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

M. Contreras-Acuña[a,b,c], T. García-Barrera[a,b,c], M.A. García-Sevillano[a,b,c], and J.L. Gómez-Ariza[a,b,c]*

[a]Department of Chemistry and Materials Science, Faculty of Experimental Sciences, University of Huelva, Campus de El Carmen, 21007-Huelva, Spain

[b]Research Center on Health and Environment (CYSMA). University of Huelva. Spain

[c]International Campus of Excellence on Agrofood (ceiA3). University of Huelva. Spain

CORRESPONDING AUTHOR:

e-mail: tamara@dqcm.uhu.es.

Tel.: +34 959 219962

Fax: +34 959 219942

ABSTRACT. The present paper reports for the first time the bioaccesibility of arsenic species in *Anemonia sulcata* and its metabolization 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

22 85 % of the amount ingested is eliminated from the body by urine in 90 h. The relative abundance of
23 As in urine varies along the time, but after the first ten hours follows the order arsenobetaine
24 (AB)≈DMA^V (dimethylarsinate)>arsenocholine (AsC)> tetra-methyl-arsonium (TETRA)>trimethyl-
25 arsine oxide (TMAO)> MA^V (monomethylarsonate)>iAs^V> dimethylarsenoethanol (DMAE), while
26 the major specie in cooked anemones is AB followed by DMA^V> dimethylmonothioarsinic acid
27 ($DMAS^V$)>AsC>TETRA>TMAO>iAs^V>glycerylphosphorylarsenocholine(GPAsC)>MA^V>DMA.
28 After 30 minutes from the ingestion, DMA^V is the most abundant in human serum, followed by AB,
29 MA^V and AsC. Finally, the use of organic mass spectrometry allowed the standarless identification
30 of AsC, GPAsC, $DMAS^V$ and DMAE.

31

32 KEYWORDS. Arsenic, speciation, *Anemonia sulcata*, seafood, bioaccessibility, mass spectrometry

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34 # 1. Introduction

35 Arsenic has been recognized as a global toxin that affects human health [1] and can be the 36 promoter of some types of cancers such as skin, lung, urinary bladder and liver [2]. Humans can be 37 exposed to arsenic by contaminated water, soil, atmosphere and food, especially seafood [3], as well 38 as products or supplements based on algae (especially *Hijiki seaweed*) and cereals or cereals derived 39 foods [4]. The levels of arsenic in marine organisms can vary widely, but almost samples fall within 40 the range of about 5-100 mg As kg^{-1} dry mass while terrestrial foods, in contrast almost invariably, 41 have low levels of arsenic with most samples having less than 0.05 mg As kg^{-1} dry mass, except rice 42 that can typically contain 0.1-0.4 mg As kg^{-1} dry mass, 500-1000 times lower than in seafood [3]. In 43 addition, in some countries, such as, Japan, China and Korea, food from marine sources constitutes 44 an important part of the diet and a series of studies has been undertaken to determine the chemical 45 form of arsenic in the organisms that contribute to the human diet [5].

46

47 On the other hand, As toxicity is strongly related to the chemical species in which it is present 48 and seafood, that is the main source of total As in the human diet, contains mainly organic As species 49 which are less toxic than inorganic arsenic (iAs). In mammals, inorganic forms of arsenic (arsenate 50 (As (V)) and arsenite (As (III)) are more toxic, while methylated forms, MA^V (monomethylarsonate) 51 and DMA^V (dimethylarsinate), are considered only moderately toxic [6, 7]. In addition, other arsenic 52 species, like trimethyl-arsine oxide (TMAO) and tetra-methyl-arsonium (TETRA) are also 53 considered moderately toxic, whereas arsenobetaine (AB), arsenocholine (AsC) and other 54 arsenosugars (AsS) show no toxicity [8]. In addition, As (III) is more toxic than (As (V)) most 55 probably due to enhanced cellular uptake and accumulation of the former (LD50 for mice are 4.5 mg 56 kg^{-1} and 14-18 mg kg^{-1} for the arsenite and arsenate, respectively) [9, 10]. Metabolites of As (III), in 57 particular trivalent methylated metabolites, are considered the main determinant of arsenic-induced

toxicities [11], as they have a high binding affinity for sulphydryl 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^{III} and DMA^{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 arsenocholine [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 gluthation, 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

83 significantly [3, 18, 21]. Moreover, DMA^V is the major metabolite produced from inorganic arsenic,
84 and the intermediates produced *in route* to DMA^V are thought to play a role in arsenic´s mode of
85 toxic action [22]. Thus, the possibility exists for inorganic arsenic and arsenosugars to have at least
86 some common intermediate metabolites, in which case arsenosugars might also be capable of
87 producing toxic effects [3]. Like in the case of arsenosugars and iAs, arsenolipids are also converted
88 to DMA^V by humans [23]. Then, although the biomethylation of iAs has been considered as a
89 detoxification mechanism during the last decades, intermediate toxic methylated trivalent arsenicals
90 make it as an intoxication process [24, 25]. On the other hand, thioarsenicals are new-found sulfur
91 containing arsenic metabolites, which has been identified in urine of animals as well as in marine
92 organisms. Methylation of inorganic arsenic resulted in the formation of thiolated metabolites;
93 dimethylarsinothioic acid ($DMAS^V$), dimethyldithioarsinic acid ($DMDTA^V$) and
94 monomethylmonothioarsonic acid ($MMMTA^V$) commonly detected in mammals urine (including
95 human) after long term exposure to inorganic or other arsenic species (MA^V, DMA^V or TMAO) [26].
96 However, how these thioarsenicals are produced during the metabolism of As (III) and the source of
97 sulfur remains unknown [26].
98
99 In order to study the metabolism in humans after food consumption it is important to know the
100 effect of cooking on arsenic species in the food, which can considerably change the total content of
101 arsenic and the ratios between species. Traditional washing and soaking of *Hizikia fusiforme*
102 seaweed, which has very high inorganic arsenic contents, may reduce the contents by up to 60% [27]
103 that is in good agreement with the 64 % of total arsenic lost in cooked *Anemonia sulcata*, which is
104 battered in flour and fried in olive oil [28]. *Anemonia sulcata* is a marine organism very appreciated
105 delicacy in Spain, especially in the south, where it is served as delicious meal. The speciation of
106 arsenic shows that DMA^V is the dominant specie in *Anemonia sulcata* followed by AB [28].
107

108 In the present paper, speciation of arsenic has been carried out in human urine and serum after
109 consumption of the anemones using a combination of inductively coupled plasma mass spectrometry
110 and organic mass spectrometry with anion/cation exchange high performance liquid chromatography
111 ((IC)-ICP-MS and –MS). The speciation data allows to deep inside into arsenic metabolism in human
112 body after the consumption of this particular food that contains unusual arsenic species as GPAsC
113 and $DMAS^V$.

114

115 2. Experimental

116 2.1. Standard solutions, reagents and seafood samples

117 Analytical grade reagents were used throughout. The chemical standards used were AB (95
118 %, Fluka, Steinheim, Germany), As (III) and As(V) (99%, Merck, Darmstadt, Germany), DMA, MA,
119 TETRA and TMAO (99%, Supelco, Bellefonte, USA). Tetramethylarsonium ion (TETRA) and
120 trimethylarsine oxide (TMAO) (Sigma Aldrich, Steinheim, Germany) were donated by Dr. Riansares
121 Muñoz (Universidad Complutense de Madrid). All the standards were dissolved in doubly deionized
122 water (18 MΩ cm) obtained with a Milli-Q Gradient system (Millipore, Watford, UK) and stored at 4
123 ºC in the dark. Working solutions were prepared daily by appropriate dilution of the stocks solutions
124 at 1000 mg l^{-1}. For total element determination, nitric acid (65 mass %) and hydrogen peroxide (30
125 mass %) of Suprapur® grade (Merck, Darmstadt, Germany) were used for mineralization of the
126 samples. Certified Reference Materials of dogfish muscle (DORM-2) and lobster hepatopancreas
127 (TORT-2) (National Research Council Canada) were used for quality control assays. Reference
128 materials were also used for lyophilized urine (Clinchek of urine control, Level II) and serum (Level
129 I) (Recipe Chemicals, Munich, Germany). Digestive enzymes (porcine pepsin, porcine pancreatin,
130 amylase) and bile salts (approx. 50% sodium cholate and 50% sodium deoxycholate) were obtained

131 from Sigma Aldrich (Sigma Aldrich, St Louis, MO, USA). The creatinine was determined with the
132 Jaffe´s reaction using a commercial Kit (Spinreact, Barcelona, Spain).

133 Ammonium carbonate $(NH_4)_2CO_3$ (Fluka, Seelze, Germany), ammonium hydroxide (Sigma
134 Aldrich, St Louis, MO, USA) and pyridine (Sigma Aldrich, St Louis, MO, USA) were used in the
135 mobile phases for the chromatographic separation. Calibration of the mass spectrometer was daily
136 performed using the standards kit from AB Sciex (AB Sciex, Foster City, CA, USA). Methanol was
137 purchased from Aldrich (Aldrich, Steinheim, Germany). A tuning solution containing Li, In, U, Co
138 and Ce (1 μg L^{-1} each) used for the ICP-MS optimization was purchased from Thermo Fisher
139 Scientific (Bremen, Germany). Calibration of the mass spectrometer was daily performed using the
140 standards kit from Applied Biosystems (AB Sciex, Foster City, CA, USA). Samples of raw
141 anemones were purchased from a local restaurant (Southwest Spain). Cooked anemones were
142 prepared from the whole animal that was battered in wheat flour and fried with olive oil.

143

144 ## 2.2. Apparatus

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

151

152 A centrifuge model 5804 R (Eppendorf AG, Hamburg, Germany) was used for the separation
153 of phases. The extraction of the arsenic species was also carried out in a focused microwave model
154 Discover equipped with an automatic arm model Explorer (CEM Corporation, Matthews, USA). On
155 the other hand, the mineralization of the samples was carried out in a microwave model MARS5.

156 The elemental detection was performed in a Thermo Scientific XSeries2 ICP-MS fitted with a
157 collision cell and nickel sampler and skimmer cones. The exit of the column was connected to the
158 Miramist nebulizer (Burgener, Mississauga, Ontario, Canada) of the ICP-MS by means of
159 polyetheretherketone (PEEK) tube. For total metal determination Micromist nebulizer (Glass
160 Expansion, Romainnotier, Switzerland) was used. The standardless identification of arsenic species
161 were performed by means of an HPLC coupled to a triple quadrupole instrument model API 2000
162 (AB Sciex, Concord, Canada) via an electrospray (ESI) interface using a PEEK tube.

163

164 ## 2.3. Human exposure experiments and samples collection

165

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

181 and frozen at -80 °C until analysis. The investigation was performed after approval by the Ethical
182 Committee of the University of Huelva (Spain).
183

184 ## 2.4. Analytical procedures

185 ### 2.4.1. Determination of total arsenic in human biological fluids and anemones

186 The serum samples were weighed (0.2000 g) into 5-mL teflon microwave vessels and 800 µL
187 of a 4:1 (v/v) mixture of nitric acid and hydrogen peroxide was added. The mineralisation was
188 carried out with a microwave accelerated reaction system at 400 W from ambient temperature
189 ramped to 160 °C within 15 min, held at this temperature for 20 min and finally, the solutions were
190 made up to 2 mL to reduce matrix effects. The anemone samples (raw or cooked) were pooled (12
191 anemones), lyophilized and weighed (0.5000 g) into 50-mL teflon microwave vessels and digested
192 with 2.5 mL of a 4:1 (v/v) mixture of nitric acid and hydrogen peroxide. The mineralisation was
193 carried out at 800 W from ambient temperature ramped to 160 °C within 15 min, held at this
194 temperature for 30 min and finally, the solutions were made up to 10 mL. Urine samples were only
195 diluted 1:5 with ultrapure water, centrifuged at 4.000g during 5 minutes and analyzed by ICP-MS.
196 All the samples were filtered through Iso-Disc poly(vinylidene difluoride) filters (25 mm diameter,
197 0.45 µm pore-size) and analysed by ICP-MS using rhodium (1 µg L^{-1}) as the internal standard. All
198 the analyses were repeated three times. A blank with the reagents used for total arsenic determination
199 was run simultaneously to the sample preparation.

200 The detection limit of the instrumental method determined as three times the standard deviation
201 of a blank (3σ) for total arsenic is 11 ng L^{-1}. The recovery of total arsenic is 95 % (n = 5) of the
202 certified value (18.0 ± 1.1 µg g^{-1}) in dogfish muscle and 98% (n=5) of the certified value (21.6 ± 1.8)
203 in lobster hepatopancreas. The recovery of total arsenic is 101 % (n=5) of the certified value (82 ± 16
204 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

205 microwave mineralization conditions previously described by serum and anemone samples.

206

207 ### 2.4.2. Study of the bioaccessible fraction by in vitro gastrointestinal digestion

208 First to all, cooked anemones were pooled (12 anemones) and lyophilized during 24 hours.
209 The in-vitro digestion was performed in triplicate by exact weighing of 1.0000 g of cooked anemone
210 into 100 mL Erlemeyer flasks according to the procedure described elsewhere by Mounicou et al.
211 [30]. Briefly, for a gastric digestion an aliquot of 10 mL of gastric juice (100 mg pepsin + 10mL 150
212 mM NaCl, pH 2.5) was added to a cooked anemone sample. Then, the sample was incubated during
213 4 hours at 37 ºC with orbital-horizontal shaking at 150 rpm. On the other hand, for the intestinal
214 extraction, pH was adjusted to 7.4, and 10 mL of intestinal juice was added (3% (w/v) pancreatin +
215 1% (w/v) amylase + 1.5 g L^{-1} bile salts in ultrapure water). The sample was incubated during 4 hours
216 at 37ºC with orbital-horizontal shaking at 150 rpm. Then, the extract was centrifugated at 8.000g
217 during 30 minutes at 4ºC and the supernatant was separated for the total As determination and
218 speciation.

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

222

223 ### 2.4.3. Sample Preparation and analysis by HPLC-ICP-MS and -MS

224 Urine samples were diluted 1:5 with ultrapure water, centrifuged at 4.000 g during 5 minutes,
225 filtered through 0.45 µm and injected directly in the IC-ICP-MS or -MS for the analysis. On the
226 other hand, 0.1000 g of serum samples were weighted and arsenic species were extracted by protein
227 precipitation with 500 µl of a 2:1 (v/v) methanol/water mixture by vigorous vortex shaking during 5
228 minutes, followed by centrifugation at 4000 g during 10 min at 4 °C. The supernatant was evaporated

229 to dryness under a nitrogen stream and stored at -80 ºC until analysis. The extract was reconstituted
230 to 200µL with mobile phase, stirred, centrifuged again (4000 g, at 4 °C for 10 minutes) and finally,
231 the clean supernatant was collected for the analysis.

232 The procedure for the speciation of arsenic species in raw and cooked *Anemonia sulcata* has
233 been published elsewhere [28]. Briefly: 5 mL of a (1:1, v/v) methanol/water mixture was added to
234 0.1000 g of a lyophilized pool of 20 anemones (raw or cooked) and arsenic species were extracted
235 using a focused microwave at 50 ºC and 150 W during 5 minutes with magnetic stirring. After that,
236 the extract was centrifuged at 10000 g during 10 minutes and the supernatant evaporated until
237 dryness under vacuum at 50 ºC in a rotary evaporator. Finally, 1 mL of ultrapure water was added,
238 filtered through Iso-Disc poly-(vinylidene difluoride) filters (10-mm diameter, 0.2-µm pore size) and
239 analyzed. All the filters were cleaned with 5 mL of the extracting solution before the use to avoid the
240 contamination of the samples. The bioaccessible fraction of the arsenic species was obtained by exact
241 weighing of 0.5000 g of the extract after gastrointestinal digestion that were extracted using the same
242 procedure than for the serum samples, but reconstituted to 1000 µL with mobile phase. All the
243 extracts were filtered through Iso-Disc poly-(vinylidene difluoride) filters (10-mm diameter, 0.2-µm
244 pore size) to avoid column overloading or clogging and the filters were cleaned with 5 mL of
245 ultrapure water before use to avoid the contamination of the samples

246 The chromatographic separation and detection of arsenic species have been carried out using
247 anion/cation exchange liquid chromatography coupled to ICP-MS. The analytical method for the
248 chromatographic separations and detection has been previously optimized and published elsewhere
249 [28]. Briefly, the separation of the arsenic species was performed by anion exchange
250 chromatography, using as mobile phase 50 mM of $(NH_4)_2CO_3$ at pH = 8.5 (adjusted with 5 M
251 ammonium hydroxide) flushed at 1.2 mL min^{-1} through the column. Since AB, TETRA and TMAO
252 coelute in this column, the separation was carried out using a cation exchange column with 20 mM
253 pyridine at pH 2.5 as mobile phase at 1.2 mL min^{-1}. The HPLC-ICP-MS online coupling was

254 performed by connecting the outlet of the chromatographic column to ICP-MS nebulizer through a
255 PEEK tube. The ICP-MS parameters using He as collision gas were optimized with an aqueous
256 solution of 1 µg L^{-1} of As with 2 vol. % HCl and 4 mL min^{-1} of He was enough to avoid the
257 interference of $^{40}Ar^{35}Cl^{+}$. Signal intensity at m/z 77 was monitored and used to correct this
258 interference. Table 1 shows the experimental conditions used in the HPLC-ICP-MS coupling.

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

271 # 3. Results and discussion

272 ## 3.1. Stability of the arsenic species in the samples

273 In order to ascertain the integrity of species and prevent (inter)conversion during the
274 experiment, the stability in the samples under the storage conditions should be evaluated. Feldmann
275 et al. [31] evaluated the stability of arsenic species in freshly collected urine samples and they found
276 that the freshly collected urine samples are stable for at least 2 months when stored at temperatures
277 under either 4 °C or -20 °C. Once the samples were obtained, they were stored at -80 ºC in 10

278 different aliquots of 100 µL using Eppendorf tubes. Replicated aliquots of these samples were placed
279 in separate eppendorf tubes and were supplemented with AB, As (III), As (V), MA^V and DMA^V (20
280 µg L^{-1} as arsenic). The evaluation of arsenic species stability was carried out each fifteen days during
281 two months by AEC-ICP-MS and CEC-ICP-MS using the procedures above described. The analysis
282 was carried out by duplicate.

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

296

297 ## 3.2. Arsenic speciation and bioaccessible fraction in cooked Anemonia sulcata

298 The detection and quantification limits (LODs and LOQs, respectively) were calculated with
299 the data generated in the linearity studies. After obtaining the calibration function, LOD and LOQ
300 were calculated as the analyte concentration that corresponds to a signal equal to “a + 3 Sy/x” and “a
301 + 10 Sy/x” respectively, where “a” is the origin ordinate, and “Sy/x” indicates the random errors in

302 the values for the slope and intercept. The detection limits using HPLC-ICP-MS are shown in the
303 Table 3. Due to the absence of the standards, the concentration of AsC, $DMAS^V$ and GPAsC were
304 calculated using the calibration curve of DMA^V, since these arsenicals have an arsenic atom per
305 metabolite and have similar structures, therefore present similar ionization efficiency in the plasma
306 making possible the quantification. In this study, the total concentration of arsenic in *Anemonia*
307 *sulcata* is 12.2 ± 0.54 and 6.62 ± 0.31 μg g^{-1} in raw and cooked samples, respectively (n=12
308 anemones pooled per group) suggesting that 54 % of the arsenic is lost after cooking. These
309 concentrations are higher than those previously reported in these anemones (4.9 ± 0.21 and 1.7 ±
310 0.11 μg g^{-1} in raw and cooked samples, respectively) [28], which can be related with different
311 arsenic concentrations in the water where they were captured. In this study, the dominant specie in
312 cooked *Anemonia sulcata* is AB representing about 48 % of the total arsenic as in most marine
313 organisms [32]. AB represents the end point of the arsenic cycle in the marine ecosystems and it is
314 largely inert, non-toxic and rapidly excreted [33]. The presence of DMA^V is also important and
315 accounts for around 26 % of the total arsenic, followed by $DMAS^V$ (12 %), AsC (5 %), TETRA (4
316 %), TMAO (2 %) and low concentrations of iAs^V, GPAsC and MA^V. Although inorganic arsenic,
317 mainly as arsenate (iAs^V), is the predominant form of arsenic in seawater, inorganic compounds
318 comprise only a small proportion of total cooked seafood sample (about 1-2 %) [34]. On the other
319 hand, generally little or no MA^V and DMA^V is found in seafood, but DMA^V is the second abundant
320 specie in these anemones. In this sense, trace levels of MA^V (0.4 % of the total) were detected in this
321 organism but in the case of DMA^V the concentration of this arsenical was 1.7 (μg g^{-1}). Besides these
322 methylated forms, TMAO and TETRA were also observed, which have been found in some species
323 of marine organisms at trace levels [33], but may be major species in some mollusks [33]. In
324 addition, $DMAS^V$ has been previously reported in vegetation, animals and human beings [35-41]
325 while GPAsC has been found in yelloweye mullet (*Aldrichetta forsteri*) and mussels after oral
326 administration of arsenocholine [42]. However, the presence of GPAsC and $DMAS^V$ in *Anemonia*

327 *sulcata* is curious since the former was not previously identified in a natural sample (without
328 exposure to AsC) and the latter was never found in marine food before [28]. On the other hand, it is
329 well known that AsC is a metabolic precursor of AB (48 % in the anemones) in marine organisms
330 [43], but in this study low levels have been found (5%) in cooked anemones.

331

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

342

343 ## 3.3. Speciation of arsenic in human urine and serum after consumption of
344 ## cooked Anemonia sulcata

345 Arsenic metabolites were identified and quantified in urine and serum samples after the
346 consumption of this seafood by HPLC-ICP-MS. Total arsenic concentration in human urine and
347 serum from five volunteers was evaluated during five days before the ingestion of cooked anemones.
348 All seafood products and other foods of daily consumption in which arsenic can occur at significant
349 concentrations (e.g. rice with about 500 $ng.g^{-1}$ of arsenic) [44] were avoided during the experiment,
350 and the results (8 μg of total arsenic L^{-1} of urine) were within the reference interval for people not

351 consuming seafood [45]. In the case of serum, the concentration of arsenic of the volunteers before
352 the beginning of the experience was 1.92 ± 0.32 ng As g^{-1}, which is also in good agreement with
353 normal concentrations in previously published works. On the other hand, higher concentrations in
354 human serum have been related by reduced renal excretion of arsenic or insufficient dialysis of the
355 arsenic species [46, 47].

356

357 ### 3.4. Human urine samples

358 Figure 2 shows typical HPLC-ICP-MS chromatograms (Fig. 2a and 2c with AEC, Fig. 2b and
359 2d with CEC) of an urine sample from one subject before (Fig. 2a and Fig. 2b) and 20 hours after
360 consumption of the anemones (Fig. 2c and Fig. 2d). An unknown compound was determined in urine
361 using HPLC-(CEC)-ICP-MS, which is non-detectable by HPLC-(AEC)-ICP-MS. This peak was
362 observed in cooked anemone extracts when the same chromatography conditions are applied (Fig. 1),
363 and was identified by HPLC-(CEC)-ESI-QqQ-MS as DMAE (Fig. 3). The presence of DMAE in
364 urine may be explained by its presence in this seafood since DMAE has been found a significant
365 metabolite in urine after ingestion of arsenosugars from seafood [48], but these arsenicals are not
366 detected in *Anemonia sulcata*.

367 Before consumption of the anemones, AB and DMA^V are the major arsenic metabolites in
368 human urine, as stated by other authors [49]. The relative abundance of the arsenic species in urine
369 along the experiment is represented in Figure 4 as a mean of the five volunteers, and corrected with
370 creatinine levels. The standard deviations show that the concentrations of arsenic species in the urine
371 samples from the different volunteers are similar among them. As can be observed, the relative
372 abundance of As varies along the time, but after the first ten hours follows the order
373 AB≈DMA^V>AsC>TETRA>TMAO>MA^V>As(V)>DMAE, while the major specie in cooked
374 anemones is AB followed by DMA^V>$DMAS^V$>AsC>TETRA>TMAO>As(V)>
375 GPAsC>MA^V>DMAE (Table 3, Fig. 1). Thus, the dominant specie in urine after the first 20 h is AB,

376 with a mean value of total AB excreted during this period of 140 µg As g^{-1} of creatinine (Figure 4a),
377 which accounts about 40 % of the total AB in 100 g of cooked anemones, and the concentration
378 decreases to 23 µg As µg g^{-1} of creatinine after 80 hours (88 % of the total AB in 100 g of cooked
379 anemones is excreted during 90 hours). These results are in good agreement with other experiments
380 after ingestion of shrimp, in which 66% of arsenobetaine is eliminated from the body after 36 hours
381 [50]. Other arsenic species can be transformed into AB such as AsC, which after oral administration
382 is rapidly absorbed or transformed into AB, with little or no degradation to inorganic arsenic, MA^V,
383 DMA^V [43, 51]. As previously reported, DMA^V is not totally eliminated from the body [52], which is
384 in good agreement with the almost constant concentration determined along the experiment. About
385 130 % of DMA^V present in 100 g of the anemones is excreted by urine during 90 h (408 µg g^{-1} of
386 creatinine) (Fig. 4b). This fact suggests the biotransformation of other arsenic species, like $DMAS^V$
387 into DMA^V, which is supported by the absence of the former in urine and its presence in *Anemonia*
388 *Sulcata*. In addition, after *in vitro* gastrointestinal digestion, the concentration of As (V) is high,
389 which can be also transformed into DMA^V as previously described [25]. Other arsenicals are less
390 abundant in urine as can be observed in Figures 4b and 4c, especially As (V), MA^V and DMAE.
391 Some of them are quickly eliminated from the body, such as AsC, TETRA, TMAO and As (V), or
392 progressively, like MA^V and DMAE. In this experiment, 89 % and 93 % of the TMAO and TETRA
393 present in 100 g of cooked anemones, respectively, are excreted by urine during the period 10-30
394 hours without biotransformation (Fig. 4b). The excretion of DMAE in urine is slower than for the
395 discussed arsenicals and about 80% of the total DMAE ingested is excreted during the period 20-50
396 hours (Fig. 4c). In summary, about 95 % of total arsenic in cooked anemones is bioaccessible (Table
397 3) and 85% of the amount ingested is eliminated from the body by urine in 90 h.

398

399 ## 3.5. Human serum samples

400 Figure 5 shows typical HPLC-ICP-MS overlapped chromatograms of serum samples before
401 consumption and 1 and 2 hours after, using AEC (Fig. 5a) and CEC (Fig. 5b). In contrast to the
402 results obtained for the anemones and urine, in human serum samples, only AB, As (V), MA^V,
403 DMA^V and AsC are detected. As can be observed in the chromatograms (Fig. 5a and b), the peak at
404 2.5 minutes in CEC (Fig. 5b) is about 5 fold higher than the peak at 11.7 minutes in AEC (Fig. 5a).
405 The explanation is the $^{40}Ar^{35}Cl$ interference, which coelutes with As (V) in CEC (Fig. 5b), as
406 suggested by other authors [53].

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

413 Fig. 6 shows the evolution of the different arsenicals during 2 hours after the ingestion. After
414 30 minutes from the ingestion, DMA^V is the most abundant in serum, followed by AB, MA^V and
415 AsC. After this period of time, the amount of AsC in human serum is constant during 2 hours (Fig.
416 6), but the concentration of other arsenicals increased progressively with a common pattern and As
417 (V) can be only detected after 1 hour. This fact supports the idea that in humans, after the absorption
418 of As (V) by the gastrointestinal tract, it is quickly reduced to As (III) in the blood, which easily
419 cross the cell membrane at physiological pH and is methylated and later oxidized [57].

420

421 ## 4. Conclusions

422 Although the obvious interest about the complete knowledge of human arsenic metabolism,
423 exposure experiments are very difficult and some organisms have been proved to be poor models
424 because of their different behavior compared to humans, like in the case of rats. A bioaccessibility

425 study of an arsenic containing seafood, namely *Anemonia sulcata*, and later monitoring of human
426 biological fluids after consumption have been used in this paper to show the real exposure to arsenic,
427 providing a deeper insight into the arsenic metabolism of humans after the consumption of these sea
428 organisms that are not usually studied. Speciation in tissue extracts and human fluids (urine and
429 serum) was carried out by anion/cation exchange chromatography with inductively coupled plasma
430 mass spectrometry (IC)-ICP-MS). The ICP-MS constitutes a powerful technique for the speciation of
431 arsenic since it provides very low detection limits, tolerance to matrix and large linearity range. In
432 addition, the use of the collision cell eliminates suitably the interferences on the arsenic signal some
433 of them caused by the complex matrix that has been analyzed. However, this technique should be
434 combined with organic mass spectrometry since in real samples the presence of unidentified
435 arsenicals is very common. The use of organic mass spectrometry in this work allowed the
436 standarless identification of AsC, GPAsC, $DMAS^{V}$ and DMAE in the analyzed samples.
437

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449 # References

450 [1] T. Grossman, C.B. Klein, Genetic and epigenetic effects of environmental arsenicals,
451 Metallomics 3 (2011) 1397.

452 [2] A.H. Smith, M. Goycolea, R. Haque, M.L. Biggs, Marked increase in bladder and lung cancer
453 mortality in a region of Northern Chile due to arsenic in drinking water, Am. J. Epidemiol. 147
454 (1998) 660.

455 [3] K. Francesconi, Arsenic species in seafood: Origin and human health implications, Pure Appl.
456 Chem. 82 (2010) 373.

457 [4] EFSA Panel on Contaminants in the Food Chain (CONTAM); Scientific Opinion on Arsenic in
458 Food. EFSA Journal 2009; 7(10):1351. [199 pp.].

459 [5] S. McSheehy, J. Szpunar, R. Morabito, P. Quevauviller, The speciation of arsenic in biological
460 tissues and the certification of reference materials for quality control, Trends Anal. Chem., 22 (2003)
461 191.

462 [6] A.E. Geiszinger, W. Goessler,K.A. Francesconi, The marine polychaete Arenicola marina: its
463 unusual arsenic compound pattern and its uptake of arsenate from seawater, Marine Environ. Res.
464 53 (2002) 37.

465 [7] D. Fattorini, F. Regoli, Arsenic speciation in tissues of the Mediterranean polychaete Sabella
466 spallanzanii, Environ. Toxicol. Chem. 23 (2004) 1881.

467 [8] D. Fattorini, A. Notti, A., F. Regoli, Characterization of arsenic content in marine organisms from
468 temperate, tropical, and polar environments, Chem. Ecol. 22 (2006) 405.

469 [9] H.V. Aposhian, M.M. Aposhian, Arsenic toxicology: five questions, Chem. Res. Toxicol., 19
470 (2006) 1.

471 [10] H. Yamauchi, B.A. Fowler, in: J.O. Nriagu (editor), Arsenic in the environment, part II: Human
472 health and ecosystems. J.Wiley and Sons, Inc., New York, USA, 1994, p.35.

473 [11] F. Ebert, A. Weiss, M. Bültemeyer, I. Hamann, A. Hartwig, T. Schwerdtle, Arsenicals affect
474 base excision repair by several mechanisms, Mutat. Res. Fundam. Mol. Mech. Mutagen., 715 (2011)
475 32.
476 [12] H. Naranmandura, N. Suzuki, T.K. Suzuki, Trivalent arsenicals are bound to proteins during
477 reductive methylation, Chem. Res. Toxicol., 19 (2006) 1010.
478 [13] S.M. Wnek, T.J. Jensen, P.L. Severson, B.W. Futscher, A.J. Gandolfi, Monomethylarsonous
479 acid produces irreversible events resulting in malignant transformation of a human bladder cell line
480 following 12 weeks of low-level exposure, Toxicol. Sci. 116 (2010) 44.
481 [14] H. Naranmandura, Y. Ogra, K. Iwata, J. Lee, K.T. Suzuki, M. Weinfeld, X.C. Le, Systemic
482 distribution and speciation of diphenylarsinic acid fed to rats, Toxicol. Appl. Pharmacol. 237 (2009)
483 214.
484 [15] S. Hirata, H. Toshimitsu, M. Aihara, Determination of arsenic species in marine samples by
485 HPLC-ICP-MS, Anal. Sci. 22 (2006) 39.
486 [16] P.B. Tchounwou, J.A. Centeno, A.K. Patlolla, Arsenic toxicity, mutagenesis, and
487 carcinogenesis--a health risk assessment and management approach, Mol. Cell Biochem., 255 (2004)
488 47.
489 [17] M. Vahter, in: W. R Chappel (Editor), Arsenic Exposure and Health effects, Elsevier,
490 Amsterdam, 1999, pp. 267.
491 [18] X.C. Le, in: W.T. Marcel Dekker (Editor), Arsenic Speciation in the Environment and Humans,
492 in Environment Chemistry of Arsenic, Frankenberger, Inc., New York, USA, 2000, pp. 95.

493 [19] M.F. Hughes, V. Devesa, B.M. Adair, S.D. Conklin, J.T. Creed, M.Y. Styblo, E.M. Kenion, D.
494 J. Thomas, Tissue dosimetry, metabolism and excretion of pentavalent and trivalent dimethylated
495 arsenic in mice after oral administration, Toxicol. Appl. Pharmacol. 227 (2008) 26.

496 [20] Concha G, Vogler G, Lezeano D, Nermell B, Vahter M, Exposure to inorganic arsenic
497 metabolites during early human development, Toxicol. Sci. 44 (1998) 185.

498 [21] X.C. Le, W.R. Cullen, K.J. Reimer, Human urinary arsenic excretion after one-time ingestion of
499 seaweed, crab, and shrimp, Clin. Chem. 40 (1994) 617.

500 [22] M. Vahter, Mechanisms of arsenic biotransformation, Toxicol. 181-182 (2002) 211.

501 [23] E. Schmeisser, A. Rumpler, M. Kollroser, G. Rechberger, W. Goessler, K.A. Francesconi,
502 Arsenic fatty acids are human urinary metabolites of arsenolipids present in cod liver, Angew Chem.
503 Int. Ed. Engl., 45 (2006) 150.

504 [24] J.S. Petrick, B. Jagadish, E.A. Mash, H.V. Aposhian, Monomethylarsonous acid (MMA(III))
505 and arsenite: LD(50) in hamsters and in vitro inhibition of pyruvate dehydrogenase, Chem. Res.
506 Toxicol. 14 (2001) 651.

507 [25] D.H. Thomas, J. Li, S.B. Waters, W. Xing, B.M. Adair, Z. Drobna, V. Devesa, M. Styblo,
508 Arsenic (+ 3 Oxidation State) Methyltransferase and the Methylation of Arsenicals in the
509 Invertebrate Chordate *Ciona intestinalis,* Exp. Biol. Med. 232 (2007) 3.

510 [26] K. Rehman, H. Naranmandura, Arsenic metabolism and thioarsenicals, Metallomics. 4 (2012)
511 881.

512 [27] V. Devesa, D. Velez, R Montoro, Effect of thermal treatments on arsenic species contents in
513 food, Food Chem. Toxicol. 46 (2008) 1.

514 [28] M. Contreras-Acuña, T. García-Barrera, M.A. García-Sevillano, J.L. Gómez-Ariza, Speciation
515 of arsenic in marine food (*Anemonia sulcata*) by liquid chromatography coupled to inductively
516 coupled plasma mass spectrometry and organic mass spectrometry, J. Chromatogr. A. 1282 (2013)
517 133.

518 [29] WHO, Biological Monitoring of chemical Exposure in the Workplace, 1996, vol 1

519 [30] S. Mounicou, J. Szpunar, R. Lobinski, D. Andrey, C. J. Blake, Bioavailability of cadmium and
520 lead in cocoa: comparison of extraction procedures prior to size-exclusion fast-flow liquid
521 chromatography with inductively coupled plasma mass spectrometric detection (SEC-ICP-MS), J.
522 Anal. At. Spectrom. 17 (2002) 880.

523 [31] J. Feldmann, V. W-M. Lai, W.R. Cullen, M. Ma, X. Lu, X.C. Le, Sample Preparation and
524 Storage Can Change Arsenic Speciation in Human Urine, Clin. Chem. 45 (1999) 1988.

525 [32] J.S. Edmonds, K.A. Francesconi, The origin of arsenobetaine in marine animals, Appl.
526 Organomet. Chem. 2 (1988) 297.

527 [33] W.R. Cullen, K.J. Reimer, Arsenic speciation in the environment, Chem. Rev. 89 (1989) 713.

528 [34] K.A. Francesconi, J.S. Edmonds, Arsenic species in marine samples, Croactica. Chem. Acta. 71
529 (1998) 343.

530 [35] H.R. Hansen, A. Raab, M. Jaspars, B.F. Milne, J. Feldmann, Sulfur-containing arsenical
531 mistaken for dimethylarsinous acid [DMA(III)] and identified as a natural metabolite in urine: major
532 implications for studies on arsenic metabolism and toxicity, Chem. Res. Toxicol. 17 (2004) 1086.

533 [36] R. Raml, A. Rumpler, W. Goessler, M. Vahter, L. Li, T. Ochi, K.A, Francesconi, Thio-
534 dimethylarsinate is a common metabolite in urine samples from arsenic-exposed women in
535 Bangladesh, Toxicol. Appl. Pharmacol. 222 (2006) 374.

536 [37] K.T. Suzuki, K. Iwata, H. Naranmadura, N. Suzuki., Metabolic differences between two
537 dimethylthioarsenicals in rats., Toxicol. Appl. Pharmacol. 218 (2007) 166.

538 [38] H. Naranmandura, K. Suzuki, Formation of dimethylthioarsenicals in red blood cells, Toxicol.
539 Appl. Pharmacol. 227 (2008) 390.
540 [39] S.K.V. Yathavakilla, M. Fricke, P.A. Creed, D.T. Heitkemper, N.V. Shockey, C. Schwegel, J.A.
541 Caruso, J.T. Creed, Arsenic speciation and identification of monomethylarsonous acid and
542 monomethylthioarsonic acid in a complex matrix, Anal. Chem. 80 (2008) 775.
543 [40] K.M. Kubachka, M.C. Kohan, S.D. Conklin, K. Herbin-Davis, J.T. Creed, D.J. Thomas, In vitro
544 biotransformation of dimethylarsinic acid and trimethylarsine oxide by anaerobic microflora of
545 mouse cecum analyzed by HPLC-ICP-MS and HPLC-ESI-MS, J. Anal. At. Spectrom. 24 (2009)
546 1062.
547 [41] R. Raml, W. Goessler, K.A. Francesconi, Improved chromatographic separation of thio-arsenic
548 compounds by reversed-phase high performance liquid chromatography-inductively coupled plasma
549 mass spectrometry, J. Chromatogr. A 1128 (2006) 164.
550 [42] K. A. Francesconi, R. V. Stick, J. S. Edmonds, Glycerylphosphorylarsenocholine and
551 phosphatidylarsenocholine in yelloweye mullet (*Aldrichetta forsteri*) following oral administration of
552 arsenocholine, Cell. Mol. Life Sci. 46 (1990) 464.
553 [43] E. Marafante, M. Vahter, L. Dencker, Metabolism of arsenocholine in mice, rats and rabbits,
554 Sci. Total Environ., 34 (1984) 223.
555 [44] G.X. Sun, P.N. Williams, A.M. Carey, Y.G. Zhu, C. Deacon, A. Raab, J. Feldmann, R.M. Islam,
556 A.A. Meharg, Inorganic arsenic in rice bran and its products are an order of magnitude higher than in
557 bulk grain, Environ. Sci. Technol. 42 (2008) 7542.
558 [45] Anonymous, in: R.E. Crossgrove (Editor), Arsenic in drinking water. National Academy Press,
559 Washington, 1999, pp. 83.
560 [46] J. De Kimpe, R. Cornelis, L. Mees, R. Vanholder. In: M. Anke, D. Meissner, C.F. Mills
561 (Editors), Trace elements in man and animals, Verlag Media Touristik, Gersforf, Germany, 1993,
562 pp. 845.

563 [47] A. Salvadeo, C. Minola, S. Segagni, G. Villa, Trace metal changes in dialysis fluid and blood of
564 patients on hemodialysis, Int. J. Artif. Organs 2 (1979) 17.
565 [48] K.A. Francesconi, R. Tanggaard, C.J. McKenzie, W. Goessler, Arsenic Metabolites
566 in Human Urine after ingestion of an Arsenosugar, *Clin. Chem.* 48 (2002) 92.
567 [49] V.W.-M Lai, Y. Sun, E. Ting, W.R. Cullen, K.J. Reimer, Arsenic speciation in human urine: are
568 we all the same?, Toxicol. Appl. Pharmacol. 198 (2004) 297.
569 [50] X.C. Le, M. Ma, V. W-M Lai, in: W.R. Chappell, CO Abernathy and RL Calderon (editors),
570 Arsenic Exposure and human health effects, Elsevier, Science BV, 1999, pp. 69.
571 [51] T. Kaise, S. Fukui, The chemical form and acute toxicity of arsenic compounds in marine
572 organisms, Appl. Organomet. Chem. 6 (1992) 155.
573 [52] L. Benramdane, M. Accominotti, L. Fanton, D. Malicier, J.J. Vallon, Arsenic speciation in
574 human organs following fatal arsenic trioxide poisoning--a case report, Clin. Chem. 45 (1999) 301.
575 [53] R. Raml, G. Raber, A. Rumpler, T. Bauernhofer, W. Goessler, K.A. Francesconi, Individual
576 Variability in the Human Metabolism of an Arsenic-Containing Carbohydrate, 2′,3′-Dihydroxypropyl
577 5-deoxy-5-dimethyl-arsinoyl-D-riboside, a Naturally Occurring Arsenical in Seafood, Chem. Res.
578 Toxicol. 22 (2009) 1534.
579 [54] J. Gailer, Arsenic-selenium and mercury-selenium bonds in biology, Coord. Chem. Rev. 251
580 (2007) 234.
581 [55] X. Zhang, L. Mees, R. Cornelis, R. Vanholder, N. Lameire, Chemical speciation of arsenic in
582 serum of uraemic patients, Analyst. 123 (1998) 13.
583 [56] Z. Slejkovec, I. Falgona, W. Goessler, JT van Elteren, R. Raml, H Podgornik, P. Cernelc,
584 Analytical artefacts in the speciation of arsenic in clinical samples, Anal. Chim. Acta. 607 (2008)
585 83.

586 [57] C.H. Tseng, A review on environmental factors regulating arsenic methylation in humans,
587 Toxicol. Appl. Pharmacol. 235 (2009) 338.

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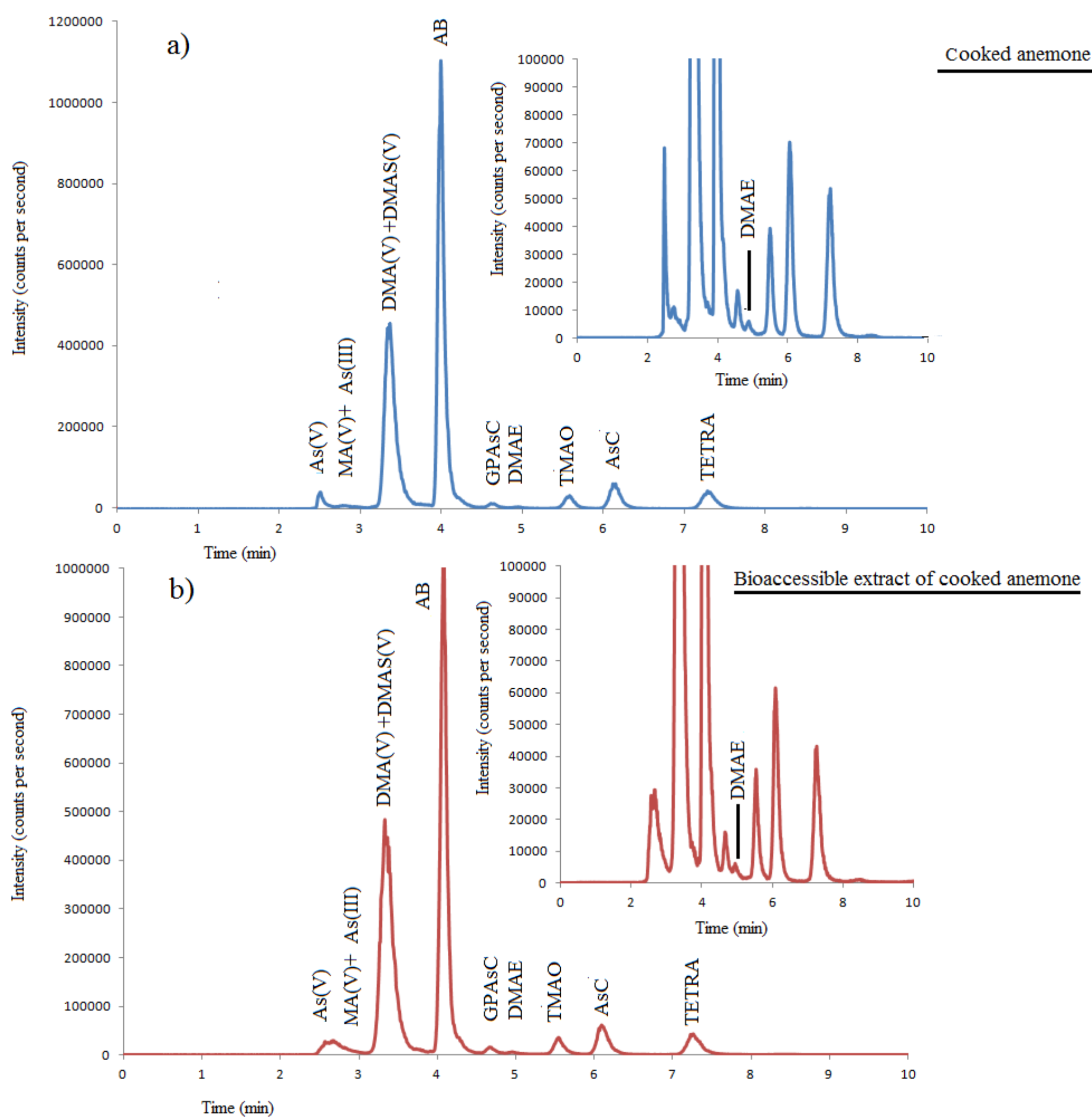
611 **Figure 1.** (a) Typical HPLC-CEC-ICP-MS chromatogram of cooked anemones. (b) As-metabolites

612 in the bioaccessible extract of cooked anemones. Chromatographic conditions are given in table 1.



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621 **Figure 2**. Speciation of arsenic in human urine (n= 3) before (a,b) and 20 hours after consumption of
622 cocked anemones (c,d) by HPLC-(CEC)-ICP-MS (a,c) and HPLC-(AEC)-ICP-MS (b,d).
623 Chromatographic conditions are given in Table 1.

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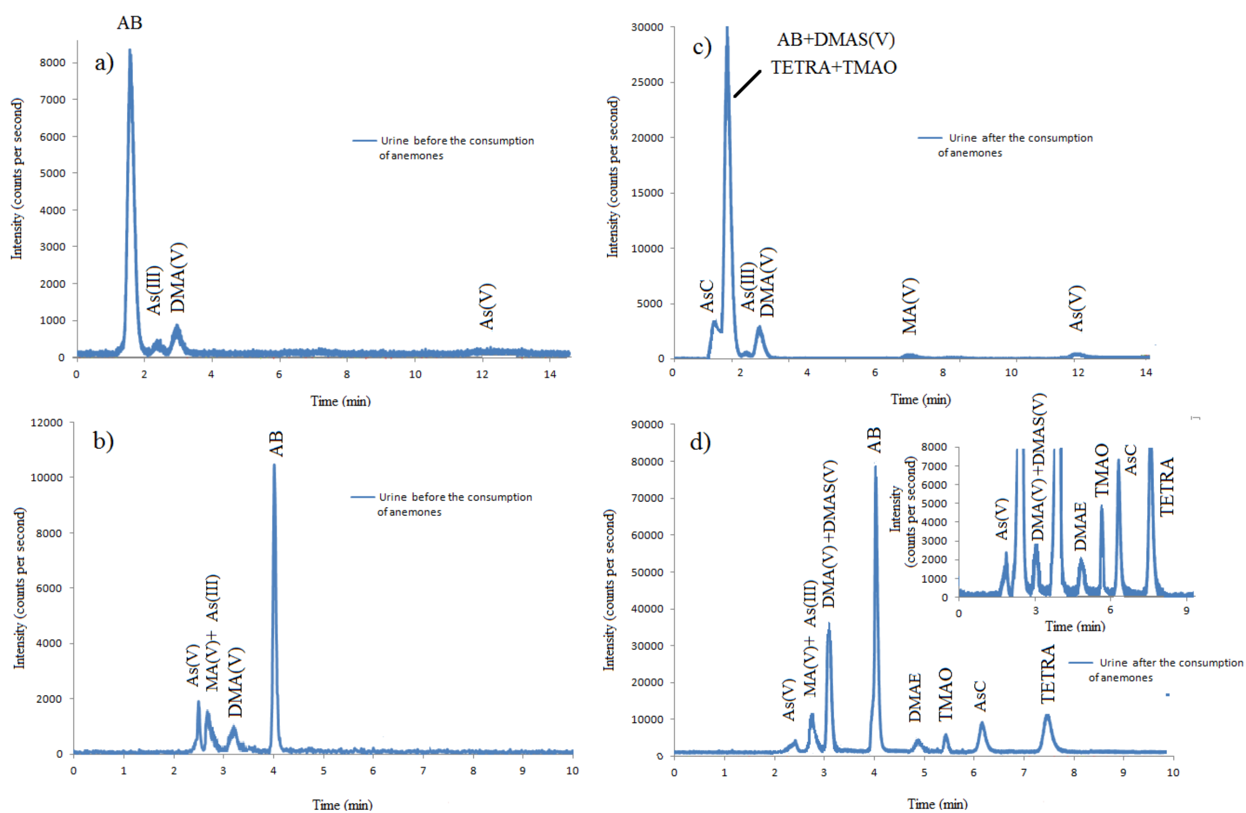

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635 **Figure 3.** (a) MS/MS spectrum of peak at 4.9 minutes obtained from an human urine 20 hours after
636 consumption of anemones using by HPLC-(CEC)-ESI-QqQ-MS (Multiple Channel Accumulation
637 (MCA) = 20, Collision Energy (CE) =30V and Declustering Potential (DP) = 80V). (b) MRM
638 chromatogram of the 167→121 *m/z* transition obtained from the same urine sample by HPLC-
639 (CEC)-ESI-QqQ-MS (CE=25V and DP=80V). The chromatographic conditions are given in Table 1.
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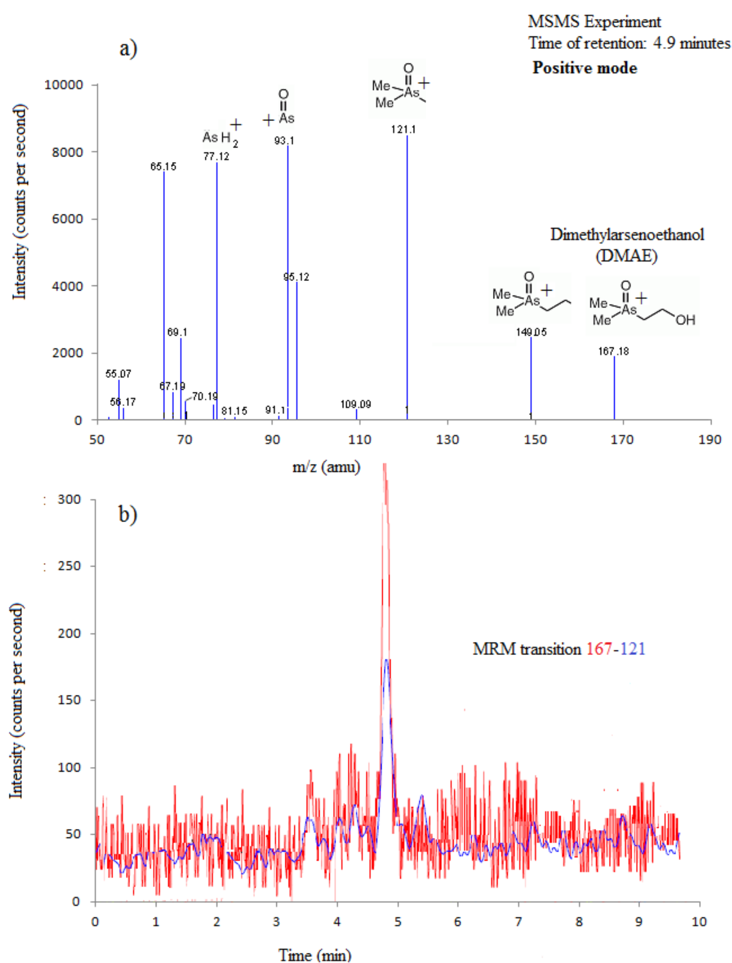

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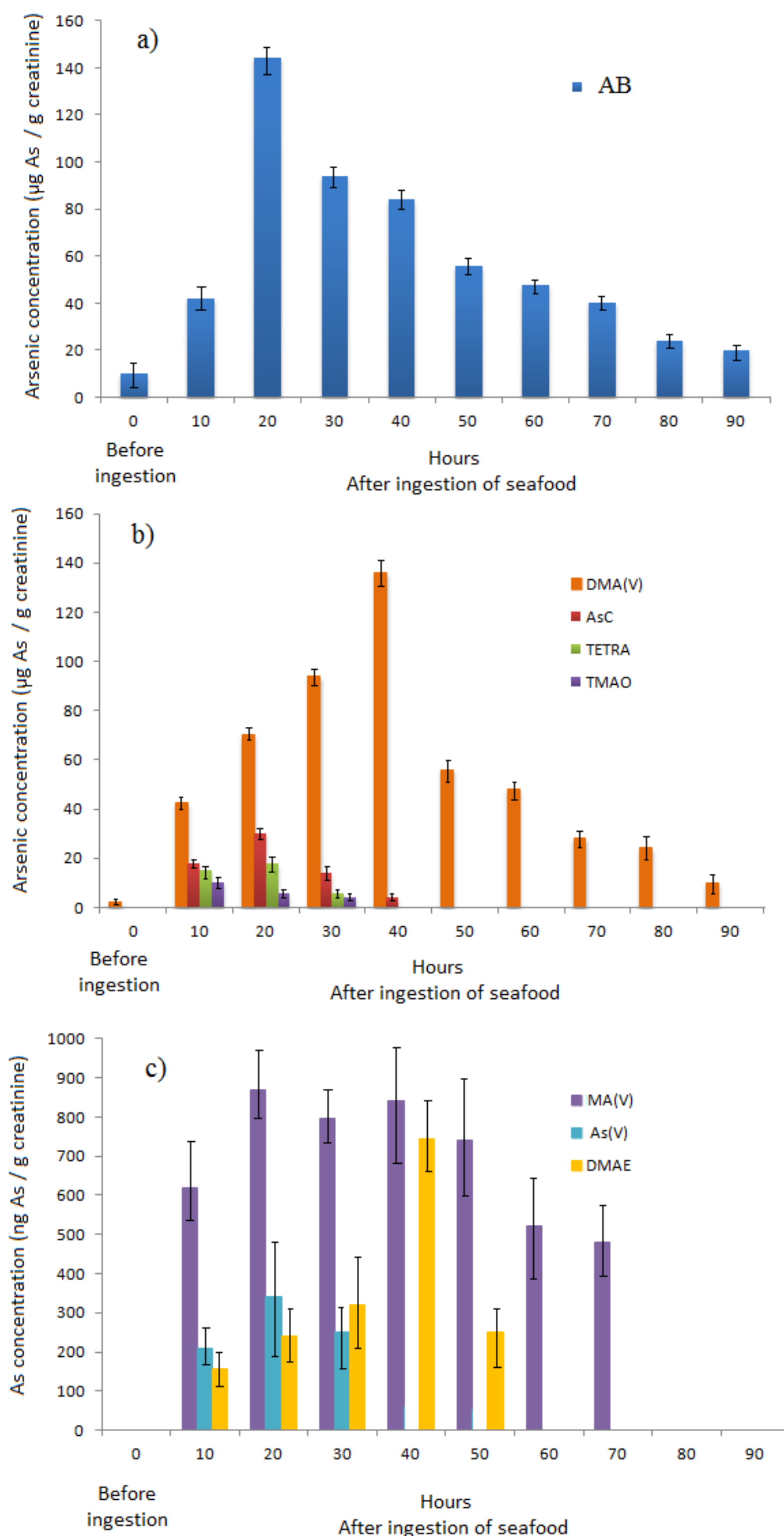
648 **Figure 4.** Relative concentrations of arsenic metabolites in urinary samples (n = 5 volunteers)
649 normalized against the concentration of creatinine, before and after consumption of 100 g of cooked
650 anemones per volunteer.



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652 **Figure 5**. Speciation of arsenic in a serum sample (n= 3) before and after consumption of cocked
653 anemones by (a) HPLC-AEC-ICP-MS and (b) HPLC-CEC-ICP-MS. Chromatographic conditions
654 are given in Table 1.

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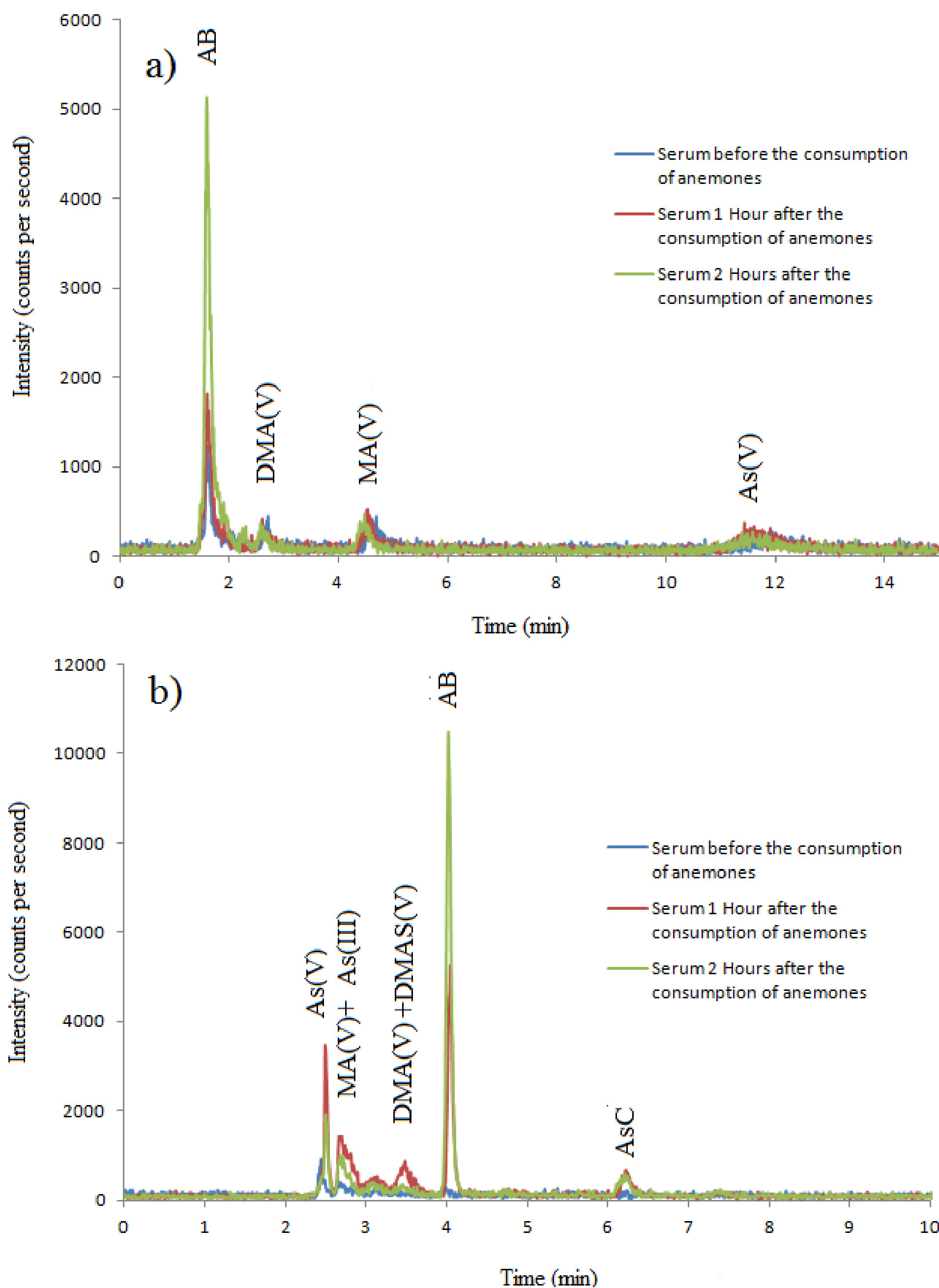

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Figure 6. Relative concentration of arsenic metabolites in serum samples collected for 5 volunteers before and after consumption of 100 g of cooked anemones per volunteer.

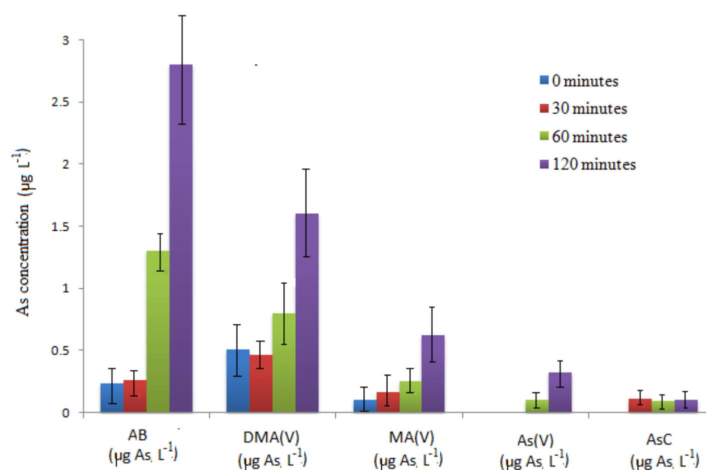

Table 1. Experimental conditions used in HPLC-ICP-MS.

HPLC conditions		
Anionic Exchange Chromatography	Column	Hamilton PRP X-100 (250 mm × 4.6 mm × 5 μm)
	Mobile phase	Ammonium carbonate, 50 mM (pH 8.5)
	Flow-rate	1.2 mL min^{-1}
	Injection volume	50 μL
Cationic Exchange Chromatography	Column	Supelcosil SCX (250 mm × 4.6 mm × 5 μm)
	Mobile phase	Pyridine, 20 mM (pH 2.5)
	Flow-rate	1.2 mL min^{-1}
	Injection volume	50 μL
ICP-MS conditions		
Forward power		1300 W
Plasma gas flow rate		13.0 L min^{-1}
Auxiliary gas flow rate		1.0 L min^{-1}
Carrier gas flow rate		1.0 L min^{-1}
Sampling and skimmer cones		Xi Interface of Nickel
He flow		4.0 mL min^{-1}
Q_{oct}		-19 V
Q_p		-17 V
Focus		-9.0 V
Dwell time		0.1 per isotope
Isotopes monitored		^{75}As, $^{77}ArCl^+$

Table 2. Evaluation of arsenic species concentrations in human urine and serum samples during storage period.

Human urine	**As(III) (%)**	**As(V) (%)**	**MA^{V} (%)**	**DMA^{V} (%)**	**AB (%)**	**As(III) (%)**	**As(V) (%)**	**MA^{V} (%)**	**DMA^{V} (%)**	**AB (%)**
	Volunteer urine samples stored at two months at -80ºC					**Spiked volunteer urine samples stored at two months at -80ºC**				
15 Days	99	<LOD	100	101	99	98	101	102	101	103
30 Days	102	<LOD	96	99	96	104	105	98	97	98
45 Days	98	<LOD	97	97	97	99	111	97	95	96
60 Days	94	<LOD	91	93	89	105	118	93	91	88
Human serum	**As(III) (%)**	**As(V) (%)**	**MA^{V} (%)**	**DMA^{V} (%)**	**AB (%)**	**As(III) (%)**	**As(V) (%)**	**MA^{V} (%)**	**DMA^{V} (%)**	**AB (%)**
	Volunteer urine samples stored at two months at -80ºC					**Spiked urine samples stored at two months at -80ºC**				
15 Days	101	98	102	98	99	102	100	101	99	98
30 Days	99	106	99	100	101	100	101	97	102	100
45 Days	100	106	101	96	98	97	104	99	98	96
60 Days	98	110	97	94	94	99	110	95	96	93

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.

	As(III)	**As(V)**	**MA^{V}**	**DMA^{V}**	**AB**	**AsC**
Cooked anemone (µg As g^{-1})	<LOD	0.086	0.027	1.7	3.2	0.34
Standard deviation (µg As g^{-1})	-----	0.004	0.003	0.1	0.3	0.05
Bioaccessible extract from cooked anemone (µg As g^{-1})	<LOD	0.11	0.031	1.5	3.1	0.32
Standard deviation (µg As g^{-1})	-----	0.005	0.002	0.09	0.4	0.03
Limit of detection (LOD, ng As g^{-1})	3.5	2.4	2.2	1.8	0.91	
Bioaccessibility (%)	-----	128	115	88	97	94

	$DMAS^{V}$	**GPAsC**	**TMAO**	**TETRA**	**TOTAL As**	**TOTAL As (as sum of the species)**
Cooked anemone (µg As g^{-1})	0.76	0.035	0.12	0.26	6.6	6.5
Standard deviation (µg As g^{-1})	0.02	0.003	0.009	0.02	0.1	0.2
Bioaccessible extract from cooked anemone (µg As g^{-1})	0.72	0.034	0.12	0.25	6.4	6.2
Standard deviation (µg As g^{-1})	0.03	0.002	0.007	0.03	0.2	0.2
Limit of detection (LOD, ng As g^{-1})			0.72	3.5	0.098	-----
Bioaccessibility (%)	95	97	100	96	96	**95**