APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2002; **16**: 123–132

Speciation Analysis AOC

Determination of arsenic species in a freshwater crustacean *Procambarus clarkii*

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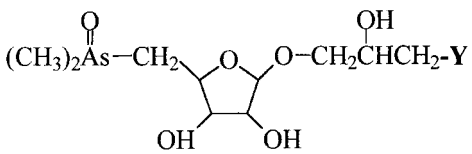
Received 9 July 2001; Accepted 5 November 2001

The arsenic species present in samples of the crayfish *Procambarus clarkii* caught in the area affected by the toxic mine-tailing spill at Aznalcóllar (Seville, Southern Spain) were analyzed. The total arsenic contents ranged between 1.2 and 8.5 μg g^{-1} dry mass (DM). With regard to the different species of arsenic, the highest concentrations were for inorganic arsenic (0.34–5.4 μg g^{-1} DM), whereas arsenobetaine, unlike the situation found in marine fish products, was not the major arsenic species (0.16 ± 0.09 μg g^{-1} DM). Smaller concentrations were found of arsenosugars 1a (0.18 ± 0.11 μg g^{-1} DM), 1b (0.077 ± 0.049 μg g^{-1} DM), 1c (0.080 ± 0.089 μg g^{-1} DM), and 1d (0.14 ± 0.13 μg g^{-1} DM). The presence of two unknown arsenic species was revealed (U1: 0.058 ± 0.058 μg g^{-1} DM; U2: 0.12 ± 0.12 μg g^{-1} DM). No significant differences were seen with respect to the total arsenic contents between the sexes. However, significant differences in the total arsenic contents were revealed between the area affected by the spill and the area not affected, the contents being greater in the affected area.

KEYWORDS: Arsenic; arsenic species; arsenobetaine; arsenosugars; freshwater; crustacean; crayfish

The study of arsenic in the marine environment is undoubtedly an area that has been greatly developed and on which there is much information, not only in terms of quantity but also in terms of speciation.[1–4] On the basis of this information, Edmonds and Francesconi[5] have suggested a plausible pathway for the formation of most known arsenic species, although the precise details are somewhat uncertain.

Studies of arsenic speciation in the freshwater environment are significantly less numerous. As far as total arsenic is concerned, the concentration of arsenic in freshwater organisms is much lower than that observed in marine organisms of the same kind.[6] The few speciation studies performed indicated a substantial difference from marine animals; arsenobetaine (AB) is considered a species that is present in all marine fish and marine fish products, but studies on freshwater organisms are contradictory concerning the distribution of this species. Shiomi *et al.*[6] reported that AB is the major species in freshwater fish; Lawrence *et al.*[7] did not detect AB in freshwater fish; and Šlejkovec *et al.*[8]



Y	
1a	–OH
1b	$-OPO_3HCH_2CH(OH)CH_2OH$
1c	$-SO_3H$
1d	$-OSO_3H$

Figure 1. Principal arsenosugars present in nature.

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Contract/grant sponsor: FEDER; Contract/grant number: 1FD97 1421-C03-03.
Contract/grant sponsor: Generalitat Valenciana (Conselleria de Cultura, Educació i Ciència).
Contract/grant sponsor: Ministerio de Educación y Cultura.

DOI:10.1002/aoc.269

observed that AB was not the predominant species in freshwater mussel and freshwater fish. A recent study carried out by Koch *et al.*[9] on samples of freshwater mussel and freshwater fish revealed the presence of AB and the most common arsenosugars (Fig. 1), although AB was the minor species present.

With regard to the arsenic cycle in the freshwater environment, Chen and Folt[10] studied a freshwater contaminated ecosystem and concluded that, as in the marine environment, there is no biomagnification of arsenic. The same observation was made by Mason *et al.*[11] Both studies only quantified total arsenic. Kaise *et al.*[12] analyzed the mono-, di-, and tri-methylated arsenic species present in water and in various organisms in an arsenic-rich freshwater environment. After making the analysis, they suggested a cycle for arsenic similar to the cycle in marine environments, i.e. the arsenic present in the water, mainly as inorganic arsenic, is dimethylated in algae and subsequently trimethylated in fish.

Contamination of the Earth's water resources as a result of toxic spills of heavy metals and arsenic was a frequent problem with a worldwide impact throughout the 20th century. In Spain, the ecological disaster that had the greatest impact in recent decades occurred on 25 April 1998. The breach of a tailings dam at mines in Aznalcóllar (Seville, Southern Spain) caused a spill of 5×10^6 m^3 of toxic residues in the environment of the Doñana National Park, the largest reserve of bird species in Europe. A key element in the ecosystem of aquatic organisms in the area is undoubtedly the red crayfish, *Procambarus clarkii*. Although introduced in the wetlands of the Guadalquivir only three decades ago, the rapid proliferation of populations of this species has led to notable alterations in the structure and functioning of the aquatic ecosystems; at the same time it has become an environmental and socioeconomic resource of great importance in Doñana and the surrounding area. Commercial exploitation of this crustacean in the region has generated a food industry that sells live or processed crayfish in Spain, in other European countries and in the USA.[13]

The ability of *P. clarkii* to accumulate metals has been shown in numerous studies[11,14–17]. Anderson *et al.*[14] studied *P. clarkii* and suggested that the concentration of non-essential metals in crayfish tissues in a wetland environment reflects its concentration of metals in the sediment in which it dwells. Data are available for lead,[15–17] cadmium,[15,17] aluminum,[15] iron,[16] copper[17] and mercury.[14,17] However, there is little information on the concentration of arsenic in this organism,[11] and there are no studies on the chemical speciation. Consequently, in addition to the ecological and socioeconomic aspects, special attention must be paid to the question of public health, since this is the raw material for various food products that are consumed in Spain and abroad. Knowledge of the arsenic species present in the red crayfish could help in the evaluation of the food safety of this product, especially after the toxic spill.

The present study provides an analysis of the arsenic species present in samples of the crayfish *P. clarkii* obtained from areas affected by the toxic spill from the mines at Aznalcóllar and from adjacent areas.

MATERIALS AND METHODS

Chemicals

Deionized water (18 MΩ cm) was used for the preparation of reagents and standards. All chemicals were of *pro analysi* quality or better. For high-performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC–ICP-MS) quantification, the standards were prepared in an aqueous medium from the following compounds: arsenic(V) from sodium arsenate heptahydrate ($Na_2HAsO_4{\cdot}7H_2O$, Sigma); arsenic(III) from As_2O_3 (Alfa Products); monomethylarsonic acid (MMAA) and dimethylarsinic acid (DMAA) from $CH_3AsO(OH)_2$ (Pfalz and Bauer) and $(CH_3)_2AsO(OH)$ (Aldrich) respectively; AB was obtained from trimethylarsine following the method described by Edmonds and Francesconi.[18] For inorganic arsenic quantification by the acid digestion–chloroform/HCl method, a commercial standard solution of arsenic(V) (Merck) was used.

Oyster tissue (1566a, NIST, USA) was employed as certified reference material in the determination of total arsenic. For identification of arsenosugar species, oyster tissue reference material and kelp powder sample, a commercially available algae product from Eastern Canada, were employed. The arsenic species present in both samples had previously been characterized by other authors.[19,20]

Glass- and plastic-ware were cleaned by soaking in 2% extran solution overnight, rinsing with water, then deionized water. This was followed by soaking in 0.1 mol l^{-1} HNO_3 solution overnight, rinsing with deionized water and air-drying.

Collection and preparation of samples

The crayfish *P. clarkii* was collected during various periods in the year 2000 (February, May, and June) from sampling stations situated in different aquatic ecosystems. Figure 2 shows the sampling stations, which were selected from the area directly affected by the spill — the River Guadiamar (Las Doblas and Aznalcázar stations) and the area of Entremuros (Puente de los Vaqueros and Aguas Mínimas Canal) — and areas not affected by the spill in the Doñana National Park itself (Acebuche). The Puente de Gerena station was selected as a reference point, on the supposition that, as it is situated upstream from the mine at Aznalcóllar, it was not affected by the spill. Samples of crayfish were also taken from ponds at an experimental installation where this species is bred. The specimens of crayfish were caught by the use of Dutch traps or pots. The pots were baited to maximize the catch and checked every 24 h, remaining in place 1 or 2 days, depending on the catch obtained. Subsequently, the

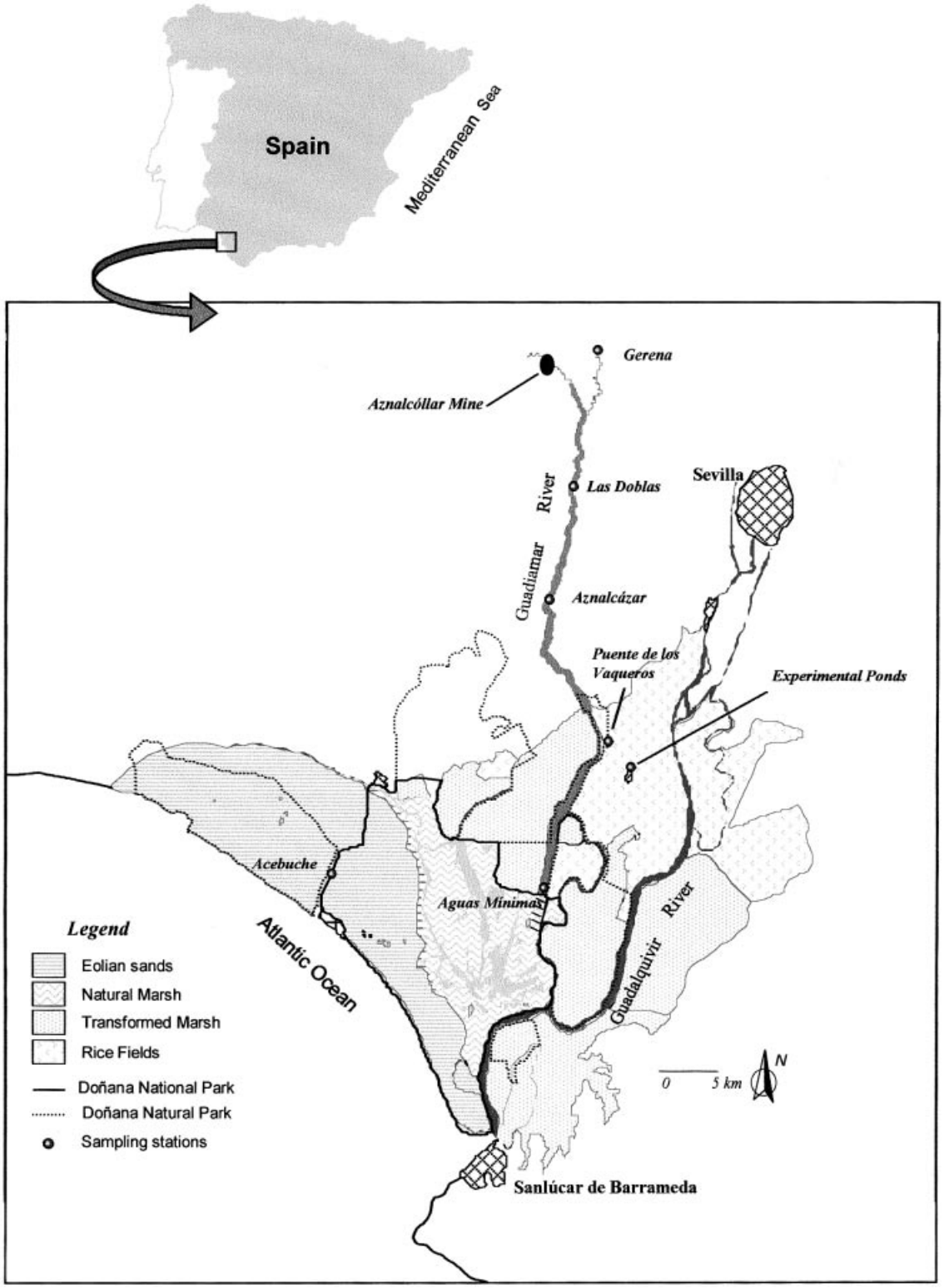

Figure 2. Map of the area studied.

crayfish were washed on site and taken to the laboratory, where they were weighed, measured and sexed. In total, 22 samples of the whole animal were used for analysis of total arsenic and arsenic species: 13 samples from the area affected by the spill, four samples from areas not affected, and five samples from the experimental installation. The

samples from the affected areas and from the areas assumed not to have been affected consisted of a sexed pool of specimens (nine male samples and eight female samples). The five samples from the experimental installation consisted of a pool of specimens that were not differentiated in terms of sex.

Each sample of whole crayfish was frozen at −20 °C and freeze dried, and then crushed to a fine powder in a mill. The resulting powder was stored in previously decontaminated twist-off flasks and kept at 4 °C until analysis.

Determination of total arsenic by ICP-MS

Samples of the freeze-dried crayfish were weighed accurately into 20 ml glass test tubes (outer diameter of 16 mm). Subsamples of 0.1–0.2 g dry mass were used. 2 ml of concentrated nitric acid and two or three Teflon boiling chips were added to each tube. The tubes were transferred to a block heater (Standard Heatblock by VWR Scientific Products) and the temperature increased by 10 °C steps every hour, starting at 70 °C and reaching 150 °C. At 150 °C the samples were evaporated to dryness over the next 1–2 days. The samples were then redissolved in 1% nitric acid containing rhodium at 5 ng ml^{-1} to a final volume of 5 ml. The rhodium served as an internal standard during ICP-MS analysis. The redissolved digests were vortex-mixed, filtered (25 mm Acrodisc Syringe Filter 0.45 μm, HT Tuffryn membrane, Pall, Gelman Laboratory) into storage vials using disposable syringes, and then stored at 4 °C.

The redissolved digests were diluted appropriately with the rhodium–acid solution and analyzed for total arsenic using ICP-MS. A VG Plasma Quad 2 Turbo Plus inductively compled plasma mass spectrometer (VG Elemental, Fisons Instrument), equipped with an SX 300 quadrupole mass analyzer, a standard ICP torch, and a de Galan V-groove nebulizer, was used. Samples were analyzed in the 'peak jump' mode. Standard interference corrections for $^{40}Ar^{35}Cl^{+}$ at *m/z* 75, i.e. at the same mass as ^{75}As, were made by monitoring *m/z* 75, 77 ($^{40}Ar^{37}Cl^{+}$ and $^{77}Se^{+}$) and 82 ($^{82}Se^{+}$). Signals were corrected according to the internal rhodium standard.

The limit of detection (LD) of this methodology is 1.3 ng g^{-1} dry mass (DM). The result obtained for oyster tissue NIST 1566a (13.7 ± 1.2 μg g^{-1} DM) was similar to the certified value for total arsenic in this reference material (14.0 ± 1.2 μg g^{-1} DM).

Arsenic speciation analysis by HPLC–ICP-MS

Lyophilized samples of whole crayfish were weighed into 15 ml centrifuge tubes. Dry masses of approximately 0.5–1.0 g were used. One extraction round included the addition of 5 ml of MeOH/H_2O (1:1, v/v) per 0.5 g of dry tissue, vigorous mixing, sonication for 10 min, centrifugation at 3000 rpm for 10 min, and transferring of the supernatant by a Pasteur pipette to a 100 or 250 ml round-bottom flask. The residue was extracted an additional four times following the same procedure. After the final extraction round the residue was freeze dried for total and inorganic arsenic determination. The five supernatant fractions were combined in the round-bottom flask, evaporated to near dryness (~1–2 ml) and made up to an exact volume of 10 ml by the addition of deionized water. Extracts were stored at −20 °C until analysis.

The HPLC system consisted of a Waters Model 510 delivery pump, a Rheodyne Model 7010 injector valve with a 20 μl sample loop, and a reverse phase C_{18}-column (Inertsil ODS, GL Sciences, Japan, 250 mm × 4.6 mm). A guard column (C_{18}, Supelco) was used preceding the analytical column. The HPLC system was connected to the ICP nebulizer via a PTFE tube (20 cm × 0.4 mm) and appropriate fittings. The mobile phase contained 10 mmol l^{-1} tetraethylammonium hydroxide, 4.5 mmol l^{-1} malonic acid, 0.1% methanol and 5 ng ml^{-1} rhodium. The pH was adjusted to 6.8 by using 3% nitric acid. The ICP-MS setup was as described previously, with the exception that the instrument was operated in the 'time-resolved analysis' mode. Assignment of arsenic compounds to the peaks in the chromatograms was performed by matching the retention times to the arsenic species in the extracts of the oyster tissue standard reference material and the kelp powder, and to standards containing arsenic(III), arsenic(V), MMA, DMA and AB. A series of standards containing arsenic concentrations of 10, 25, 50 and 100 ng ml^{-1} of each of the five arsenic compounds [arsenic(III), arsenic(V), MMA, DMA and AB] was used to quantify the resulting chromatograms. The arsenosugars were quantified by using the calibration curve of DMA. All samples were filtered (25 mm Acrodisc Syringe Filter 0.45 μm, HT Tuffryn membrane, Pall, Gelman Laboratory) prior to injection onto the column.

The analytical characteristics of the method are as follows. LD: 1 ng g^{-1} DM for AB, 9.7 ng g^{-1} DM for arsenosugar 1a, and 4.8 ng g^{-1} DM for arsenosugar 1b; precision: 3%.

Determination of inorganic arsenic

The method employed was developed previously by Muñoz *et al.*[21] 0.5 g of lyophilized sample was weighed into a screw-top centrifuge tube and 4.1 ml of water was added and the mixture was agitated until completely moistened. After the addition of 18.4 ml of concentrated HCl, the solution was agitated for 1 h and it was left to stand for 12–15 h. A reducing agent (2 ml of HBr and 1 ml of hydrazine sulfate) was added and, after agitation for 30 s, 10 ml of $CHCl_3$ was added and the mixture was agitated for 3 min. The phases were separated by centrifuging at 2000 rpm for 5 min. The chloroform phase separated by aspiration was poured into another tube. The extraction process was repeated two more times and the chloroform phases were combined and centrifuged again. The remnants of the acid phase were eliminated by aspiration, and possible remnants of solid material in the chloroform phase by passing it through Whatman GD/X syringe filters with 25 mm PTFE membrane

Table 1. Total and arsenic species contents in the samples. Results expressed as micrograms of arsenic per gram of dry mass

Sampling area	Sex	Total As[a]	Inorganic As[b]	AB[c]	1a[c]	1b[c]	1c[c]	1d[c]	U1[c]	U2[c]	Others[c]
Area affected	Female	2.6	1.8	0.059	0.13	0.12	0.052	0.046	0.020	nd	nd
	Female	8.5	5.4	0.14	0.22	0.062	0.050	0.20	0.024	nd	nd
	Female	5.8	3.6	0.11	0.065	0.074	0.032	0.030	0.033	nd	nd
	Female	6.3	na	0.12	0.056	0.048	0.033	0.065	nd	0.11	nd
	Female	6.3	na	0.28	0.098	0.044	0.030	0.035	0.019	nd	nd
	Female	5.0	na	0.17	0.064	0.042	nd	0.091	nd	nd	nd
	Male	1.6	0.75	0.056	0.068	0.033	0.083	0.070	0.013	0.043	nd
	Male	2.0	0.87	0.25	0.27	0.076	0.44	0.40	0.023	nd	nd
	Male	3.8	2.2	0.17	0.16	0.076	0.023	0.094	0.076	nd	nd
	Male	3.1	2.1	0.12	0.070	0.057	0.034	0.044	0.059	nd	nd
	Male	2.6	2.4	0.27	0.15	0.059	0.022	0.033	0.018	0.017	0.21
	Male	5.1	2.8	0.15	0.21	0.068	0.031	0.15	nd	0.14	nd
	Male	3.0	2.2	0.27	0.061	0.058	0.013	0.087	nd	nd	0.42
Area not affected	Female	1.2	na	0.19	0.17	0.063	0.053	0.064	0.15	0.068	nd
	Female	1.3	na	0.080	0.24	0.023	0.087	0.080	0.22	nd	nd
	Male	1.8	1.1	0.072	0.15	0.050	0.073	0.030	0.019	0.31	0.28
	Male	1.6	0.34	0.13	0.41	0.10	0.097	0.091	0.084	0.081	nd
Experimental installation	Not sexed	3.8	na	0.085	0.10	0.073	0.067	0.30	nd	0.31	0.14
	Not sexed	4.1	na	nd	0.46	0.28	0.13	0.17	0.14	nd	nd
	Not sexed	2.2	na	0.067	0.22	0.078	0.066	0.63	nd	nd	0.063
	Not sexed	7.2	4.3	nd	0.23	0.076	0.047	0.22	nd	0.20	nd
	Not sexed	2.5	0.73	0.11	0.37	0.064	0.068	0.17	0.037	0.38	nd

na: not analyzed; nd: not detected.
[a] Concentration obtained by total acid digestion method.
[b] Concentration obtained from acid digestion–chloroform/HCL method.
[c] Concentration obtained from MeOH/H_2O extraction method.

(Merck Farma y Química S.A., Barcelona, Spain). The chloroform phase, containing the species arsenic(III), arsenic(V), and MMA, was back-extracted by agitating for 3 min with 10 ml of HCl (1 mol l^{-1}). The phases were separated by centrifuging and the aqueous phase was aspirated and poured into a beaker. This stage was repeated once more and the back-extraction phases obtained were combined. Subsequently, 2.5 ml of ashing aid suspension [20% m/v $Mg(NO_3)\cdot 6H_2O$ and 2% m/v MgO] and 10 ml of concentrated HNO_3 were added to the 1 mol l^{-1} HCl phase. After evaporation on a sand bath (PL 5125 model, Raypa, Scharlau S.L., Barcelona, Spain) until total dryness, a dry-ashing of the sample was made.

Ashes from the mineralized samples were dissolved in 5 ml of 50% v/v HCl, and pre-reduced with 5 ml of solution containing 5% m/v KI and 5% m/v ascorbic acid. After 30 min, the solution was diluted to volume with water and filtered through Whatman No.1 filter-paper into a 25 ml volumetric flask, and quantified by flow injection–hydride generation (FI–HG; Perkin–Elmer FIAS-400, Perkin–Elmer, PE, Norwalk, CT, USA) atomic absorption spectrometry (AAS; PE Model 3300) using an autosampler (PE AS-90). The instrumental conditions used for arsenic determination were as follows. *FI–HG*: loop sample 0.5 ml; reducing agent 0.2% (w/v) $NaBH_4$ in 0.05% (w/v) NaOH, 5 ml min^{-1} flow rate; HCl solution 10% (v/v), 10 ml min^{-1} flow rate; carrier gas argon, 100 ml min^{-1} flow rate; *AAS*: wavelength 193.7 nm; spectral bandpass 0.7 nm; electrodeless discharge lamp system 2, lamp current setting 400 mA; cell temperature 900 °C.

The residues obtained from the MeOH/H_2O extraction were also treated for the determination of inorganic arsenic. After extraction with MeOH/H_2O, the residues were lyophilized in the same extraction tube because of the difficulty of extracting them without losses. Once lyophilized, 0.5 g of the residues was weighed and treated using the same procedure as described previously for the samples.

The analytical characteristics of the method, evaluated in a previous study,[21] are as follows. LD: 13 ng g^{-1} DM or 3 ng g^{-1} wet mass (WM); precision: 3–5%; and quantitative recovery for both arsenic(III) (99%) and arsenic(V) (96%). The application of the methodology was carried out with a sample certified for total arsenic, TORT-2 (Lobster Hepatopancreas), because of the non-existence of samples certified for inorganic arsenic. The results obtained (0.581 ± 0.055 μg g^{-1} DM) were similar to those obtained by another

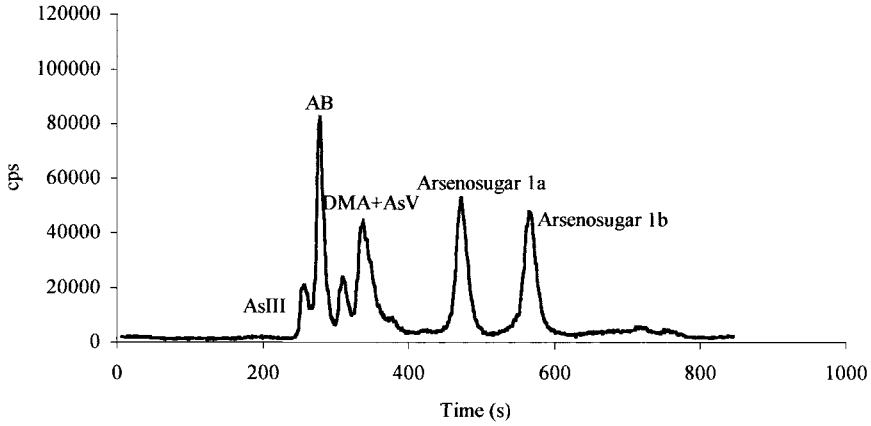

Figure 3. Reverse-phase C_{18} chromatographic separation of arsenical species in oyster tissue sample.

methodology, in which the extraction was performed by microwave-assisted distillation (0.506 ± 0.031 μg g^{-1} DM).[22]

RESULTS AND DISCUSSION

Total arsenic contents

The concentrations of total arsenic obtained by acid digestion–ICP-MS are shown in Table 1. The arsenic concentration values range from 1.2 to 9 μg g^{-1} DM (0.3 to 2.3 μg g^{-1} WM). A one-way ANOVA test showed that there were no significant differences ($P = 0.16$, $F = 2.14$, Df = 1) between the total arsenic contents of males ($n = 9$, 3.0 ± 1.4 μg g^{-1} DM) and females ($n = 8$, 4.4 ± 2.6 μg g^{-1} DM). As for the total arsenic contents in the affected area, the area supposedly not affected, and the experimental installation, a one-way ANOVA test revealed the existence of significant differences ($P = 0.05$, $F = 3.50$, Df = 2). A multiple range test for total arsenic by areas showed a statistically significant difference between affected areas (mean: 4.3 μg g^{-1} DM) and areas not affected (mean: 1.5 μg g^{-1} DM).

Comparison of the concentrations obtained with previous data in the literature is difficult because of the scarcity of information about freshwater organisms. In most cases the total arsenic contents reported refer to the abdominal muscle or the hepatopancreas, or else to complete organisms bred in the presence of high concentrations of arsenic. The data available for arsenic concentrations in marine crustaceans in which the whole animal is analyzed show, as indicated earlier, that concentrations are much higher in the marine environment (crab and shrimp: 15–118 μg g^{-1} DM).[23]

Arsenic species contents

Figures 3–6 respectively show the HPLC–ICP-MS chromatograms of oyster tissue sample, kelp powder sample, standards solution, and one of the samples of crayfish analyzed. AB and the four most common arsenosugars in

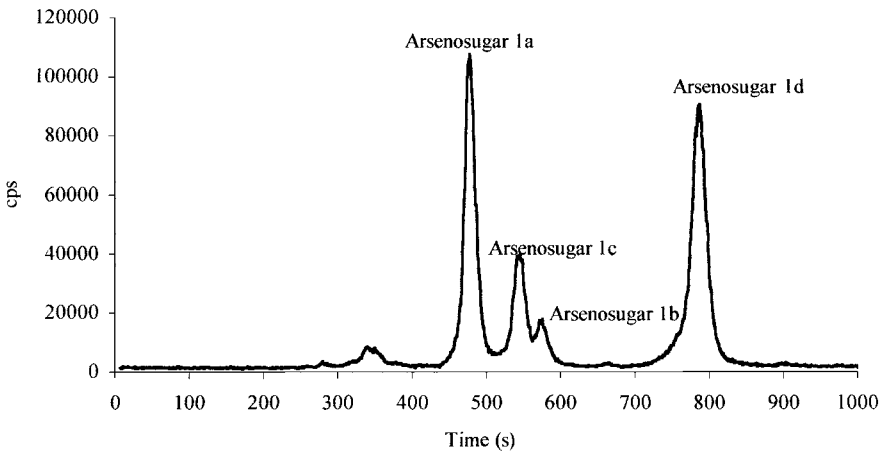

Figure 4. Reverse-phase C_{18} chromatographic separation of arsenical species in kelp powder sample.

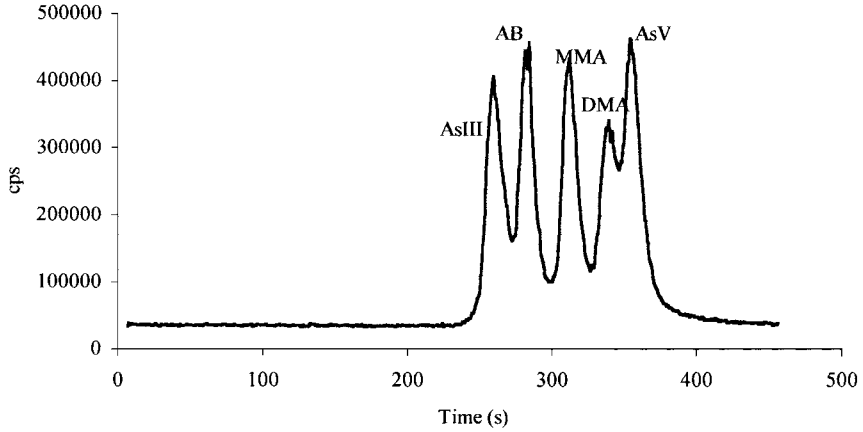

Figure 5. Reverse-phase C_{18} chromatographic separation of arsenical species solution.

nature (Figure 1) can be identified in crayfish by comparison of the retention times with oyster reference material and kelp powder sample. Comparison of the chromatograms of oyster tissue and kelp powder shows that the retention times of the common arsenosugars in these two samples are conserved in time and are independent of the matrix used. By comparison of the crayfish chromatogram with the standards solution chromatogram [AB, arsenic(III), arsenic(V), DMA and MMA] it was possible to identify in the sample of *P. clarkii* an unresolved signal corresponding to the co-elution of arsenic(V) and DMA, and also two peaks that do not correspond to any of those in the samples used as references and that also do not match the standards available. These two unknown compounds eluted immediately before (unknown 1, U1) and immediately after (unknown 2, U2) arsenosugar 1d. Using an analytical system analogous to that used in the present study, Lai *et al.*[24] reported the presence in samples of scallops of an arsenic compound that, like U1, eluted before arsenosugar 1d, and those authors suggested the possibility that it was a sugar derivative.

In three of the crayfish samples analyzed, a peak was detected at a retention time of 313 s. This is similar to the retention time of the MMA standard (305 s). When the samples were analyzed by means of column switching in HPLC as developed by Súñer *et al.*,[25] however, it was seen that it was not MMA. This has already been described by Lai *et al.*,[24] who reported the existence of a peak that eluted at the same time as MMA in the ion-pairing chromatographic system; after ion-exchange chromatography, it was seen by Lai *et al.* that it did not correspond to MMA but to a cationic species that did not coincide with any of the available standards, and they suggested that it was a sugar derivative.

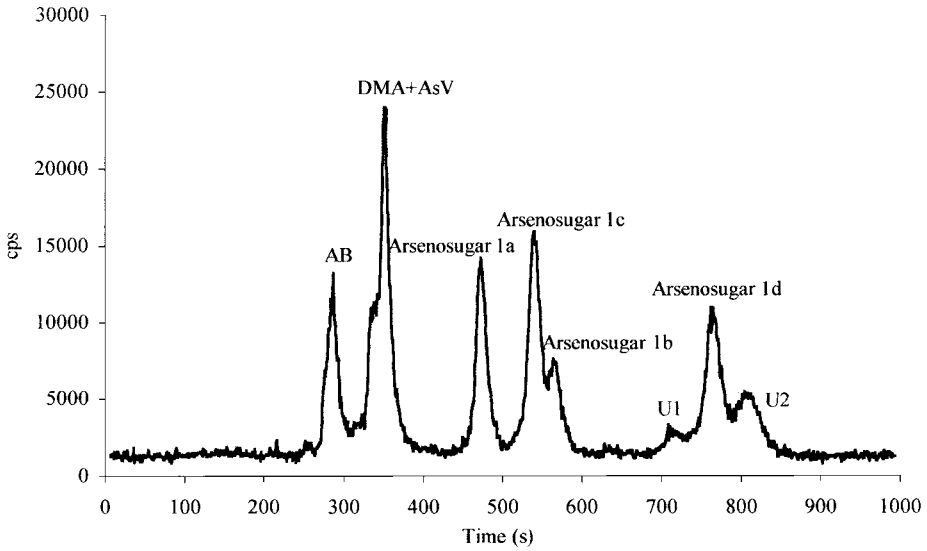

Figure 6. Reverse-phase C_{18} chromatographic separation of a crayfish sample.

The contents of arsenic species extracted by MeOH/H_2O in the whole crayfish samples are shown in Table 1 and indicate a fairly constant pattern of composition throughout the sampling. Arsenosugars 1a, 1b, and 1d, and the overlapping peak of arsenic(V) and DMA appear in all the samples analyzed; arsenosugar 1c appears in 99% of the samples, and AB in 92%. The presence of other species is less widespread: U1 in 62% of the samples, U2 in 46%. None of these arsenic species appears in high concentrations in all the samples analyzed. The values ($x \pm s$) of the arsenic species were as follows: DMA and arsenic(V), 0.37 ± 0.30 μg g^{-1} DM; arsenosugar 1a, 0.18 ± 0.11 μg g^{-1} DM; AB, 0.16 ± 0.09 μg g^{-1} DM; arsenosugar 1d, 0.14 ± 0.13 μg g^{-1} DM; arsenosugar 1b, 0.08 ± 0.05 μg g^{-1} DM; U2, 0.12 ± 0.12 μg g^{-1} DM; U1, 0.06 ± 0.06 μg g^{-1} DM.

The percentages ($x \pm s$) represented by the various arsenic species with respect to the total arsenic extracted are: arsenic(V) + DMA, $29 \pm 13\%$; arsenosugar 1a, $16 \pm 7\%$; AB, $16 \pm 8\%$; arsenosugar 1d, $11 \pm 9\%$; arsenosugar 1c, $7 \pm 6\%$; arsenosugar 1b, $7 \pm 3\%$. In the samples that contained unknown species, U1 represented $6 \pm 7\%$, and U2 was $10 \pm 8\%$. These results show that, after extraction with MeOH/H_2O, there is no species with a concentration much higher than the others.

Comparison of the arsenic species present in freshwater crustaceans with those found in marine crustaceans, specifically shrimps,[23] showed very different patterns of composition. AB is the species with the highest concentration in all the samples of shrimps, where in *P. clarkii* one can speak of a fairly even distribution of the species of arsenic. Consequently, unlike the situation in marine organisms, where AB is the predominant species, in freshwater crayfish samples one cannot speak of a major species among those that can be extracted with MeOH/H_2O. The high concentration of AB in marine organisms might be attributable to a possible osmoregulatory activity performed by AB, chemically similar to the osmoregulatory compound betaine.[26] The lower concentration of salt in the freshwater environment by comparison with the marine environment might be translated into a lesser need for osmoregulation and, consequently, a lesser accumulation of AB. The difference between the arsenical composition of freshwater and marine organisms might also be a consequence of the different arsenical composition of the food that they ingest, or of a possible difference in metabolization of species of arsenic. These organisms in the freshwater environment present not only AB but also a wide range of arsenosugars and inorganic arsenic, just as marine organisms do, although the relative proportions in which the arsenic species are found is different.

Inorganic arsenic contents

For all the samples analyzed, the contents of inorganic arsenic [arsenic (III) + arsenic (V)] obtained by acid digestion–chloroform/HCl extraction, range from 0.34 to 5.4 μg g^{-1} DM (0.11–1.7 μg g^{-1} WM) (Table 1). The concentrations of inorganic arsenic found reached 50% of the total arsenic in some samples, making it the predominant species in most of the samples analyzed (21–84%). Comparison of the results obtained with previous data about inorganic arsenic in freshwater crustaceans is not possible because of the absence of other data.

From a health viewpoint, inorganic arsenic is considered to be a carcinogen, and the FAO/WHO uses its concentration to establish the provisional tolerable weekly intake (PTWI) for the contaminant arsenic (2.1 μg of inorganic arsenic per kilogram of body weight per week). Consequently, the determination of inorganic arsenic is of the utmost interest from the viewpoint of health and legislation. Spain does not have maximum limits for inorganic arsenic in seafood products, and, therefore, all the samples analyzed could be sold commercially. On a global level, only Australia and New Zealand have legislation for this contaminant (1 μg g^{-1} WM),[27] and the legislation in those countries therefore serves as a model to be followed by other countries. Several of the samples analyzed in this study exceed that concentration. However, it must be borne in mind that the entire animal was analyzed. Further studies are needed to ascertain concentrations of inorganic arsenic in the edible parts of crayfish in order to clarify the toxicological risks associated with its consumption. From the viewpoint of health, interpretation of the results would require evaluation of intake, an aspect not included in the present study.

Efficiency of the extraction

For 14 of the 22 samples analyzed the extraction of arsenic species by MeOH/H_2O was less than 50% of the total arsenic, and for 20 samples it was less than 80%. This is not an uncommon observation for samples other than fish muscle. The retention of arsenic species might be because of a strong bond between arsenic and proteins in the matrix. Moreover, Mason *et al.*[11] reported that most of the arsenic present in crayfish accumulates in the carapace. This carapace contains chitin, a substance not dissolved even by hot caustic alkalis, which is soluble in cold concentrated hydrochloric acid without change, and may be precipitated from its solution by the addition of water.[28] As the carapace was included in the crayfish analyzed, the arsenic may seem to be bound to the chitin, given that its extraction with MeOH/H_2O is low. Koch *et al.*[29] suggest that arsenic is bound to this kind of component in fungus, and that it is difficult to extract arsenic bound to chitin because of the characteristics of the latter.

Comparison of the inorganic arsenic values obtained by this methodology with those obtained by MeOH/H_2O extraction shows that the sum of the arsenic (III) and arsenic (V) contents detected by HPLC would be much less than the inorganic arsenic obtained by acid digestion–chloroform/HCl extraction. This suggests that a substantial part of the inorganic arsenic was not extracted by using the MeOH/

H_2O mixture. Therefore, inorganic arsenic was determined in the lyophilized residues of the MeOH/H_2O extraction by the acid digestion–chloroform/HCl method. The results obtained show that a substantial part of the inorganic arsenic was not extracted (~19–77%) by the MeOH/H_2O method. Part of the inorganic arsenic that was not extracted may be bound to the chitin present in the carapace, as mentioned earlier, and part may be bound to the —SH groups of cytosolic proteins in the various parts of the body. The inorganic arsenic in the residue represents 28–100% of the total; the remainder of the arsenic in the residue might partly correspond also to arsenosugar 1a not being extracted quantitatively, given that Kuehnelt *et al.*[30] reported the extraction of these arsenosugars from lichens is greater when water is used as the extractant than when a mixture of MeOH/H_2O is used. It must also not be forgotten that this arsenic may correspond to species such as arsenolipids, which would not be soluble in an MeOH/H_2O mixture.

Arsenical composition and diet

The red crayfish is defined as a polytrophic species, so that it is omnivorous and detritivorous at the same time.[31,32] Gutiérrez-Yurrita *et al.*[33] performed a study on the wetlands of the Doñana National Park in which they analyzed the stomach contents of crayfish in order to determine the diet of this species. They observed that non-green vegetable matter, green plants, and organic sediment constituted the greater part of the stomach content. Animals and animal larvae of the meiobenthos and zooplankton, filamentous algae and seeds were associated with the organic sediment. The animal diet consisted of insects, insect larvae and *Cladocera*, but to a lesser extent than with the categories mentioned previously. In general, both immature and mature crayfish consumed mainly plants and detritus, but in the young specimens there was a greater proportion of food of animal origin than in the adults. Other studies carried out in natural habitats[34,35] or in the laboratory[36] also showed the diversity of the feeding habits of the crayfish. These feeding habits might, to some extent, explain the variety of arsenic species found in all the samples of crayfish.

The high percentages of inorganic arsenic found in the samples of crayfish might be due to the herbivorous diet. Most speciation studies on plants showed that arsenic is mainly present as inorganic arsenic.[30,37] One can also not rule out the possibility that this form of arsenic may partly come from the sediment that is also ingested by *P. clarkii*. Finally, there is evidence that the freshwater shrimp *Neocardia denticulata*[38] is able to demethylate MMA and DMA to inorganic arsenic, so that possibly a quantity of the inorganic arsenic observed — small in comparison with the quantity contributed by the diet — may come from this demethylation process.

As for the arsenosugars present, we may suppose that they come from organic sediments, which contain filamentous algae, and from the periphyton, the main source of arsenosugars in any food chain. The possibility that some of them may come directly from plants also cannot be ruled out.

With regard to AB, the origins might be of various kinds. Although *P. clarkii* is mainly herbivorous, it does ingest a certain quantity of insects and larvae of aquatic insects, *Cladocera*, and copepods, as well as other animals present in the organic sediment. There is evidence to show that certain insects are able to trimethylate arsenic, as is the case with the freshwater flea *Daphnis magna*,[12] although it is not clear what species of arsenic it is able to produce, so that ingested insects might be a direct source of AB.

The AB could be synthesized by the animal itself from the arsenosugars that it ingests with the diet, as in the Edmonds and Francesconi pathway.[2] In this case, methylation might be performed by the bacterial biomass existing in the sediment and periphyton that the crayfish itself ingests. However, some studies indicate that AB cannot be synthesized from arsenosugars, and that the food chain or water supply are the only sources of AB.[3]

In any case, it is difficult to determine the origin of the various species of arsenic present in crayfish, although the diet seems to be the most acceptable candidate. Further studies on this aspect are required.

CONCLUSIONS

The arsenical composition of *P. clarkii* indicates that approximately 50% of the arsenic is in the form of inorganic arsenic. In some cases the concentrations of this form of arsenic are sufficiently high to be considered problematic from a health viewpoint. But it must not be forgotten that, in all cases, the complete, non-depurated organism was analyzed. This is not the form in which this organism is normally consumed, with the exception of certain minor sectors of the population. In the food industry, crayfish are normally cleaned before being processed, and subsequently the consumer removes the outer covering after cooking and consumes the muscle and, to a lesser extent, the hepatopancreas and part of the head. In order to make a more reliable toxicological evaluation, it would be advisable to carry out speciation studies of the various edible portions of crayfish, both in the raw state and after industrial processing.

Acknowledgements

The authors are grateful to Sergio Algora, Concepción Almela, Victoria Benito, María Jesús Clemente, Maite de la Flor, Corinne Lehr and Kevin Mark for their technical support and Bert Mueller for his help with ICP-MS measurements. This research was supported by the project FEDER 1FD97 1421-C03-03, for which the authors are deeply indebted. V. Devesa and M. A. Súñer received Spanish Research Personnel Training Grants from the Generalitat Valenciana (Conselleria de Cultura, Educació i Ciència) and the Ministerio de Educación y Cultura respectively.

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