Arsenic metabolites in human serum and urine after seafood 

(Anemonia sulcata) consumption and bioaccessibility 

assessment using liquid chromatography coupled to inorganic 

and organic mass spectrometry

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ABSTRACT. The present paper reports for the first time the bioaccessibility of arsenic species in Anemonia sulcata and its metabolism in the human body after the ingestion of this seafood, a delicacy highly appreciated in the south of Spain. Speciation in tissue extracts and human fluids was carried out by anion/cation exchange chromatography with inductively coupled plasma mass spectrometry ((IC)-ICP-MS). Results obtained show that about 54\% of the arsenic present in the anemones is lost after cooking, around 95\% of total arsenic in cooked anemones is bioaccessible and
85% of the amount ingested is eliminated from the body by urine in 90 h. The relative abundance of As in urine varies along the time, but after the first ten hours follows the order arsenobetaine (AB) ≈ DMA\textsuperscript{V} (dimethylarsinate) > arsenocholine (AsC) > tetra-methyl-arsonium (TETRA) > trimethyl-arsine oxide (TMAO) > MA\textsuperscript{V} (monomethylarsonate) > iAs\textsuperscript{V} > dimethylarsenoethanol (DMAE), while the major specie in cooked anemones is AB followed by DMA\textsuperscript{V} > dimethylmonothioarsinic acid (DMAS\textsuperscript{V}) > AsC > TETRA > TMAO > iAs\textsuperscript{V} > glycerylphosphorylarsenocholine (GPAsC) > MA\textsuperscript{V} > DMA. After 30 minutes from the ingestion, DMA\textsuperscript{V} is the most abundant in human serum, followed by AB, MA\textsuperscript{V} and AsC. Finally, the use of organic mass spectrometry allowed the standarless identification of AsC, GPAsC, DMAS\textsuperscript{V} and DMAE.

KEYWORDS. Arsenic, speciation, \textit{Anemonia sulcata}, seafood, bioaccessibility, mass spectrometry
1. Introduction

Arsenic has been recognized as a global toxin that affects human health [1] and can be the promoter of some types of cancers such as skin, lung, urinary bladder and liver [2]. Humans can be exposed to arsenic by contaminated water, soil, atmosphere and food, especially seafood [3], as well as products or supplements based on algae (especially *Hijiki seaweed*) and cereals or cereals derived foods [4]. The levels of arsenic in marine organisms can vary widely, but almost samples fall within the range of about 5-100 mg As kg\(^{-1}\) dry mass while terrestrial foods, in contrast almost invariably, have low levels of arsenic with most samples having less than 0.05 mg As kg\(^{-1}\) dry mass, except rice that can typically contain 0.1-0.4 mg As kg\(^{-1}\) dry mass, *500-1000 times lower than in seafood* [3]. In addition, in some countries, such as, Japan, China and Korea, food from marine sources constitutes an important part of the diet and a series of studies has been undertaken to determine the chemical form of arsenic in the organisms that contribute to the human diet [5].

On the other hand, As toxicity is strongly related to the chemical species in which it is present and seafood, that is the main source of total As in the human diet, contains mainly organic As species which are less toxic than inorganic arsenic (iAs). In mammals, inorganic forms of arsenic (arsenate (As (V)) and arsenite (As (III)) are more toxic, while methylated forms, MA\(^V\) (monomethylarsonate) and DMA\(^V\) (dimethylarsinate), are considered only moderately toxic [6, 7]. In addition, other arsenic species, like trimethyl-arsine oxide (TMAO) and tetra-methyl-arsonium (TETRA) are also considered moderately toxic, whereas arsenobetaine (AB), arsenocholine (AsC) and other arenosugars (AsS) show no toxicity [8]. In addition, As (III) is more toxic than (As (V)) most probably due to enhanced cellular uptake and accumulation of the former (LD50 for mice are 4.5 mg kg\(^{-1}\) and 14-18 mg kg\(^{-1}\) for the arsenite and arsenate, respectively) [9, 10]. Metabolites of As (III), in particular trivalent methylated metabolites, are considered the main determinant of arsenic-induced
toxicities [11], as they have a high binding affinity for sulphhydryl and thiol groups of proteins in various organs as compared with As (V) [12]. In this sense, methylated arsenic has much lower toxicity compared to inorganic arsenic (LD50 for mice are 1800 mg kg\(^{-1}\) and 1200 mg kg\(^{-1}\) for the MA\(^V\) and DMA\(^V\), respectively) and the trivalent methylated forms MA\(^{\text{III}}\) and DMA\(^{\text{III}}\) have been recently identified as cancer promoters [13-15]. In contrast, arsenobetaine has the least toxicity of the typical arsenicals investigated in human biological fluids (LD50 for mice is 10000 mg kg\(^{-1}\)). Thus, due to the different toxicities between the arsenicals, analysis of total arsenic is insufficient for complete risk assessment evaluation [16].

In humans, arsenic compounds can be easily absorbed by the gastrointestinal tract and transported to the blood. It has been reported that the absorption respect to the dose is 50 % for As (III) and more than 95 % for arsenobetaine and arsenochoine [17]. In the human body, inorganic arsenic (iAs) is metabolized to methylated forms; As (V) is reduced to As (III) and later methylated by oxidative addition. The reduction is carried out by glutathion, cysteine and dithiothreitol and the methyl groups are provided by S-Adenosyl methionine. The main specie is DMA, however the metabolic pathway can continue to trimethylated species. The DMA\(^V\) and MA\(^V\) can be easily identified in urine and individuals that do not consume a high quantity of seafood present 10-30% inorganic arsenic, 10-20% MA\(^V\) and 55-80 % DMA\(^V\) [18]. The metabolism and excretion of pentavalent and trivalent dimethylated arsenic in mice after oral administration has also been reported [19]. The proportion of arsenic compounds varies with the age since the urine from children can contain until 50 % more than the urine from adults [20]. All evidence to date indicates that although AB is bioavailable to humans, it is excreted directly without metabolization and the presence in urine indicates the consumption of fish and crustaceous. When the arsenic comes from the consumption of algae, mussels, or ingested as a pure synthesized compound, that contains arsenosugars, they are metabolized to DMA\(^V\) and the proportion of this compound increases
significantly [3, 18, 21]. Moreover, DMA\textsuperscript{V} is the major metabolite produced from inorganic arsenic, and the intermediates produced in route to DMA\textsuperscript{V} are thought to play a role in arsenic’s mode of toxic action [22]. Thus, the possibility exists for inorganic arsenic and arsenosugars to have at least some common intermediate metabolites, in which case arsenosugars might also be capable of producing toxic effects [3]. Like in the case of arsenosugars and iAs, arsenolipids are also converted to DMA\textsuperscript{V} by humans [23]. Then, although the biomethylation of iAs has been considered as a detoxification mechanism during the last decades, intermediate toxic methylated trivalent arsenicals make it as an intoxication process [24, 25]. On the other hand, thioarsenicals are new-found sulfur containing arsenic metabolites, which has been identified in urine of animals as well as in marine organisms. Methylation of inorganic arsenic resulted in the formation of thiolated metabolites; dimethylarsinothioic acid (DMAS\textsuperscript{V}), dimethylidithioarsinic acid (DMDTA\textsuperscript{V}) and monomethylmonothioarsenic acid (MMMTA\textsuperscript{V}) commonly detected in mammals urine (including human) after long term exposure to inorganic or other arsenic species (MA\textsuperscript{V}, DMA\textsuperscript{V} or TMAO) [26]. However, how these thioarsenicals are produced during the metabolism of As (III) and the source of sulfur remains unknown [26].

In order to study the metabolism in humans after food consumption it is important to know the effect of cooking on arsenic species in the food, which can considerably change the total content of arsenic and the ratios between species. Traditional washing and soaking of Hizikia fusiforme seaweed, which has very high inorganic arsenic contents, may reduce the contents by up to 60% [27] that is in good agreement with the 64 % of total arsenic lost in cooked Anemonia sulcata, which is battered in flour and fried in olive oil [28]. Anemonia sulcata is a marine organism very appreciated delicacy in Spain, especially in the south, where it is served as delicious meal. The speciation of arsenic shows that DMA\textsuperscript{V} is the dominant specie in Anemonia sulcata followed by AB [28].
In the present paper, speciation of arsenic has been carried out in human urine and serum after consumption of the anemones using a combination of inductively coupled plasma mass spectrometry and organic mass spectrometry with anion/cation exchange high performance liquid chromatography ((IC)-ICP-MS and –MS). The speciation data allows to deep inside into arsenic metabolism in human body after the consumption of this particular food that contains unusual arsenic species as GPAsC and DMASV.

2. Experimental

2.1. Standard solutions, reagents and seafood samples

Analytical grade reagents were used throughout. The chemical standards used were AB (95%, Fluka, Steinheim, Germany), As (III) and As(V) (99%, Merck, Darmstadt, Germany), DMA, MA, TETRA and TMAO (99%, Supelco, Bellefonte, USA). Tetramethylarsonium ion (TETRA) and trimethylarsine oxide (TMAO) (Sigma Aldrich, Steinheim, Germany) were donated by Dr. Riansares Muñoz (Universidad Complutense de Madrid). All the standards were dissolved in doubly deionized water (18 MΩ cm) obtained with a Milli-Q Gradient system (Millipore, Watford, UK) and stored at 4 ºC in the dark. Working solutions were prepared daily by appropriate dilution of the stocks solutions at 1000 mg l⁻¹. For total element determination, nitric acid (65 mass %) and hydrogen peroxide (30 mass %) of Suprapur® grade (Merck, Darmstadt, Germany) were used for mineralization of the samples. Certified Reference Materials of dogfish muscle (DORM-2) and lobster hepatopancreas (TORT-2) (National Research Council Canada) were used for quality control assays. Reference materials were also used for lyophilized urine (Clinchek of urine control, Level II) and serum (Level I) (Recipe Chemicals, Munich, Germany). Digestive enzymes (porcine pepsin, porcine pancreatin, amylase) and bile salts (approx. 50% sodium cholate and 50% sodium deoxycholate) were obtained
from Sigma Aldrich (Sigma Aldrich, St Louis, MO, USA). The creatinine was determined with the Jaffe’s reaction using a commercial Kit (Spinreact, Barcelona, Spain).

Ammonium carbonate \((\text{NH}_4)_2\text{CO}_3\) (Fluka, Seelze, Germany), ammonium hydroxide (Sigma Aldrich, St Louis, MO, USA) and pyridine (Sigma Aldrich, St Louis, MO, USA) were used in the mobile phases for the chromatographic separation. Calibration of the mass spectrometer was daily performed using the standards kit from AB Sciex (AB Sciex, Foster City, CA, USA). Methanol was purchased from Aldrich (Aldrich, Steinheim, Germany). A tuning solution containing Li, In, U, Co and Ce (1 \(\mu\)g L\(^{-1}\) each) used for the ICP-MS optimization was purchased from Thermo Fisher Scientific (Bremen, Germany). Calibration of the mass spectrometer was daily performed using the standards kit from Applied Biosystems (AB Sciex, Foster City, CA, USA). Samples of raw anemones were purchased from a local restaurant (Southwest Spain). Cooked anemones were prepared from the whole animal that was battered in wheat flour and fried with olive oil.

2.2. Apparatus

Chromatographic separations were performed using a Model 1100 high-performance liquid chromatography (HPLC) pump (Agilent Technologies, Wilmington, DE, USA) as delivery system. Injections were performed using a Rheodyne valve with a 50 \(\mu\)L or 100 \(\mu\)L sample loop and arsenic species were separated using two chromatographic columns, based on anion exchange chromatography (25 cm x 4.6 mm, 5 \(\mu\)m Hamilton PRP X-100 column) and cation exchange chromatography (Supelcosil LC-SCX, 25 cm x 4.6 mm, 5 \(\mu\)m).

A centrifuge model 5804 R (Eppendorf AG, Hamburg, Germany) was used for the separation of phases. The extraction of the arsenic species was also carried out in a focused microwave model Discover equipped with an automatic arm model Explorer (CEM Corporation, Matthews, USA). On the other hand, the mineralization of the samples was carried out in a microwave model MARS5.
The elemental detection was performed in a Thermo Scientific XSeries2 ICP-MS fitted with a collision cell and nickel sampler and skimmer cones. The exit of the column was connected to the Miramist nebulizer (Burgener, Mississauga, Ontario, Canada) of the ICP-MS by means of polyetheretherketone (PEEK) tube. For total metal determination Micromist nebulizer (Glass Expansion, Romainnotier, Switzerland) was used. The standardless identification of arsenic species were performed by means of an HPLC coupled to a triple quadrupole instrument model API 2000 (AB Sciex, Concord, Canada) via an electrospray (ESI) interface using a PEEK tube.

2.3. Human exposure experiments and samples collection

The samples of human urine and plasma were collected from 5 volunteers before and after the consumption of 100 g of cooked anemones. Before consumption of the anemones, the analyses of urine samples were repeated until the presence of arsenic was minimal and constant. During the experiment other seafood was eliminated from the diet of the volunteers. The volunteers consumed cooked anemones in the morning, as well as all urines between the first 90 h, were collected. Serum samples were collected immediately after the ingestion of 100 g of cooked anemones and every 30 minutes to 2 hours. All the subjects have creatinine values inside the range of 0.3-3 g L\textsuperscript{-1}, recommended by the World Health Organization (WHO) for the acceptability of urine samples for biological monitoring [29] and all the data have been corrected with the creatine levels. On the other hand, blood samples from 5 volunteers were obtained by venipuncture of the antecubital region at Juan Ramón Jiménez Hospital (Huelva, Spain), before consumption of seafood and every 30 minutes during 2 hours after consumption. All samples were collected in BD Vacutainer SST II tubes with gel separator and Advance vacuum system, previously cooled in a refrigerator. The samples were immediately cooled and protected from light for 30 minutes to allow clot retraction to obtain serum after centrifugation (4000 g for 10 minutes). The serum was divided into aliquots in Eppendorf tubes.
and frozen at -80 °C until analysis. The investigation was performed after approval by the Ethical Committee of the University of Huelva (Spain).

2.4. Analytical procedures

2.4.1. Determination of total arsenic in human biological fluids and anemones

The serum samples were weighed (0.2000 g) into 5-mL teflon microwave vessels and 800 µL of a 4:1 (v/v) mixture of nitric acid and hydrogen peroxide was added. The mineralisation was carried out with a microwave accelerated reaction system at 400 W from ambient temperature ramped to 160 °C within 15 min, held at this temperature for 20 min and finally, the solutions were made up to 2 mL to reduce matrix effects. The anemone samples (raw or cooked) were pooled (12 anemones), lyophilized and weighed (0.5000 g) into 50-mL teflon microwave vessels and digested with 2.5 mL of a 4:1 (v/v) mixture of nitric acid and hydrogen peroxide. The mineralisation was carried out at 800 W from ambient temperature ramped to 160 °C within 15 min, held at this temperature for 30 min and finally, the solutions were made up to 10 mL. Urine samples were only diluted 1:5 with ultrapure water, centrifuged at 4.000g during 5 minutes and analyzed by ICP-MS. All the samples were filtered through Iso-Disc poly(vinylidene difluoride) filters (25 mm diameter, 0.45 µm pore-size) and analysed by ICP-MS using rhodium (1 µg L\(^{-1}\)) as the internal standard. All the analyses were repeated three times. A blank with the reagents used for total arsenic determination was run simultaneously to the sample preparation.

The detection limit of the instrumental method determined as three times the standard deviation of a blank (3σ) for total arsenic is 11 ng L\(^{-1}\). The recovery of total arsenic is 95 % (n = 5) of the certified value (18.0 ± 1.1 µg g\(^{-1}\)) in dogfish muscle and 98% (n=5) of the certified value (21.6 ± 1.8) in lobster hepatopancreas. The recovery of total arsenic is 101 % (n=5) of the certified value (82 ± 16 µg L\(^{-1}\)) in urine and 99% (n=5) of the certified value (11.3 ± 2.3 µg L\(^{-1}\)) in serum, using the same
microwave mineralization conditions previously described by serum and anemone samples.

2.4.2. Study of the bioaccessible fraction by in vitro gastrointestinal digestion

First to all, cooked anemones were pooled (12 anemones) and lyophilized during 24 hours. The in-vitro digestion was performed in triplicate by exact weighing of 1.0000 g of cooked anemone into 100 mL Erlemeyer flasks according to the procedure described elsewhere by Mounicou et al. [30]. Briefly, for a gastric digestion an aliquot of 10 mL of gastric juice (100 mg pepsin + 10mL 150 mM NaCl, pH 2.5) was added to a cooked anemone sample. Then, the sample was incubated during 4 hours at 37 °C with orbital-horizontal shaking at 150 rpm. On the other hand, for the intestinal extraction, pH was adjusted to 7.4, and 10 mL of intestinal juice was added (3% (w/v) pancreatin + 1% (w/v) amylase + 1.5 g L⁻¹ bile salts in ultrapure water). The sample was incubated during 4 hours at 37°C with orbital-horizontal shaking at 150 rpm. Then, the extract was centrifugated at 8.000g during 30 minutes at 4°C and the supernatant was separated for the total As determination and speciation.

For the determination of total arsenic bioaccessible fraction, 1.0000 g of the obtained extract was weighted and digested using the same procedure than for the total arsenic determination in anemones above described.

2.4.3. Sample Preparation and analysis by HPLC-ICP-MS and -MS

Urine samples were diluted 1:5 with ultrapure water, centrifuged at 4.000 g during 5 minutes, filtered through 0.45 µm and injected directly in the IC-ICP-MS or -MS for the analysis. On the other hand, 0.1000 g of serum samples were weighted and arsenic species were extracted by protein precipitation with 500 µl of a 2:1 (v/v) methanol/water mixture by vigorous vortex shaking during 5 minutes, followed by centrifugation at 4000 g during 10 min at 4 °C. The supernatant was evaporated
to dryness under a nitrogen stream and stored at -80 °C until analysis. The extract was reconstituted to 200µL with mobile phase, stirred, centrifuged again (4000 g, at 4 °C for 10 minutes) and finally, the clean supernatant was collected for the analysis.

The procedure for the speciation of arsenic species in raw and cooked *Anemonia sulcata* has been published elsewhere [28]. Briefly: 5 mL of a (1:1, v/v) methanol/water mixture was added to 0.1000 g of a lyophilized pool of 20 anemones (raw or cooked) and arsenic species were extracted using a focused microwave at 50 °C and 150 W during 5 minutes with magnetic stirring. After that, the extract was centrifuged at 10000 g during 10 minutes and the supernatant evaporated until dryness under vacuum at 50 °C in a rotary evaporator. Finally, 1 mL of ultrapure water was added, filtered through Iso-Disc poly-(vinylidene difluoride) filters (10-mm diameter, 0.2-µm pore size) and analyzed. All the filters were cleaned with 5 mL of the extracting solution before the use to avoid the contamination of the samples. The bioaccessible fraction of the arsenic species was obtained by exact weighing of 0.5000 g of the extract after gastrointestinal digestion that were extracted using the same procedure than for the serum samples, but reconstituted to 1000 µL with mobile phase. All the extracts were filtered through Iso-Disc poly-(vinylidene difluoride) filters (10-mm diameter, 0.2-µm pore size) to avoid column overloading or clogging and the filters were cleaned with 5 mL of ultrapure water before use to avoid the contamination of the samples.

The chromatographic separation and detection of arsenic species have been carried out using anion/cation exchange liquid chromatography coupled to ICP-MS. The analytical method for the chromatographic separations and detection has been previously optimized and published elsewhere [28]. Briefly, the separation of the arsenic species was performed by anion exchange chromatography, using as mobile phase 50 mM of (NH₄)₂CO₃ at pH = 8.5 (adjusted with 5 M ammonium hydroxide) flushed at 1.2 mL min⁻¹ through the column. Since AB, TETRA and TMAO coelute in this column, the separation was carried out using a cation exchange column with 20 mM pyridine at pH 2.5 as mobile phase at 1.2 mL min⁻¹. The HPLC-ICP-MS online coupling was
performed by connecting the outlet of the chromatographic column to ICP-MS nebulizer through a PEEK tube. The ICP-MS parameters using He as collision gas were optimized with an aqueous solution of 1 µg L\(^{-1}\) of As with 2 vol. % HCl and 4 mL min\(^{-1}\) of He was enough to avoid the interference of \(^{40}\text{Ar}^{35}\text{Cl}^+\). Signal intensity at m/z 77 was monitored and used to correct this interference. Table 1 shows the experimental conditions used in the HPLC-ICP-MS coupling.

For the identification of unknown arsenic species, the samples were also analyzed by organic mass spectrometry. Data were collected in positive ion mode (ES\(^+\)) and the mass analyzer scanned from m/z 50 to 400. The ionspray voltage (IS) was set at 5500 V and the declustering and focusing potentials (ring potentials) at 30 and 400 V, respectively. The curtain gas and nebulizer gas (high purity nitrogen) were fixed at 15 and 10 units, respectively. The ion energy (IE) was fixed at 0.5 V with a channel electron multiplier (CEM) of 2500 V. In the experiment, the entrance potential (EP) was set at 10 V and collision cell rod offset (RO2) at -20 V. The scan time was equal to 0.5 s. Data were treated with the manufacturer’s Analyst 1.4.1. Software. The collision energy and declustering potential in the product ion mode varied between the experiments and therefore was optimized as appropriate. In all the experiments, collision cell exit potential (CXP) was set at 2 V and collision gas (CAD) at 5 units. Optimal conditions for tandem mass spectrometry and ion transitions used for Multiple Reaction Monitoring (MRM) have been previously described elsewhere [28].

3. Results and discussion

3.1. Stability of the arsenic species in the samples

In order to ascertain the integrity of species and prevent (inter)conversion during the experiment, the stability in the samples under the storage conditions should be evaluated. Feldmann et al. [31] evaluated the stability of arsenic species in freshly collected urine samples and they found that the freshly collected urine samples are stable for at least 2 months when stored at temperatures under either 4 °C or -20 °C. Once the samples were obtained, they were stored at -80 °C in 10
different aliquots of 100 µL using Eppendorf tubes. Replicated aliquots of these samples were placed in separate eppendorf tubes and were supplemented with AB, As (III), As (V), MA V and DMA V (20 µg L⁻¹ as arsenic). The evaluation of arsenic species stability was carried out each fifteen days during two months by AEC-ICP-MS and CEC-ICP-MS using the procedures above described. The analysis was carried out by duplicate.

In general, processing or storage by freezing neither change the arsenic species concentration nor alter the speciation pattern greatly; however, a significant decrease of AB in urine and serum concentration after storage was observed. Decreased levels of AB were found at the end of the experiment that maybe related with degradation to DMA V during storage. Moreover, insignificant degradations of MA V and DMA V concentrations were observed after 45 days of storage at -80°C in both biological fluids (Table 2). In addition, increased levels of iAs V were observed along the storage time at -80 °C, more pronounced in urine that in serum. Finally, insignificant increase of As (III) was observed after 60 days from the beginning of the experience, similar results were obtained by Feldmann et al.[31]. In conclusion, low temperatures conditions (-80 °C) are suitable for the storage of human urine and serum samples for up to 2 months without substantial changes of arsenicals without the addition of any additive. It may be possible that with higher storage times, the stability of arsenic species varies with the sample matrix. A similar stability study for the anemones extracts were performed and published elsewhere [28].

3.2. Arsenic speciation and bioaccessible fraction in cooked Anemonia sulcata

The detection and quantification limits (LODs and LOQs, respectively) were calculated with the data generated in the linearity studies. After obtaining the calibration function, LOD and LOQ were calculated as the analyte concentration that corresponds to a signal equal to “a + 3 Sy/x” and “a + 10 Sy/x” respectively, where “a” is the origin ordinate, and “Sy/x” indicates the random errors in
the values for the slope and intercept. The detection limits using HPLC-ICP-MS are shown in the Table 3. Due to the absence of the standards, the concentration of AsC, DMA\textsuperscript{V} and GPAsC were calculated using the calibration curve of DMA\textsuperscript{V}, since these arsenicals have an arsenic atom per metabolite and have similar structures, therefore present similar ionization efficiency in the plasma making possible the quantification. In this study, the total concentration of arsenic in \textit{Anemonia sulcata} is 12.2 ± 0.54 and 6.62 ± 0.31 µg g\textsuperscript{-1} in raw and cooked samples, respectively (n=12 anemones pooled per group) suggesting that 54 % of the arsenic is lost after cooking. These concentrations are higher than those previously reported in these anemones (4.9 ± 0.21 and 1.7 ± 0.11 µg g\textsuperscript{-1} in raw and cooked samples, respectively) [28], which can be related with different arsenic concentrations in the water where they were captured. In this study, the dominant specie in cooked \textit{Anemonia sulcata} is AB representing about 48 % of the total arsenic as in most marine organisms [32]. AB represents the end point of the arsenic cycle in the marine ecosystems and it is largely inert, non-toxic and rapidly excreted [33]. The presence of DMA\textsuperscript{V} is also important and accounts for around 26 % of the total arsenic, followed by DMAS\textsuperscript{V} (12 %), AsC (5 %), TETRA (4 %), TMAO (2 %) and low concentrations of iAs\textsuperscript{V}, GPAsC and MA\textsuperscript{V}. Although inorganic arsenic, mainly as arsenate (iAs\textsuperscript{V}), is the predominant form of arsenic in seawater, inorganic compounds comprise only a small proportion of total cooked seafood sample (about 1-2 %) [34]. On the other hand, generally little or no MA\textsuperscript{V} and DMA\textsuperscript{V} is found in seafood, but DMA\textsuperscript{V} is the second abundant specie in these anemones. In this sense, trace levels of MA\textsuperscript{V} (0.4 % of the total) were detected in this organism but in the case of DMA\textsuperscript{V} the concentration of this arsenical was 1.7 (µg g\textsuperscript{-1}). Besides these methylated forms, TMAO and TETRA were also observed, which have been found in some species of marine organisms at trace levels [33], but may be major species in some mollusks [33]. In addition, DMAS\textsuperscript{V} has been previously reported in vegetation, animals and human beings [35-41] while GPAsC has been found in yelloweye mullet (\textit{Aldrichetta forsteri}) and mussels after oral administration of arsenuocholine [42]. However, the presence of GPAsC and DMAS\textsuperscript{V} in \textit{Anemonia}}
**sulcata** is curious since the former was not previously identified in a natural sample (without exposure to AsC) and the latter was never found in marine food before [28]. On the other hand, it is well known that AsC is a metabolic precursor of AB (48 % in the anemones) in marine organisms [43], but in this study low levels have been found (5%) in cooked anemones.

Figure 1 shows the chromatogram obtained by HPLC-CEC-ICP-MS from cooked anemones and bioaccessible extracts (Fig. 1a and 1b, respectively). As can be seen, nine main arsenic species were separated. In a previous work, only eight arsenic species were found in *Anemonia sulcata* [28], which can be explained by the low concentration of this new specie that made impossible the identification by organic mass spectrometry. This new arsenical has been identified in present work by HPLC-(CEC)-ESI-QqQ-MS as dimethylarsenoethanol (DMAE) (see next section). As can be seen in Table 3, around 95 % of the total arsenic is bioaccessible after in vitro gastric and intestinal digestion. In general, the bioaccessibility of all arsenic species is high, being the more remarkable As (V) and MA^V_. The percentages higher than 100 % may indicate the transformation of other species into them during the gastrointestinal digestion.

### 3.3. Speciation of arsenic in human urine and serum after consumption of cooked *Anemonia sulcata*

Arsenic metabolites were identified and quantified in urine and serum samples after the consumption of this seafood by HPLC-ICP-MS. Total arsenic concentration in human urine and serum from five volunteers was evaluated during five days before the ingestion of cooked anemones. All seafood products and other foods of daily consumption in which arsenic can occur at significant concentrations (e.g. rice with about 500 ng g⁻¹ of arsenic) [44] were avoided during the experiment, and the results (8 µg of total arsenic L⁻¹ of urine) were within the reference interval for people not
consuming seafood [45]. In the case of serum, the concentration of arsenic of the volunteers before the beginning of the experience was $1.92 \pm 0.32 \text{ ng As g}^{-1}$, which is also in good agreement with normal concentrations in previously published works. On the other hand, higher concentrations in human serum have been related by reduced renal excretion of arsenic or insufficient dialysis of the arsenic species [46, 47].

3.4. Human urine samples

Figure 2 shows typical HPLC-ICP-MS chromatograms (Fig. 2a and 2c with AEC, Fig. 2b and 2d with CEC) of an urine sample from one subject before (Fig. 2a and Fig. 2b) and 20 hours after consumption of the anemones (Fig. 2c and Fig. 2d). An unknown compound was determined in urine using HPLC-(CEC)-ICP-MS, which is non-detectable by HPLC-(AEC)-ICP-MS. This peak was observed in cooked anemone extracts when the same chromatography conditions are applied (Fig. 1), and was identified by HPLC-(CEC)-ESI-QqQ-MS as DMAE (Fig. 3). The presence of DMAE in urine may be explained by its presence in this seafood since DMAE has been found a significant metabolite in urine after ingestion of arsenosugars from seafood [48], but these arsenicals are not detected in *Anemonia sulcata*.

Before consumption of the anemones, AB and DMA$^V$ are the major arsenic metabolites in human urine, as stated by other authors [49]. The relative abundance of the arsenic species in urine along the experiment is represented in Figure 4 as a mean of the five volunteers, and corrected with creatinine levels. The standard deviations show that the concentrations of arsenic species in the urine samples from the different volunteers are similar among them. As can be observed, the relative abundance of As varies along the time, but after the first ten hours follows the order $AB \approx DMA^V > AsC > TETRA > TMAO > MA^V > As(V) > DMAE$, while the major specie in cooked anemones is $AB$ followed by $DMA^V > DMAS^V > AsC > TETRA > TMAO > As(V) > GPAAsC > MA^V > DMAE$ (Table 3, Fig. 1). Thus, the dominant specie in urine after the first 20 h is $AB$,
with a mean value of total AB excreted during this period of 140 µg As g⁻¹ of creatinine (Figure 4a), which accounts about 40 % of the total AB in 100 g of cooked anemones, and the concentration decreases to 23 µg As µg g⁻¹ of creatinine after 80 hours (88 % of the total AB in 100 g of cooked anemones is excreted during 90 hours). These results are in good agreement with other experiments after ingestion of shrimp, in which 66% of arsenobetaine is eliminated from the body after 36 hours [50]. Other arsenic species can be transformed into AB such as AsC, which after oral administration is rapidly absorbed or transformed into AB, with little or no degradation to inorganic arsenic, MAV, DMAV [43, 51]. As previously reported, DMAV is not totally eliminated from the body [52], which is in good agreement with the almost constant concentration determined along the experiment. About 130 % of DMAV present in 100 g of the anemones is excreted by urine during 90 h (408 µg g⁻¹ of creatinine) (Fig. 4b). This fact suggests the biotransformation of other arsenic species, like DMASV into DMAV, which is supported by the absence of the former in urine and its presence in Anemonia Sulcata. In addition, after in vitro gastrointestinal digestion, the concentration of As (V) is high, which can be also transformed into DMAV as previously described [25]. Other arsenicals are less abundant in urine as can be observed in Figures 4b and 4c, especially As (V), MAV and DMAE. Some of them are quickly eliminated from the body, such as AsC, TETRA, TMAO and As (V), or progressively, like MAV and DMAE. In this experiment, 89 % and 93 % of the TMAO and TETRA present in 100 g of cooked anemones, respectively, are excreted by urine during the period 10-30 hours without biotransformation (Fig. 4b). The excretion of DMAE in urine is slower than for the discussed arsenicals and about 80% of the total DMAE ingested is excreted during the period 20-50 hours (Fig. 4c). In summary, about 95 % of total arsenic in cooked anemones is bioaccessible (Table 3) and 85% of the amount ingested is eliminated from the body by urine in 90 h.

3.5. Human serum samples
Figure 5 shows typical HPLC-ICP-MS overlapped chromatograms of serum samples before consumption and 1 and 2 hours after, using AEC (Fig. 5a) and CEC (Fig. 5b). In contrast to the results obtained for the anemones and urine, in human serum samples, only AB, As (V), MA\textsuperscript{V}, DMA\textsuperscript{V} and AsC are detected. As can be observed in the chromatograms (Fig. 5a and b), the peak at 2.5 minutes in CEC (Fig. 5b) is about 5 fold higher than the peak at 11.7 minutes in AEC (Fig. 5a). The explanation is the $\text{^{40}Ar^{35}Cl}$ interference, which coelutes with As (V) in CEC (Fig. 5b), as suggested by other authors [53].

As in the case of urine, human serum samples from 5 volunteers were collected before and after the consumption of 100 g of cooked anemones every 30 minutes. The mean total concentration of arsenic in serum before the consumption is $1.92 \pm 0.32 \text{ ng g}^{-1}$ which accounts for $81 \pm 17$ % of the total arsenic determined as sum of the species. The reason may be that As (III) is likely bound to proteins and peptides in serum [54, 55] which precipitate during the methanol/water extraction procedure, thus causing losses of As (III) in the pellet upon centrifugation [56].

Fig. 6 shows the evolution of the different arsenicals during 2 hours after the ingestion. After 30 minutes from the ingestion, DMA\textsuperscript{V} is the most abundant in serum, followed by AB, MA\textsuperscript{V} and AsC. After this period of time, the amount of AsC in human serum is constant during 2 hours (Fig. 6), but the concentration of other arsenicals increased progressively with a common pattern and As (V) can be only detected after 1 hour. This fact supports the idea that in humans, after the absorption of As (V) by the gastrointestinal tract, it is quickly reduced to As (III) in the blood, which easily cross the cell membrane at physiological pH and is methylated and later oxidized [57].

4. Conclusions

Although the obvious interest about the complete knowledge of human arsenic metabolism, exposure experiments are very difficult and some organisms have been proved to be poor models because of their different behavior compared to humans, like in the case of rats. A bioaccessibility
study of an arsenic containing seafood, namely *Anemonia sulcata*, and later monitoring of human biological fluids after consumption have been used in this paper to show the real exposure to arsenic, providing a deeper insight into the arsenic metabolism of humans after the consumption of these sea organisms that are not usually studied. Speciation in tissue extracts and human fluids (urine and serum) was carried out by anion/cation exchange chromatography with inductively coupled plasma mass spectrometry (IC)-ICP-MS). The ICP-MS constitutes a powerful technique for the speciation of arsenic since it provides very low detection limits, tolerance to matrix and large linearity range. In addition, the use of the collision cell eliminates suitably the interferences on the arsenic signal some of them caused by the complex matrix that has been analyzed. However, this technique should be combined with organic mass spectrometry since in real samples the presence of unidentified arsenicals is very common. The use of organic mass spectrometry in this work allowed the standarless identification of AsC, GPAsC, DMAS\textsuperscript{V} and DMAE in the analyzed samples.

**Acknowledments**

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References


[29] WHO, Biological Monitoring of chemical Exposure in the Workplace, 1996, vol 1


Figure 1. (a) Typical HPLC-CEC-ICP-MS chromatogram of cooked anemones. (b) As-metabolites in the bioaccessible extract of cooked anemones. Chromatographic conditions are given in table 1.
Figure 2. Speciation of arsenic in human urine (n= 3) before (a,b) and 20 hours after consumption of cocked anemones (c,d) by HPLC-(CEC)-ICP-MS (a,c) and HPLC-(AEC)-ICP-MS (b,d). Chromatographic conditions are given in Table 1.
Figure 3. (a) MS/MS spectrum of peak at 4.9 minutes obtained from an human urine 20 hours after consumption of anemones using by HPLC-(CEC)-ESI-QqQ-MS (Multiple Channel Accumulation (MCA) = 20, Collision Energy (CE) = 30V and Declustering Potential (DP) = 80V). (b) MRM chromatogram of the 167→121 m/z transition obtained from the same urine sample by HPLC-(CEC)-ESI-QqQ-MS (CE=25V and DP=80V). The chromatographic conditions are given in Table 1.
Figure 4. Relative concentrations of arsenic metabolites in urinary samples (n = 5 volunteers) normalized against the concentration of creatinine, before and after consumption of 100 g of cooked anemones per volunteer.
Figure 5. Speciation of arsenic in a serum sample (n= 3) before and after consumption of cocked anemones by (a) HPLC-AEC-ICP-MS and (b) HPLC-CEC-ICP-MS. Chromatographic conditions are given in Table 1.
Figure 6. Relative concentration of arsenic metabolites in serum samples collected for 5 volunteers before and after consumption of 100 g of cooked anemones per volunteer.

Table 1. Experimental conditions used in HPLC-ICP-MS.

<table>
<thead>
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<th>HPLC conditions</th>
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<td>Anionic Exchange Chromatography</td>
<td>Column</td>
<td>Hamilton PRP X-100 (250 mm × 4.6 mm × 5 µm)</td>
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<td></td>
<td>Mobile phase</td>
<td>Ammonium carbonate, 50 mM (pH 8.5)</td>
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<td>Flow-rate</td>
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<tr>
<td></td>
<td>Injection volume</td>
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<tr>
<td>Cationic Exchange Chromatography</td>
<td>Column</td>
<td>Supelcosil SCX (250 mm × 4.6 mm × 5 µm)</td>
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<tr>
<td></td>
<td>Mobile phase</td>
<td>Pyridine, 20 mM (pH 2.5)</td>
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<tr>
<td></td>
<td>Flow-rate</td>
<td>1.2 mL min⁻¹</td>
</tr>
<tr>
<td></td>
<td>Injection volume</td>
<td>50 µL</td>
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<tr>
<th>ICP-MS conditions</th>
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<tr>
<td>Forward power</td>
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<tr>
<td>Plasma gas flow rate</td>
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<td>Auxiliary gas flow rate</td>
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</tr>
<tr>
<td>Carrier gas flow rate</td>
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<td>Sampling and skimmer cones</td>
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<td>He flow</td>
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<tr>
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<tr>
<td>Focus</td>
<td>-9.0 V</td>
<td></td>
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<tr>
<td>Dwell time</td>
<td>0.1 per isotope</td>
<td></td>
</tr>
<tr>
<td>Isotopes monitored</td>
<td>⁷⁵As, ⁷⁷ArCl⁺</td>
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Table 2. Evaluation of arsenic species concentrations in human urine and serum samples during storage period.

<table>
<thead>
<tr>
<th>Human urine</th>
<th>As(III) (%)</th>
<th>As(V) (%)</th>
<th>MA(^\text{V}) (%)</th>
<th>DMA(^\text{V}) (%)</th>
<th>AB (%)</th>
<th>As(III) (%)</th>
<th>As(V) (%)</th>
<th>MA(^\text{V}) (%)</th>
<th>DMA(^\text{V}) (%)</th>
<th>AB (%)</th>
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<td>Volunteer urine samples stored at two months at -80°C</td>
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<tr>
<td>15 Days</td>
<td>99</td>
<td>&lt;LOD</td>
<td>100</td>
<td>101</td>
<td>99</td>
<td>98</td>
<td>101</td>
<td>102</td>
<td>101</td>
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<td>96</td>
<td>104</td>
<td>98</td>
<td>97</td>
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<td>98</td>
<td>&lt;LOD</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>99</td>
<td>111</td>
<td>97</td>
<td>95</td>
<td>96</td>
</tr>
<tr>
<td>60 Days</td>
<td>94</td>
<td>&lt;LOD</td>
<td>91</td>
<td>93</td>
<td>89</td>
<td>105</td>
<td>118</td>
<td>93</td>
<td>91</td>
<td>88</td>
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<td>Spiked volunteer urine samples stored at two months at -80°C</td>
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<td>99</td>
<td>110</td>
<td>95</td>
<td>96</td>
<td>93</td>
</tr>
</tbody>
</table>

LOD of As (III) and As (V) are 3.5 and 2.4 ng As g\(^{-1}\), respectively.

Table 3. Arsenic speciation in cooked anemones and bioaccessible fraction by in vitro digestion.

<table>
<thead>
<tr>
<th>Cooked anemone (µg As g(^{-1}))</th>
<th>As(III) (%)</th>
<th>As(V) (%)</th>
<th>MA(^\text{V}) (%)</th>
<th>DMA(^\text{V}) (%)</th>
<th>AB (%)</th>
<th>AsC (%)</th>
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<tbody>
<tr>
<td>Standard deviation (µg As g(^{-1}))</td>
<td>&lt;LOD</td>
<td>0.086</td>
<td>0.027</td>
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<td>3.2</td>
<td>0.34</td>
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<td>Bioaccessible extract from cooked anemone (µg As g(^{-1}))</td>
<td>&lt;LOD</td>
<td>0.11</td>
<td>0.031</td>
<td>1.5</td>
<td>3.1</td>
<td>0.32</td>
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<td>Standard deviation (µg As g(^{-1}))</td>
<td>-----</td>
<td>0.005</td>
<td>0.002</td>
<td>0.09</td>
<td>0.4</td>
<td>0.03</td>
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<tr>
<td>Limit of detection (LOD, ng As g(^{-1}))</td>
<td>3.5</td>
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<td>128</td>
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<th>DMAS(^\text{V})</th>
<th>GPAsC</th>
<th>TMAO</th>
<th>TETRA</th>
<th>TOTAL As (as sum of the species)</th>
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<td>0.25</td>
<td>6.4</td>
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