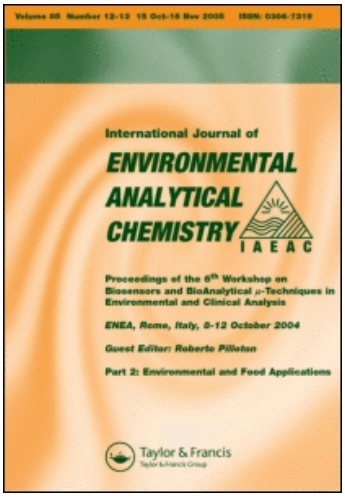

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ARSENIC SPECIATION IN BIOLOGICAL SAMPLES USING THE COUPLINGS HPLC-UV-HG-AAS AND HPLC-UV-HG-AFS

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A speciation procedure for arsenobetaine (AsB), arsenite (As^{III}), dimethylarsinic acid (DMA), monomethylarsonic acid (MMA) and arsenate (As^{V}) based on the couplings HPLC-HG-AFS and HPLC-UV-HG-AFS has been developed and results compared with HPLC-HG-AAS and HPLC-UV-HG-AAS. AFS detection improves the performance of these couplings, being the detection limits about twenty times lower (from 0.06 to 0.14 $\mu g\ l^{-1}$ for AFS against 1.1 to 3.9 $\mu g\ l^{-1}$ for AAS). The linear range extends from 0.25 to 8000 $\mu g\ l^{-1}$ for AFS in contrast to 5 to 500 $\mu g\ l^{-1}$ for AAS. This approach has been applied to the arsenic speciation in biological tissues (certified material TORT-1 and fresh bivalve tissues) in which arsenobetaine was the main species found. Extraction of the species has been carried out using repetitive extractions with methanol and methanol/water (1:1) mixtures being the later more suitable for quantitative recovery.

Keywords: Arsenic speciation; atomic fluorescence spectrometry; atomic absorption spectrometry; photooxidation; biological tissues

INTRODUCTION

Arsenic is a widely distributed element in the environment whose toxicity depends on the chemical form in which is present. Generally, the inorganic forms, arsenite and arsenate, are considered the most toxic species, occurring naturally in sediments, soils and waters, being arsenite considered more toxic than arsenate[1,2]. Different organisms, such as algae, fungus, bivalves and mammals, methylate the inorganic arsenic compounds, producing organic and less

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toxic compounds, such as methylarsonic acid (MMA) and dimethylarsinic acid (DMA)[3]. The methylation path seems to involve the reduction of arsenic due to thiol groups, acting probably the S-adenosylmethionine as methyl group donors[4]. Methylated species can be further transformed by marine organism into more complex molecules, being the main final product arsenobetaine (AsB), which is considered non toxic. AsB constitutes usually the major form of arsenic in molluscs, shell-fish and fish, although some organosugars have also been detected[5,6]. In order to evaluate the risks associated with the presence of arsenic, the identification and quantification of the different species of this element in the complex environmental matrices are necessary.

The speciation of arsenic compounds combines the separation by liquid chromatography with a suitable element detector. The chromatographic separation is generally based on the use of either ion-exchange or ion-pair reverse phase columns[7,8]. Direct couplings between HPLC and different atomic detectors such as ICP-AES[9] and ICP-MS[10] do not provide usually detection limits low enough for the analysis of biological samples or can suffer from the interference of chloride from marine samples.

Sensitivity of arsenic detection is improved using a post-column derivatization (hydride generation), converting the arsenic species into volatile arsines. In addition, this approach eliminates interferences from the sample matrix. Detection limits, in the order of $\mu g\ l^{-1}$, are achived with the couplings HPLC-HG-ICP-AES[11] and HPLC-HG-AAS[12], however, these levels are sometimes not low enough to detect the arsenic species present in water or biological samples. The sensitivity can be further increased with the HPLC-HG-ICP-MS[13] coupling, with limits of detection below $\mu g\ l^{-1}$. More recently, atomic fluorescence spectrometry (AFS) is being also employed as a suitable technique for arsenic speciation[14,15,16], with detection limits at least similar to ICP-MS detection.

Non-reducible organic compounds of arsenic, such as arsenobetaine (AsB) represent sometimes the main arsenic compound present in marine organisms, as well as in human urine due to the ingestion of marine products[17]. However, these species do not form volatile hydrides. For this reason different authors have proposed the on-line oxidation of arsenic compounds with potassium persulfate under the action of microwave[18] or ultraviolet radiation[19].

A critical aspect in arsenic speciation is the extraction of the different forms of this element from the matrix. There are not totally established procedures for the extraction of arsenic from complex samples,especially in biological tissues. The approaches proposed in the bibliography are based in the use of solvents such as water, methanol, chloroform and mixtures between them[20], but these procedures should be optimized for the samples under study.

In this work, arsenic species were separated by high performance liquid chromatography (HPLC) equipped with an anionic exchange column. Optionally, an on-line photooxidation step (UV) was included at the outlet of the column, followed by on-line hydride generation (HG). The volatile arsines were detected either by quartz cuvette atomic absorption spectrometry (AAS) or atomic fluorescence spectrometry (AFS). The coupling HPLC-HG-AAS, has been widely used for arsenic speciation in environmental and biological samples[21]. However, atomic fluorescence spectrometry has only recently been considered for arsenic speciation. Comparison of performances between both approaches has been fulfilled in this study and their application to biological tissues has been considered. Moreover, the extraction of arsenic from these tissues has been optimized using methanol and methanol/water mixtures.

EXPERIMENTAL

Standard solutions and reagents

Stock standard solutions of 1000 mg l^{-1} (as As) were prepared from arsenic trioxide (primary standard, Panreac), sodium arsenate (Merck), sodium monomethylarsonate (Carlo Erba) and dimethylarsinic acid (Sigma) in destilled water. A 10 mg l^{-1} arsenobetaine solution was donated by Dr. Foulkes (University of Plymouth). Phosphate buffers at 10 mM and 100 mM concentration (pH 5.8) were used as HPLC mobile phases, being prepared from KH_2PO_4 and $K_2HPO_4 \cdot 3\ H_2O$ (Merck). Potassium persulfate (Aldrich) 2% (w/v) in 2% (w/v) NaOH, and $NaBH_4$ (Panreac) 1.5% (w/v) in 1% (w/v) NaOH were prepared daily. Methanol was super purity solvent (Romil). The different aqueous solutions were filtered using a 0.45 μm membrane (HA type, Millipore). Certified material Tort-1 "Lobster hepatopancreas" (National Research Council, Canada) was used in the extractions.

Instrumentation

The HPLC system consisted in a Varian 9012 ternary pump. The sample is introducted via a Rheodyne 7125 injector fitted with a 200 μl loop. The separation of the arsenic species takes place in a 25 cm × 4.1 mm Hamilton PRP X-100 column. The photooxidation of the arsenic compounds takes place in a 8 m long teflon tube (i.d. 0.35 mm) wrapped around a low pressure Hg lamp (TNN 15/32, Heraeus) that emits UV radiation at λ= 254 nm, as it has been described by

Rubio *et al*[22]. Hydride generation of volatile arsines is performed adding on-line solutions of HCl and $NaBH_4$ at the outlet of the column by means of a Gilson Minipulse 3 peristaltic pump, in order to obtain volatile arsines.

Atomic absorption spectrometry (AAS) and atomic fluoresecence spectrometry (AFS) were studied as detection techniques for arsines. When using a HPLC-UV-HG-AAS coupling, the separation of the arsines was performed in a Varian VGA-76 gas-liquid separator, using nitrogen as carrier gas. The detection takes place in a Pye-Unicam SP9 Atomic Absorption Spectrophotometer equipped with a quartz cell heated with an air-acetilene flame, using as radiation source an arsenic hollow cathode lamp (Photron). On the other hand, HPLC-UV-HG-AFS detection is achieved with a PSA Excalibur 10.33 detector, using as radiation source a boosted-discharge hollow cathod lamp (Photron). In this case the separation of the arsines is performed in a PSA Type A gas-liquid separator. The analog signal output of either the AAS or the AFS detector was connected to a computer equipped with the Star Chromatography Sofware (Varian).

Speciation procedure

A volume of sample of 200 µl was injected in the HPLC system, using a gradient program that involves two phosphate buffers (10 mM and 100 mM at pH 5.8) to achieve the separation of the arsenic species in the strong anionic exchange column, following similar conditions described by other authors for this particular type of colum[19].

Volatile arsines were generated by on-line addition of 1 ml min^{-1} flow of HCl 1.5 M and 1 ml min^{-1} of $NaBH_4$ 1.5% (w/v) (stabilized with NaOH 1% (w/v)).

When using AAS detection, nitrogen was used as carrier gas for the transport of the arsines to the detector. A nitrogen flow of 90 ml min^{-1} was introduced after the hydride generation step in order to obtain a fast transport of the arsines to the gas-liquid separator. A second nitrogen flow of 90 ml min^{-1} in the gas-liquid separator carries the arsines to the detector.

For AFS detection the nitrogen was replaced by argon, which enhances the sensitivity in this type of detection. In this case, a flow of 100 ml min^{-1} of carrier gas was introduced after the hydride generation and an auxiliary flow of 200 ml min^{-1} of argon transport the gas to the liquid separator. The atomization of the arsines takes place in an air-hydrogen diffusion flame, being necessary a flow of 60 ml $min^{-1}$of H_2 to support the flame. The gas flow that carries the arsines to the flame was dried using a hygroscopic membrane before reaching the flame, because the water moisture produces instability in the flame, with the result of an

increase in the signal noise. A flow of 3 l min^{-1} of air at the outside of the hygroscopic membrane removes the water moisture.

A photooxidation step is optionally introduced after the chromatographic separation prior to the hydride generation for the determination of non reduci0ble arsenic species such as arsenobetaine. In order to achieve the oxidation of this compound to arsenate, a 0.3 ml min^{-1} flow of $K_2S_2O_8$ 2% (w/v) in NaOH 2% (w/v) was added at the outlet of the HPLC column, in a 8 m long teflon coil wrapped around the UV lamp. With this setting, is necessary to neutralize the basicity apported by the persulfate solution, being required to increase the HCl concentration up to 8 M to get an optimal signal for all the studied species. AsB coelutes with As^{III} with this type of column, due to the fact that at the working pH arsenite remains as a neutral species and arsenobetaine as a zwiterion. In order to distinguish between both species, experiences with and without the photooxidation step were carried out.

A diagram of both instrumental couplings is depicted in Figure 1.

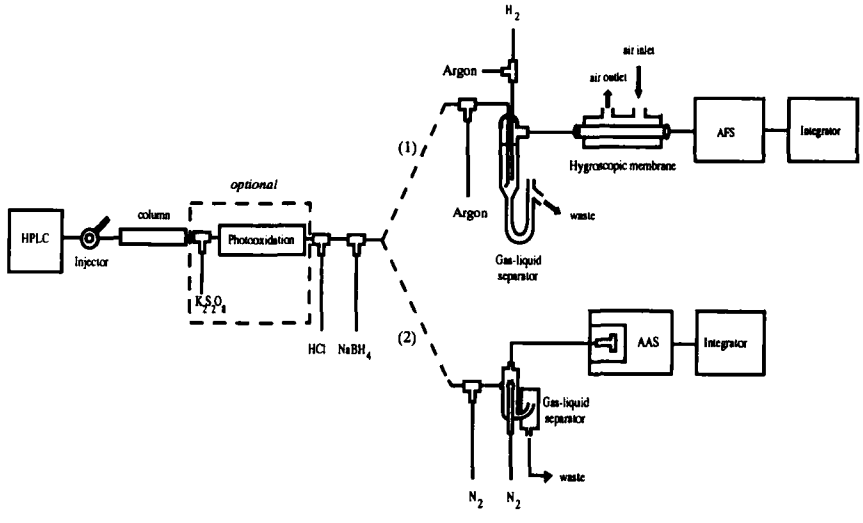

FIGURE 1 Scheme of instrumental couplings based on HPLC separation and (1) AFS detection, (2) AAS detection

Sample extraction procedure

1.0 g of biological tissue was placed in a polipropylene centrifuge tube, adding 20 ml of either methanol or a water/methanol (1:1) mixture. The extraction was performed in an ultrasonic bath at room temperature during 20 minutes. After-

wards, samples were centrifuged at 10000 rpm during 10 minutes and the supernatant transfered with a Pasteur pipette to a test tube, where methanol was evaporated using a N_2 flow. Then, distilled water was added and transfered to a volumetric flask, to a final volume of 10 ml. Extract aliquots were filtered through a C-18 cartridge before their injection in the chromatograph.

Total arsenic determination

0.5 g of sample were placed in a 100 m l Kjeldahl flask, and predigested with 20 ml of concentrated HNO_3 for 4 h, then a water cooled reflux was set on the Kjeldahl flask, placing it on a sand bath and heated up to 120°C during 1 h. Afterwards, 5 ml of $HClO_4$ were added and the temperature increased until the appearance of white fumes (210°C). The sample was then evaporated almost to dryness. Finally, the Kjeldahl flask was washed with water and the solution transfered to a volumetric flask to a final volume of 50 ml.

RESULTS AND DISCUSSION

Performance of the instrumental couplings

The optimization of experimental conditions for the arsenic speciation using the instrumental couplings studied in this paper have been previously reported [16,23], but detailed comparison of the performance among them has to be done in order to evaluate their application to real samples.

Detection limits for the couplings without photooxidation, suitable for samples of water not containing AsB, are shown in Table I. Results obtained using AAS detection show detection limits in the $\mu g\ l^{-1}$ range, whereas AFS detection provided an increment in the sensitivity, with detection limits about twenty times lower. With both detectors, As^{III} gives the lowest detection limits, followed by the organic species (DMA and MMA). As^{V}, that is the last species eluted, presents a marked derease in sensitivity, that can be attributed to both peak broadening and to the kinetic controlled reduction of As^{V} to As^{III} before hydride generation[24].

In the analysis of biological marine samples, it is necessary to introduce a photooxidation step in the instrumental coupling, with the purpouse of AsB detection, which constitutes generally the main arsenic species in this type of samples. In this case, all the arsenic species injected in the chromatograph are effectively oxidized to arsenate, prior to the hydride generation. This fact explains the very

similar values of detection limits in Table Iand the general decrease in sensitivity. Nevertheless, the detection limits achieved with AFS are again lower than those with AAS. A typical chromatogram obtained for AsB, MMA, DMA, and As^{V}, with HPLC-UV-HG-AFS can be seen in Figure 2. There is not significant influence of the matrix from biological samples in the detection limits, due to separation of these species from the biological tissue by extraction with 1:1 methanol-water mixture *(see sample extraction procedure)* followed by evaporation of methanol, dilution with water and clean up through a C-18 cartridge. Therefore, the resulting matrix for the analysis is very similar to that from water samples.

TABLE I Comparison between the couplings based on AAS or AFS detection, with and without photooxidation step. Linear ranges for all the studied species, with a least square fit ($r > 0.99$). Detection limts (DL) for each species in water, calculated as three times the standard deviation ($3\ S_{n-1}$) of a blank injected ten times (n=10), divided by the sensitivity

	DL ($\mu g\ l^{-1}$)					*Linear range* ($\mu g\ l^{-1}$)				
	AsB	As^{III}	*DMA*	*MMA*	As^{V}	*AsB*	As^{III}	*DMA*	*MMA*	As^{V}
HPLC-HG-AAS	--	1.1	2.0	1.9	3.9	--	5–100	10–300	10 – 300	25 – 500
HPLC-HG-AFS	--	0.06	0.10	0.07	0.14	--	0.25–6000	0.5–8000	0.25–6000	0.5–8000
HPLC-UV-HG-AAS	3.9	3.5	3.9	3.9	3.9	25–500	25–500	25–500	25–500	25–500
HPLC-UV-HG-AFS	0.14	0.14	0.15	0.15	0.15	0.5–8000	0.5–8000	0.5–8000	0.5–8000	0.5–8000

The linear ranges of the calibration curves are narrow for AAS detection, whereas measurements performed with AFS detection extends the linearity over several orders of magnitude (Table I). When the photooxidation step is introduced, the linear range of all the arsenic species are similar to the one of arsenate, as it can be expected. These results indicate that AFS is a more suitable detection technique for arsenic speciation than AAS, and it was therefore selected for the subsequent analysis of biological samples.

Biological tissues extraction and speciation

The procedure of arsenic speciation has been applied to a sample of bivalve *(Cassostrea angulata)* from an estuarine area of the south west of Spain, as well

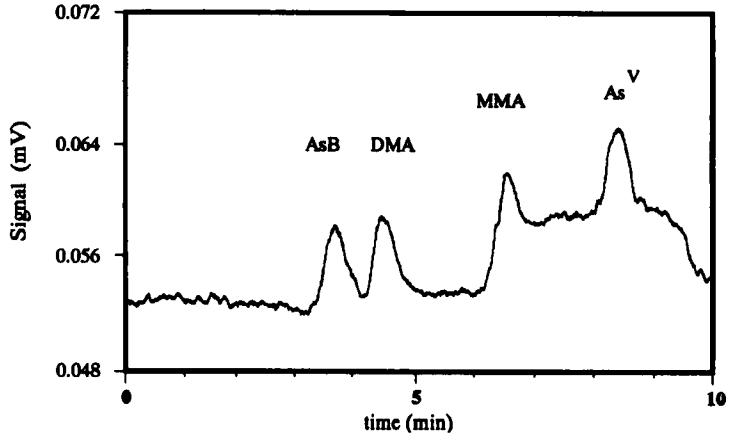

FIGURE 2 Chromatogram corresponding to the HPLC-UV-HG-AFS coupling. Concentration of each standard, 2 μg l^{-1} (as As)

as to the certified material Tort-1 "Lobster hepatopancreas", using the coupling HPLC-UV-HG-AFS.

TABLE II Repetitive extraction of 1.000 g of Tort-1 with 20 ml of methanol or methanol/water (1:1). Results are the mean ± standard deviation. of three replicates, expressed as μg g^{-1} (as As)

	n° extractions	*AsB*	*As^{III}*	*DMA*	*MMA*	*As^{v}*	*% extracted*	*Total As (certified)*	*Total As (acid digestion)*
	1	15.3 ± 0.8	n.d.	1.6 ± 0.2	n.d.	0.6 ± 0.1			
methanol/ water (1:1)	2	2.3 ± 0.1	n.d.	0.2 ± 0.0	n.d.	0.2 ± 0.0			
	3	0.3 ± 0.0	n.d.	n.d.	n.d.	n.d.			
	Total extracted	17.9 ± 0.9	n.d.	1.8 ± 0.2	n.d.	0.8 ± 0.1	83%	24.6 ± 2.2	24.8 ± 1.6
	1	15.1 ± 0.8	n.d.	1.7 ± 0.1	n.d.	n.d.			
methanol	2	2.1 ± 0.1	n.d.	0.2 ± 0.1	n.d.	n.d.			
	3	0.3 ± 0.3	n.d.	n.d.	n.d.	n.d.			
	Total extracted	17.5 ± 0.9	n.d	1.9 ± 0.2	n.d.	n.d.	78%		

The Tort-1 material has been used for the extraction optimization. Single extraction with methanol or methanol/water (1:1) mixtures do not give quantitative recoveries for AsB, DMA, and As^{V}. Other arsenic species such as As^{III} and MMA were not detected. Therefore, repetitive extractions were carried out until no more arsenic was detected in the extract (Table II). Arsenic compounds were leached in the first three extractions, being the arsenobetaine the main arsenic species in this material, followed by DMA and As^{V}.

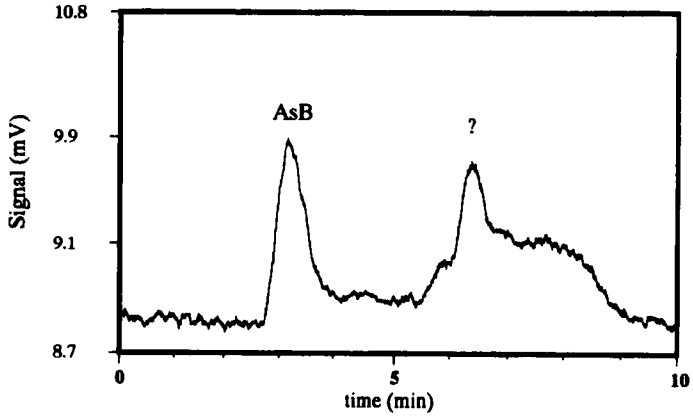

FIGURE 3 Chromatogram corresponding to the first extraction with MeOH/water (1:1) of the fresh bivalve tissue

Both extractants do not give significant differences in the recovery of AsB and DMA, but As^{V} was only extracted using the methanol/water mixture. The content of the arsenic (AsB, DMA and As^{V}) estimated with this later extractant is 20.5±1.1 μg g^{-1} (as As), that represents a 83% of the total arsenic compared with the value obtained by acid digestion. When methanol is used only a recovery of 78% is obtained (AsB and DMA). A similiar speciation study from Larsen *et al*[25] using HPLC-ICPMS and water/methanol and chloroform as extractants, on this reference material provided analogous results of arsenic content (18.67 μg g^{-1} as As) in the water/methanol phase. On the other hand, Lamble *et al*[18] using enzymatic digestion detected the presence of AsB and inorganic arsenic, but no DMA.

The water/methanol mixture was also employed for the determination of arsenic species in a bivalve sample. In this case, only AsB was detected in the first two extractions (14.1±2.2 and 0.4±0.1 μg g^{-1} (as As, dry weight), respectively), representing a 76 % of the total arsenic content obtained with the acid digestion (19.0±1.8 μg As g^{-1} of tissue, dry weight). The chromatogram of this

sample (Figure 3) revealed the presence of a peak with retention time corresponding to MMA. However, in a later experiment carried out without photooxidation this peak disappeared, which is in opposition to the usual behaviour of MMA under these conditions. Therefore, this peak should be attributed to other organic species of arsenic that could not be characterized by our approach. Taking into account that all arsenic species after the photooxidation step are oxidized to arsenate, the quantification of this unknown species, which was only present in the first extraction, resulted in a concentration of 3.9±0.2 $\mu g\ g^{-1}$ (as As, dry weight). Thus, the sum of AsB and the unknown arsenic compound represents a 97 % of the total arsenic content in the sample.

CONCLUSIONS

Analytical speciation of arsenic in biological samples can be successfully acomplished using the coupling HPLC-UV-HG-AFS. This approach represent a clear improvement in relation to the well established coupling based in AAS detection. The AFS detector allows a remarkable decrease of detection limits for AsB, As^{III}, DMA, MMA and As^{V}, and a wide calibration linear range.

These facts, especially the possibility of AsB determination, make the procedure very suitable for arsenic speciation in biological tissues, particularly those from marine occurrence. Extraction of arsenic species from these matrices is critical, and only about a 83% recovery (mainly as AsB) was obtained on a reference material (TORT-1) using repetitive extractions with methanol/water (1:1) mixture. The speciation procedure has also been applied to fresh biological tissues with similar recoveries. Therefore, additional studies to increase the extraction efficiency have to be undertaken.

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