–UV irradiation–hydride generation–atomic fluorescence detection (LC–UV–HG–AFS).[16] Extraction of the arsenic species was optimized in terms of extracting agent, extraction time and microwave power, using a factorial Doehlert[17,18] optimization.[19] The effects of the clean-up conditions, the microwave field and the IR drying process on the arsenic compounds detected in the oyster tissue (AsBet, dimethylarsinate (DMA) and an arsenic-containing ribofuranoside (AsS)), as well as on the stability of the extracts with time, are presented and discussed. A comparison between the results obtained in the extraction and speciation of arsenic in the lyophilized oyster tissue and in the fresh oyster tissue is also reported, in order to assess the possible effect of the lyophilization step on the species distribution. Furthermore, fresh material is usually analysed in food and control laboratories.

EXPERIMENTAL

Apparatus

Arsenic compounds were separated with a Perkin Elmer 250 LC binary pump (CT, USA), equipped with a Rheodyne 7125 injector (Cotati, CA, USA) and a 100 μl loop. Separation was performed with an anion-exchange Hamilton PRP X100 (Reno, NV, USA) spherical poly(styrene–divinylbenzene) trimethylammonium exchanger with a guard-column packed with the same stationary phase.

Two home-made photoreactors were used. One consisted of a PTFE tube (i.d. 0.55 mm; from Cole Parmer, Vernon Hills, IL, USA) that surrounded a water refrigerated 150 W high-pressure mercury vapour lamp (Heraeus TQ 150, Hanau, Germany).[20] The other combined a Heraeus TNN 15/32 low-pressure mercury vapour lamp (15 W) and PTFE tubing (i.d. 0.5 mm; Cole Parmer). More details are described elsewhere.[21] A computer-controlled microburette (MicroBU 2031, Crison, Parkland, FL, USA) was used to introduce the peroxodisulfate solution into the photoreactor.

Hydride generation was performed with a P.S. Analytical (Kent, UK) model 10.004 hydride generator module. The sample channel was eliminated, and a T-shaped connection was used to add the outlet from either the LC or the LC–UV system to the acid channel.[16]

Signal detection was achieved using a P.S.Analytical model Excalibur atomic fluorescence spectrometer equipped with an arsenic-boosted hollow cathode lamp (current intensities: primary, 27.5; boost, 35.0) and a Perma pure drying membrane (Perma Pure Products, Farmingdale, NJ, USA) for drying the hydride generated.

Data acquisition of the signal was performed with a microcomputer using home-made, newly developed software (Pendragon 1.0). Peak heights and areas were calculated from custom-developed software running with the Matlab language.

Extraction of arsenic species and sample digestion were performed in a Prolabo (Paris, France) microwave digester (model A301, 2.45 GHz), equipped with a TX32 programmer. Powers of 20–200 W can be applied in steps of 10 W. This system works under atmospheric pressure with microwave energy focused into the glass vessel. A Hettich (Tuttlingen, Germany) Universal 30F centrifuge and an IR lamp (Philips, Madrid, Spain) were used after extraction.

The oyster tissue was freeze-dried in a Telstar (Barcelona, Spain) lyophilization system.

Reagents and standards

Doubly deionized water (USF purelab plus, Ransbach Baumbach, Germany) of 18.2 MΩ cm resistivity was used for the preparation of all solutions.

1000 mg l^{-1} arsenic stock solutions were prepared and standardized with respect to arsenite by ICP-MS measurements. Arsenite: NIST Oxidimetric Primary Standard 83d (arsenite tri-oxide,

As_2O_3), 99.9926 ± 0.0030 wt%, was dissolved in Merck (Darmstadt, Germany) Suprapur® 4 g l^{-1} NaOH. Monomethylarsonate (MMA), $(CH_3)As(ONa)_2 \cdot 6H_2O$ (Carlo Erba), and dimethylarsinate (DMA), $(CH_3)_2AsNaO_2 \cdot 3H_2O$ (Fluka, Buchs, Switzerland), were dissolved in water. A solution of arsenobetaine (AsBet; $(CH_3)_3As^+CH_2COO^-$) was supplied by the 'Service Central d'Analyse', CNRS (Vernaison, France). Calibration solutions were prepared daily by diluting the stock standard solutions.

Phosphate buffers prepared from NaH_2PO_4 'Suprapur' (Merck) and Na_2HPO_4 'Suprapur' (Merck) were used with the anion-exchange column. Mobile phase solutions were filtered through a 0.22 μm nylon membrane.

Peroxodisulfate solution ($K_2S_2O_8$, Fluka, purity >99.5%) at 5.0% was prepared in sodium hydroxide (NaOH, Merck, 'Suprapur') at 2.5%.

A solution of hydrochloric acid (HCl 32%, Merck, 'Pro-analysi') 2.0 mol l^{-1} and a solution of sodium borohydride ($NaBH_4$ 97% 'Purum', Fluka, Buchs, Switzerland) at 1.5% prepared in 0.4% sodium hydroxide (NaOH, Merck, 'Suprapur') aqueous solution for hydride generation, were prepared daily. The $NaBH_4$ solution was filtered through a 0.45 μm cellulose acetate membrane.

Nitric acid 65% (HNO_3 'Baker Instra-Analyzed', J. T. Baker, Deventer, The Netherlands) and sulfuric acid 95–97% (H_2SO_4, Merck, 'Suprapur') were used for the digestion of the samples. Potassium iodide (KI, Merck, 'Suprapur') and Merck 'Pro-analysi' L(+)-ascorbic acid were used for reducing arsenate to arsenite. Methanol (gradient grade for HPLC Merck) was used in the extraction step. Clean-up was performed with C18 Snap-cap cartridges (600 mg of sorbent, Lida, Kenosha, WI, USA). The National institute of Standards and Technology (NIST) Standard Reference Material SRM 1566a "Oyster tissue" was analysed.

Procedure for the preparation of oyster tissue sample

Oysters from the northwest of Spain were purchased in a local market. After manually removing the shell, the sample was mechanically crushed until a homogeneous mass was obtained. A portion of the sample was stored at −20 °C for further analyses, and the rest of the material was freeze-dried for 48 h (temp. −40 °C; vacuum pressure 10.13 Pa), ground to a fine powder and then collected in polyethylene flasks. The final, freeze-dried material was stored at −20 °C until analysis.

Procedure for total arsenic determination

500 mg of lyophilized material was placed in an open reflux vessel and focused microwaves were applied, according to the following digestion programme: 10 ml HNO_3 at 40 W for 5 min; the power was then increased to 70 W for 10 min and, finally, 5 ml H_2SO_4 was added and the power was maintained at 120 W for 10 min. The resulting solution (about 5 ml) was diluted to 50 ml in 2 mol l^{-1} hydrochloric acid, 1% (w/v) potassium iodide and 0.2% (w/v) ascorbic acid. After an hour, the final solutions were analysed by HG–AFS. Moisture was determined in a separate aliquot by drying in an oven at 70 °C until constant weight was achieved (about 24 h).

Procedure for extraction

A sample of oyster tissue (0.5 g from freeze-dried sample, 3 g from fresh material) and 20 ml of a methanol/water solution (1 + 1) were placed in an open reflux vessel. 40 W of focused microwaves was applied for 5 min. After decanting, the extract was centrifuged at 2500 rpm for 10 min and the liquid phase was evaporated to complete dryness under an IR lamp ($T < 40$ °C) for approximately 4 h. The dry extract was then dissolved in 10 ml of water and filtered through a nylon membrane of 0.2 μm porosity. The filtrate was then defatted by clean-up with a C18 cartridge, which had been previously conditioned by passing methanol (5 ml) and water (5 ml) at 1 ml min^{-1}. The extract was passed through the cartridge (1 ml min^{-1}). The first 2–3 ml, mainly arising from conditioning solutions, were discarded. Finally, an aliquot of the cleaned-up extract was made up to a fixed volume. Moisture was determined in a separate aliquot of the oyster sample by drying in an oven at 70 °C for 24 h.

Procedure for speciation analysis by LC–UV–HG–AFS

A 100 μl aliquot of the extract was injected into the LC system, where mobile phases were pumped at a flow of 1 ml min^{-1}. Separation was achieved at pH 6.0 with a gradient of two phosphate mobile phases, 5 mmol l^{-1} NaH_2PO_4/Na_2HPO_4 (solution A) and 100 mmol l^{-1} NaH_2PO_4/Na_2HPO_4 (solution B). The gradient programme was 100% A for 2 min, decreasing to 50% A in 0.1 min and maintained for 3 min, then increasing to 100% A in 0.1 min and maintained for 9 min. The eluate was then carried

into the photoreactor with addition of 5% (m/v) peroxodisulfate in 2.5% (m/v) NaOH solution at 0.2 ml min^{-1} where it was UV-irradiated for 60 s with a 150 W lamp. The solution was then introduced into the hydride generation system. Addition of 2 mol l^{-1} HCl at 8 ml min^{-1} and 1.5% (m/v) $NaBH_4$ in 0.4% NaOH at 3 ml min^{-1} was then performed for hydride generation. After reaction, the final eluate was propelled by an argon flow of 300 ml min^{-1} to the AFS detector through the gas–liquid separator. An air-based Perma pure drying membrane (air flow: 2.5 l min^{-1}) was used to dry the hydride before entering the AFS detector. Quantification (peak area) of arsenic species was performed by the standard addition method over the cleaned-up samples.

RESULTS AND DISCUSSION

The accuracy of the total arsenic determination in the oyster tissue was checked by analysing the NIST 1566a 'Oyster tissue' with a certified arsenic concentration of 14 ± 1.2 mg kg^{-1}. After quantification of total arsenic, we obtained 13.72 ± 0.54 mg kg^{-1} for the NIST 1566a and 9.74 ± 0.37 mg kg^{-1} for the oyster tissue under study.

A chromatogram obtained after microwave extraction at 40 W, 5 min and 20 ml of methanol/water (1 + 1) is shown in Fig. 1 for the local oyster extract. Three arsenic compounds were detected in the non-spiked oyster: AsBet, DMA and an unidentified peak (U) with a similar retention time to that of MMA. A similar species distribution in bivalves is reported by several authors. According to Gailer *et al.*,[3] Larsen[4] and Corr and Larsen,[22] an arsenic-containing ribofuranoside such as dimethyl-arsinylriboside (AsS) is the most likely structure for such a compound U, which is very likely to be found in bivalves, such as oysters and mussels. Therefore, AsS concentration was estimated by additions of MMA in the present study.

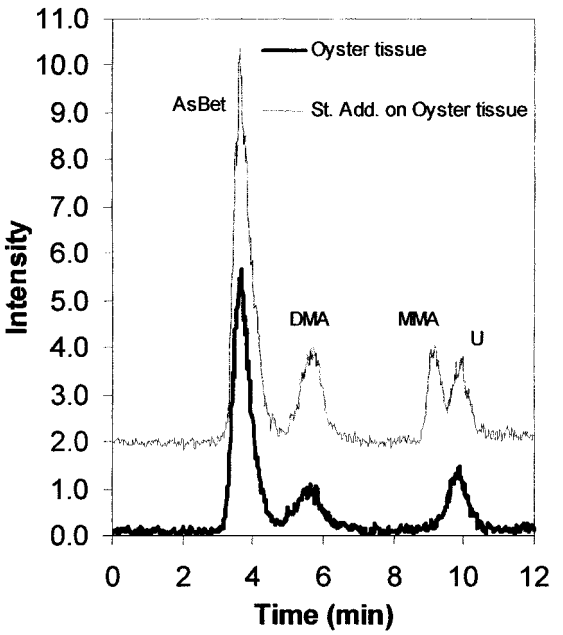

Figure 1 Chromatograms obtained of an oyster tissue extract before and after standard addition. Final concentrations of the standard additions were (expressed as As): 10 μg l^{-1} MMA, 10 μg l^{-1} DMA, and 100 μg l^{-1} AsBet. 100 μl of sample extract were injected in the LC–UV–HG–AFS system. U = unidentified peak.

Optimization of the UV irradiation and the sample amount injected

In this study it was observed that chromatographic separation and hydride generation were not influenced by the amount of sample injected. However, the efficiency for the UV photodecomposition of organic arsenic compounds was strongly dependent on the volume injected. Several combinations of UV lamps and injection loops were assayed in order to obtain the highest signal response for AsBet, MMA and DMA. Aliquots of an oyster extract were analysed with several injection loop–UV photoreactor combinations. Table 1 shows the results obtained in terms of detection limits. AsBet response was highly dependent on the sample amount, and the absolute detection limit (expressed in Table 1 as nanograms of arsenic) decreased with a decrease of sample amount and with an increase in UV power. MMA and DMA were not affected by sample amount, and it appeared that the injection of 20 μl and 150 W UV power provided the optimum conditions.

Nevertheless, relative detection limits (expressed in Table 1 as micrograms of arsenic per litre) were quite different in each case. If one takes into account that arsenic in bivalves is mainly present as AsBet, the best conditions would be those giving lower detection limits for the lowest concentration species. Thus, injection of 20 μl at 150 W UV provided too high detection limits for MMA and DMA. We chose an injection of 100 μl and a 150 W

Table 1 Detection limits (3σ) for AsBet, MMA and DMA in oyster tissue extracts, with several UV photoreactors and injection loop combinations. The system LC–UV–HG–AFS was used in all measurements

	Detection limit (ng As)			Detection limit (μg l^{-1} as As)		
	AsBet	MMA	DMA	AsBet	MMA	DMA
Loop 100 μl / 15 W UV photoreactor	n.d.	0.11	0.13	n.d.	1.1	1.3
Loop 20 μl / 15 W UV photoreactor	0.52	0.09	0.14	26.2	4.5	7.2
Loop 100 μl / 150 W UV photoreactor	0.73	0.13	0.17	7.3	1.3	1.7
Loop 20 μl / 150 W UV photoreactor	0.11	0.12	0.17	5.5	6.0	8.3

n.d. = not detected.

UV irradiation to ensure the proper quantification of the arsenic compounds in oyster tissue.

Effect of the C18 clean-up process

As previously described,[1] cleaning of the sample extracts is necessary to prevent adsorption of AsBet on the stationary phase. Therefore, a study was carried out to establish the efficiency of such a clean-up process. To assess the recovery, two series of extracts (ten replicates each) were analysed: one after C18 clean-up and the other without clean-up. A recovery of 97% for all arsenic species was observed (Fig. 2). Furthermore, the relative standard deviations (RSDs) obtained (lower than 4% for both AsBet and the sum of species) can be considered low enough to ensure accurate results for further determinations. The recovery of the sum of species was directly related to AsBet, which shows that this compound is the major species in the oyster tissue under analysis.

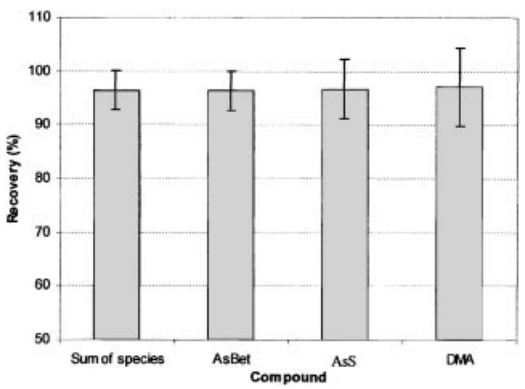

Figure 2 Arsenic species recovery after C18 clean-up of the oyster extract.

Arsenic species extraction

Good results had been obtained for arsenic species extraction from mussel tissue with 20 ml of methanol/water (55/45), microwave power of 40 W and less than 4 min of irradiation time.[8] The differences between the distribution of species in mussel and oyster tissues, and additionally the different sample preparation system (mussels were extracted from the shells by adding liquid nitrogen, whereas oysters in the present study were directly, manually extracted), required optimization of the extraction step. For this, a three-factor Doehlert design was applied, according to the values shown in Table 2. After calculation, the optimum settings were 40 W microwave power, 5 min extraction time and 20 ml of methanol/water (50/50). Such conditions may lead to 97% of total arsenic extracted, which corresponds to 87% for AsBet, 4.9% for AsS and 4.7% for DMA. The extraction yield could be considered quantitative, since the 3% loss is attributed to the clean-up process.

Since the optimal conditions were quite close to those obtained for the extraction of arsenic from mussel tissue,[8] we calculated the recovery of arsenic species in both matrices, applying the optimal values found for oyster and for mussel

Table 2 Experimental domain explored by the Doehlert design applied to the extraction of arsenic in oyster tissue

	Design factors		
	Power (W)	Time (min)	MetOH (%)
Centre value	40	5	50
Variation for two factors	20	3	0
Variation for three factors	20	3	25

Table 3 Calculated arsenic extraction yields % obtained in both mussel and oyster tissues in the optimal conditions for each one of them

Material	Optimal conditions	Sum of species (%)	AsBet (%)	AsS (%)	DMA (%)
Oyster	Oyster[a]	97	87	4.9	4.7
Oyster	Mussel[b]	92	83	4.8	4.1
Mussel	Oyster[a]	85	67	15	2.2
Mussel	Mussel[b]	85	68	14	2.3

[a] Optimal conditions for arsenic extraction from oysters: 40 W power, 5 min extraction time and 50/50 methanol/water.
[b] Optimal conditions for arsenic extraction from mussels: 40 W power, 3.6 min extraction time and 55/45 methanol/water.[7]

tissue in the two Doehlert designs optimized, as an attempt to propose a general procedure for arsenic extraction from bivalves (Table 3). Whereas the calculated arsenic extraction yield from mussels was very close under the two conditions tested in the factorial design, extraction from oyster tissue was significantly influenced by the conditions chosen. Thus, arsenic extraction was lowered by 5%, mainly attributable to the 4% loss in AsBet extraction. AsS was the only compound not affected by the variation in the extraction conditions. Therefore, as a preliminary test, the application of compromise conditions (i.e. about 40 W microwave power, 4–5 min extraction time, and 20 ml methanol/water (1 + 1)) may lead to qualitative information about arsenic species distribution in bivalves, with extraction yields around 80–100%. Such conditions could be extended to research in other bivalves, even though optimization of the extraction method is required in order to obtain the best extraction yield.

Stability of arsenic species under the microwave field and under IR irradiation

One of the main objectives in speciation analysis is the assurance of the stability of species during the whole process. The effects of both the microwave field and IR irradiation during extraction and drying on the arsenic species was studied by analysing a standard solution containing AsBet (200 μg l^{-1}), MMA (25 μg l^{-1}) and DMA (25 μg l^{-1}) (concentrations expressed as arsenic, and similar to those present in the oyster tissue extract for each arsenic compound). An aliquot of this solution was subjected to the microwave extraction and IR drying processes, under the conditions previously established. Simultaneously, an aliquot of the same standard solution was only IR dried. No significant effect was observed by applying either microwaves or IR irradiation on the arsenic compounds (Fig. 3). Thus, the stability of the species during the process is clearly assured.

Stability of arsenic species in oyster extracts with time

Several assays were carried out in order to determine the stability of the oyster extracts before measurement. For this, sub-samples of the lyophilized oyster were extracted, and aliquots of the extracts were analysed after 12, 24, 48 and 72 h. The values of the fresh extracts were taken as references. Extracts were kept at 4 °C between analyses. No decrease in the concentration of the extracts was observed after 72 h (Fig. 4). This permits a delay of 3 days before the measurement of oyster extracts if necessary.

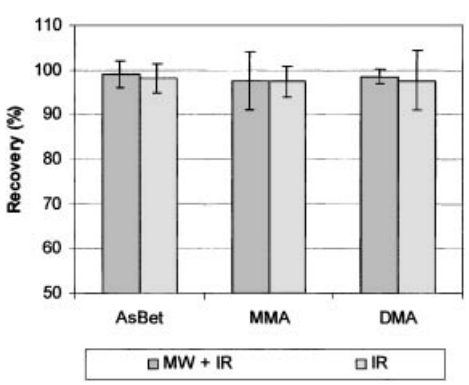

Figure 3 Arsenic compounds recovery after applying microwave irradiation and IR drying process, and after only IR drying. Concentrations are: AsBet, 200 μg l^{-1}; MMA, 25 μg l^{-1}; DMA, 25 μg l^{-1} (expressed as As).

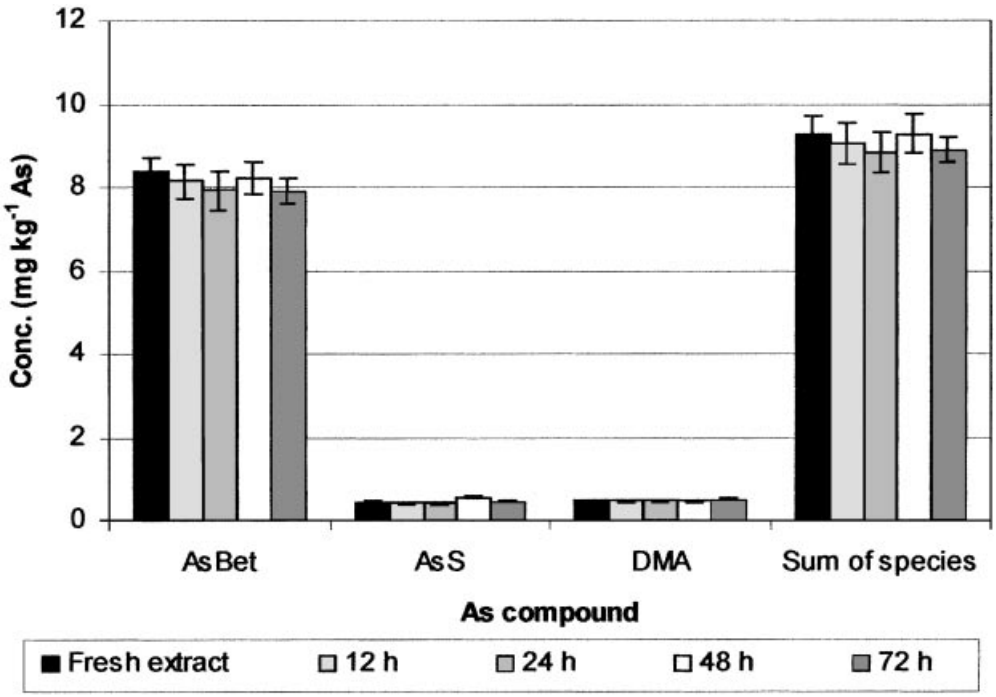

Figure 4 Stability of the arsenic species in the oyster extracts with time. Concentrations are expressed in dry weight.

Effect of the thermal pre-treatment on the oyster tissue

Several thermal pre-treatments of the fresh oyster sample were tested in order to apply the present method to routine analysis, avoiding lyophilization, and to establish an easy handling procedure for laboratories. Furthermore, by directly analysing the fresh sample, the distribution of species is identical to those received for humans in the consumption of fresh oysters. Therefore, the information on arsenic speciation in the fresh material is useful for toxicological purposes. Part of the original sample was frozen and kept until analysis, and aliquots of the frozen oyster were dried in an electric oven at 40 °C (for 24 or 48 h) and at 70 °C (24 h), in order to reduce the moisture of the aliquots and to make the sample handling easy. These aliquots were also kept at −20 °C before analysis. The results were compared with those obtained for the lyophilized oyster (Fig. 5). Only after the strongest thermal treatment (70 °C) was a signal decrease observed for all the arsenic compounds, with losses between 15% (in AsBet and AsS) and 60% (in DMA). This could suggest a significant effect of temperature used for pre-treatment. However, arsenic species distribution in the fresh sample was the same as that observed in the lyophilized material, and, therefore, the method can be applied directly to the collected sample, simplifying sample pre-treatment and shortening the analysis.

CONCLUSIONS

Arsenic speciation was carried out in oyster tissue. The extraction method is similar to those previously developed for mussel tissues, which suggests the possible application as a general extraction method for arsenic speciation in bivalves. However, some modifications in the optimal extraction conditions were done, which proved the effect of the sample matrix on the performance of the method. Thus, even for two very similar materials, slight modifications must be carried out in order to improve the method.

Near-quantitative extraction of arsenic from oyster tissue was achieved in a single step, using 5 min of microwave irradiation time at 40 W and 20 ml of a solution of methanol/water (1 + 1).

The stability of arsenic species under the

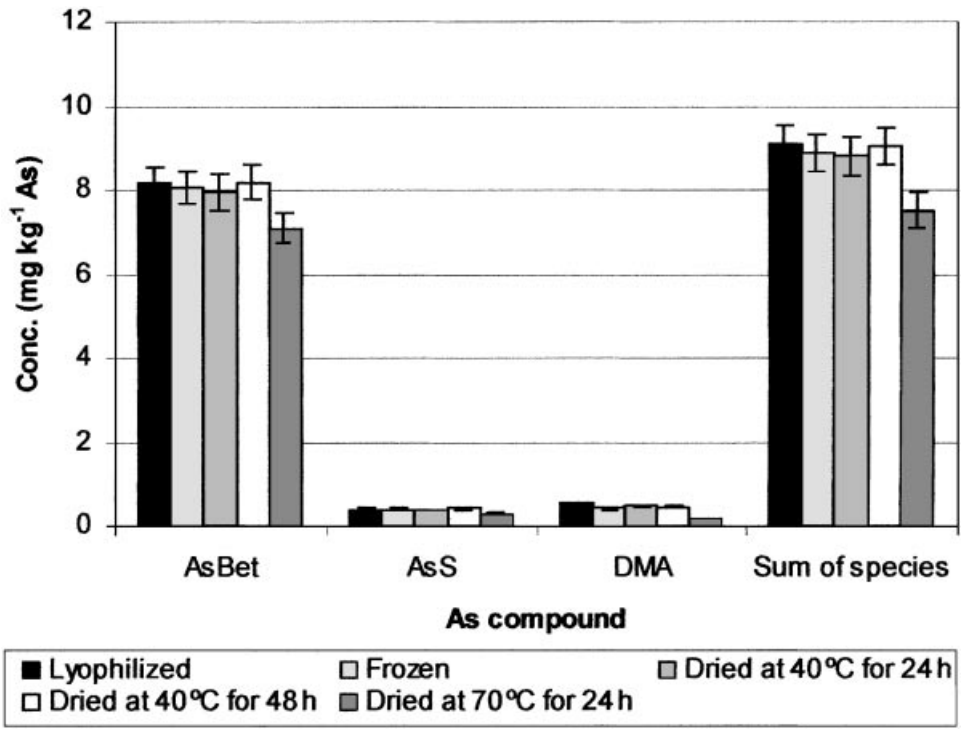

Figure 5 Effect of the thermal pre-treatment on the extraction of arsenic compounds from oyster tissue. Results are expressed in mg kg^{-1} (as arsenic) in the solid sample (dry weight).

extraction conditions was assured. Moreover, the extracts could be kept for up to 3 days at 4 °C before measurement and without any change in the arsenic speciation.

The applicability of the method to fresh oyster tissue has been proven. Whereas lyophilized samples are very useful as reference materials, because of sample stability, fresh samples are also easily handled for routine analysis.

Acknowledgements The authors thank the DGICYT, Project PB 98-1259, for the financial help received in support of this study. The authors also thank Dr Antoni Padró for his help in graphical plotting using Matlab software, and Dr J. M. Bayona (Departament de Química Ambiental, CSIC, Barcelona) for the use of the facilities of the lyophilization system.

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