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Transformation of Arsenic Species during in Vitro Gastrointestinal Digestion of Vegetables

Marta Calatayud,[†] Edi Bralatei,[‡] Jorg Feldmann,[‡] Vicenta Devesa,[†] and Dinoraz Vélez*[,†]

[†]Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Avenida Agustín Escardino no 7, 46980 Paterna (Valencia), Spain

[‡]Department of Chemistry, School of Physical Sciences, University of Aberdeen, Meston Building, AB243UE, United Kingdom

ABSTRACT: Arsenic is an element widely distributed in the environment, and the diet is the main source of arsenic exposure for most people. However, many of the processes related to steps before intestinal absorption are unknown. This study evaluates the effect of in vitro gastrointestinal digestion on pentavalent arsenic forms [As(V), MMA(V), DMA(V)] present in various vegetables (garlic, broccoli, asparagus, spinach) after soaking or boiling in aqueous solutions of these species. The results showed that the gastrointestinal digest contained trivalent or thiolated arsenic forms different from the pentavalent species added initially. Transformation percentages varied, depending on sample, treatment, and arsenic species. Results showed transformation of up to 22% to As(III), 35% to MMA(III)/MMAS, and 26% to DMA(III)/DMAS. These data indicate that more toxic arsenic species are present in the gastrointestinal digest, and they highlight the need to consider this process when evaluating the toxicological risk associated with ingestion of this metalloid.

KEYWORDS: *arsenic, transformation, gastrointestinal digestion, vegetables*

INTRODUCTION

Arsenic is an element widely distributed in the environment, and the diet is the main source of arsenic exposure for most of the population. According to a study conducted by the European Food Standard Authority (EFSA), the food categories considered as the main contributors to exposure to total arsenic are "cereal and cereal products" and "fish and seafood." The contribution made by other groups, such as "vegetables, nuts, and pulses", "fruit and vegetable juices", and "food for special dietary uses", is smaller but must also be considered.[1] The risk associated with exposure to arsenic is influenced by the species ingested. From a toxicological viewpoint, the species of greatest interest are inorganic arsenic and the monomethylated and dimethylated arsenic species. Inorganic arsenic is the major species in drinking water and is considered a group 1 carcinogen.[2] Numerous epidemiological studies have shown a direct relationship between high exposure to inorganic arsenic and various kinds of cancer.[1] Studies conducted in recent years have shown that the oxidation state is one of the factors that condition processes of uptake, transport, metabolism, and toxic effects that take place in animal or human cell lines.[3−5] It has been shown that As(III), As(V), and pentavalent methylated species [MMA(V) monomethylarsonic acid, DMA(V) dimethylarsinic acid] are present in some foods. So far the presence of trivalent methylated species has only been reported in carrots grown in an area polluted with arsenic.[6]

The content in raw foods is generally used to evaluate the risk associated with intake of inorganic arsenic, but it is necessary to realize that processing of foods prior to consumption and transformations during gastrointestinal transit could introduce changes in the arsenic species that reach the intestinal epithelium and the bloodstream. This means that new variables must be taken into account when estimating the risk associated with consumption of this metalloid. In this regard, there are reports of changes in the chemical forms of arsenic present in foods after cooking or processing.[7] Laparra et al.[8] showed that changes in redox states of inorganic arsenic derived from foods can also take place during the gastrointestinal digestion process.

Moreover, studies have shown that As(V) present in aqueous solutions, soils, and rice can be transformed into As(III), MMA(V), MMA(III), and monomethylmonothioarsonic acid [MMMTA(V)] by human colonic microbiota.[9,10] These species are more toxic than the As(V) present in the samples, indicating the importance that these transformations could have when evaluating the toxicological risk associated with ingestion of arsenic.

This work evaluates changes in the chemical form of As(V), MMA(V), and DMA(V) after gastrointestinal digestion of vegetables soaked or boiled with these pentavalent arsenic species. The results provide novel information about the transformations of these arsenic forms prior to their intestinal absorption.

MATERIALS AND METHODS

Arsenical Species. The standard of As(V) (1000 mg/L) was obtained from Merck (Spain). The standard of As(III) (1000 mg/L) was prepared by dissolving 1.320 g of As_2O_3 (Riedel de Haën, Germany) in 25 mL of 20% (m/v) KOH, neutralizing with 20% (v/v) H_2SO_4, and diluting to 1 L with 1% (v/v) H_2SO_4. The standard solutions of MMA(V) and DMA(V) (1000 mg/L) were prepared by dissolving in water the appropriate amount of $CH_3AsO(ONa)_2{\cdot}6H_2O$ (Carlo Erba, Italy) or $(CH_3)_2AsNaO_2{\cdot}3H_2O$ (Fluka, Spain), respectively. The standard solutions of MMA(III) and DMA(III)

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(1000 mg/L) were prepared by dissolving in ethanol the appropriate amount of CH_3AsI_2 and $(CH_3)_2AsI$ (Argus Chemicals, Italy), respectively. These trivalent species are very unstable, and therefore the primary standards were prepared on the same day as when they were used.

Reagents. Deionized water (18.2 MΩ cm), obtained with a Milli-Q water system (Millipore Inc., Spain), was used for the preparation of standards and reagents. Enzymes and bile salts for in vitro gastrointestinal digestion were purchased from Sigma (Spain): porcine pepsin (enzymatic activity 944 U/mg protein), porcine pancreatin (activity equivalent to 4 × US Pharmacopoeia specifications/mg pancreatin: lipase, 944 USP units; protease, 11 800 USP units; amylase, 11 800 USP units), and bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts). Other reagents used in the investigation were of analytical or reagent grade. Plastic and glassware material was treated with 10% HNO_3 (v/v) for 24 h and then rinsed with deionized water before use.

Food Samples. Various batches of garlic (*Allium sativum*), broccoli (*Brassica oleracea italica*), asparagus (*Asparagus officinalis*), and spinach (*Spinacia oleracea*) were purchased at supermarkets in the city of Valencia (Spain). The samples were chopped, homogenized, and separated into three subsamples (A−C), which were subjected to different treatments. Subsample A was used for the analysis of the raw product. Subsample B was left to soak (1 mg/L, 24 h, room temperature) in an aqueous solution of As(V), MMA(V), or DMA(V), each assayed independently. Subsample C was boiled (1 mg/L, 100 °C, 15 min) in an aqueous solution of As(V), MMA(V), or DMA(V), and each assayed independently. No treatments with MMA(V) or DMA(V) were applied to the spinach. The sample/water relationship was 1:3. After soaking or boiling, excess liquid was removed by centrifugation.

In Vitro Gastrointestinal Digestion. Food subsamples B and C were digested using a simulated digestion process with two stages, gastric and intestinal.[11] Samples (10 g wet weight, ww) were weighed, and cellular-grade water (90 mL) was added. To emulate the gastric stage of digestion, the pH was adjusted to 2.0 with 6 M HCl (Merck, Spain). After 5 min, the pH value was checked and if necessary readjusted to pH 2.0. Then freshly prepared pepsin solution (0.01 g of pepsin/10 g sample) was added. The sample was made up to 100 g with water and incubated in a shaking water bath (120 strokes/min; Unitronic Orbital C, Selecta, Spain) at 37 °C for 2 h.

For intestinal digestion, the pH value was raised to 6.5 by dropwise addition of 1 M $NaHCO_3$ (Panreac, Spain). Then the pancreatin−bile extract mixture (0.0025 g of pancreatin and 0.015 g of bile extract/10 g sample) was added. Incubation at 37 °C continued for 2 h. The digests were transferred to polypropylene centrifuge tubes and centrifuged (15344 g/30 min/4 °C; RC-5B Superspeed refrigerated centrifuge, DuPont Sorvall, France) to separate soluble and precipitate. Total arsenic and arsenic species were quantified in the soluble (bioaccessible) fraction. Bioaccessibility was determined as the percentage of solubilized arsenic with respect to the arsenic content in the food.

Total Arsenic Determination. The analysis of total arsenic contents of raw and processed food, as well as bioaccessible fractions, was performed by flow injection-hydride generation-atomic absorption spectrometry (FI-HG-AAS) after a dry ashing step.[12] Triplicate analyses were performed for each sample. The analytical characteristics of the method are as follows: detection limit = 0.026 μg/g dry weight (dw); precision = 2%. Throughout the experiment, the quality assurance/quality control of the measurement was checked by analyzing a rice flour certified reference material (SRM 1568a, National Institute of Standards and Technology, NIST) with each batch of samples (certified value = 0.29 ± 0.03 μg/g).

Inorganic Arsenic Determination. Inorganic arsenic [As(V) + As(III)] was analyzed in the raw vegetables and those soaked or cooked with As(V). The analysis was performed by acid digestion, solvent extraction, and FI-HG-AAS.[12] Triplicate analyses were performed for each sample. The analytical characteristics of the method are as follows: detection limit = 0.013 μg/g dw; precision = 4%; As(III) recovery = 99%, and As(V) recovery = 96%. This method was used for the determination of inorganic arsenic in rice in IMEP-107, a proficiency test organized by the European Commission (Joint Research Centre, Institute for Reference Materials and Measurements), with optimum results.[13] Rice flour SRM 1568a was analyzed with each series of samples, and the quality assurance/quality control of the measurement was checked by comparing the values found (0.110 ± 0.003 μg/g dw) with the range reported in the literature (0.08−0.110 μg/g dw).[14]

Arsenic Species Determination by pH-Specific HG-AAS. The trivalent and pentavalent arsenic species in bioaccessible fractions were determined following a previously described procedure involving hydride-generation cryotrapping gas chromatography coupled to atomic absorption spectrometry (HG-CT-AAS).[15] Using this technique at pH 1, total inorganic, monomethylated, and dimethylated forms are quantified without distinguishing oxidation states; at pH 6 only trivalent forms [As(III), MMA(III), and DMA(III)] or methylated thioarsenicals (MMAS, DMAS) are determined.[16] Generation of arsines at pH 1 was performed in a reaction mixture containing a variable volume of sample, 0.5 mL of antifoam B silicone emulsion 1% (v/v) (Sigma), 1 mL of 6 M HCl, and the quantity of deionized water needed to make the volume up to 7 mL. For arsine generation at pH 6, the HCl was replaced with 1 mL of Tris-HCl buffer (2.5 M; Sigma). The standard addition method was used to verify the absence of matrix interference in the bioaccessible fraction obtained from each species of vegetable. For this purpose, calibration curves were obtained from standard solutions (0.5−30 ng arsenic for each standard) for quantification at both pH 1 [As(V), MMA(V), DMA(V)] and pH 6 [As(III), MMA(III), DMA(III)].

Determination of Antioxidant Capacity of the Bioaccessible Fraction. The antioxidant capacity of the solubilized fraction after gastrointestinal digestion of the samples (raw, soaked, or boiling with arsenic) was determined. For this purpose, the ferric reducing ability of plasma (FRAP) was determined, with slight modifications.[17] This colorimetric method is based on the reduction of a complex of iron and tripyridyl-*s*-triazine. The FRAP reagent was prepared daily from 25 mL of 0.3 M acetate buffer at pH 3.6, 2.5 mL of 10 mM 2,4,6-tripyridyl-*s*-triazine solution in 40 mM HCl, and 2.5 mL of 20 mM ferric chloride. In 96-well plates, 180 μL of FRAP reagent was added to 20 μL of sample or standard ($FeSO_4$ $7H_2O$), and absorbance was read at 593 nm (PolarSTAR OPTIMA, BMG-Labtech, Germany) 4 and 30 min after the start of the reaction. The concentrations were calculated by using a standard calibration curve (100−2000 μM). The results are expressed as equivalent concentration of $FeSO_4{\cdot}7H_2O$.

Reduced and Oxidized Glutathione Levels (GSH/GSSG) in the Bioaccessible Fraction. GSH and GSSG contents in the solubilized fraction of samples (raw, soaked, or boiling with arsenic) were measured according to Hissin and Hilf.[18] For GSH quantification, 10 μL of bioaccessible fraction was incubated with 10 μL of buffered formaldehyde (37−40% formaldehyde: 0.1 M Na_2HPO_4 in a proportion of 1:4 v/v; pH 8), 170 μL of 0.1 M Na_2HPO_4−5 mM EDTA buffer, and 10 μL of the phthaldialdehyde solution (OPT, 1 mg/mL). After mixing and incubation at room temperature for 45 min, the fluorescence intensity was measured at excitation−emission wavelengths of 355−460 nm (PolarSTAR OPTIMA). For GSSG quantification, bioaccessible medium (10 μL) was incubated for 30 min at room temperature with 10 μL of the buffered formaldehyde described above, and 4 μL of 40 mM *N*-ethylmaleimide. Subsequently, 166 μL of 0.1 M NaOH and 10 μL of OPT were added. After mixing and incubation at room temperature for 45 min, the fluorescence intensity was measured in the same conditions as described above. The GSH and GSSG concentrations were calculated by using independent standard calibration curves (5−200 μg/mL, Sigma).

Statistical Analysis. All the assays were performed at least in triplicate. The results were analyzed statistically by a one-factor analysis of variance (ANOVA) with Tukey's HSD post hoc multiple comparison or Student's *t* (SPSS, version 15.0). Differences were considered significant when $p < 0.05$.

RESULTS

The raw vegetable products used in this study had very low concentrations of total arsenic: garlic 5−14 ng/g ww; broccoli 4−5 ng/g ww; asparagus 7−9 ng/g ww; spinach 5−12 ng/g ww. The analysis of inorganic arsenic by acid digestion−solvent extraction−FI-HG-AAS (see Materials and Methods) showed that this arsenic consisted entirely of inorganic species. The treatments in the presence of water containing 1 mg/L of As(V) produced an increase in the inorganic arsenic concentration in the samples (soaking: 840−1237 ng/g fresh weight; boiling: 1035−1634 ng/g fresh weight) (Table 1). After soaking or boiling in the presence of 1 mg/L MMA(V) or DMA(V) the total arsenic concentrations in the vegetables ranged between 1139 and 2760 ng/g wet weight (Table 2).

Table 1. Inorganic Arsenic Concentrations in Vegetables Treated with 1 mg/L of As(V) (Soaked or Boiled) and Inorganic Arsenic and As(III) Concentrations in the Bioaccessible Fraction of the Vegetables[a]

		food	bioaccessible fraction	
		inorganic As, (ng/g) ww	inorganic As, (ng/g) ww	As(III), (ng/g) ww
garlic	soaked	1237 ± 80	1011 ± 138	1 ± 0.1
	boiled	1143 ± 324	573 ± 253	81 ± 6
broccoli	soaked	937 ± 86	993 ± 114	189 ± 22
	boiled	1059 ± 174	1037 ± 126	17 ± 10
asparagus	soaked	1035 ± 214	1097 ± 192	44 ± 8
	boiled	1526 ± 164	1589 ± 127	266 ± 65
spinach	soaked	840 ± 134	890 ± 131	62 ± 9
	boiled	1634 ± 401	1219 ± 376	51 ± 38

[a]The results, expressed in wet weight (ww), present a mean value ± standard deviation of three different samples of each vegetable.

Effect of Gastrointestinal Digestion of Food Samples on Chemical Form of Arsenic. Gastrointestinal digestion could alter the arsenic species present in food as a result of the conditions that apply during the digestion (temperature, pH, enzymes, and other reagents). In order to evaluate whether these conditions produce changes in arsenic species, the gastrointestinal digestion process was applied to standards of 1 mg/L of the pentavalent species [As(V), MMA(V), and DMA(V)] and their trivalent analogues [As(III), MMA(III), and DMA(III)]. The results obtained show that As(V), As(III), MMA(V), and DMA(V) had not altered after the digestion. However, there was a change in the chemical form of the trivalent methylated species: 83 ± 7% of the MMA(III) oxidized to MMA(V) and 71 ± 3% of the DMA(III) oxidized to DMA(V). No other qualitative changes were detected.

Samples Treated with As(V). The bioaccessibility of the arsenic in the vegetables that were soaked with As(V) was high (91−116%). Neither MMA nor DMA was detected in the soluble fractions of asparagus, spinach, and broccoli; inorganic arsenic was the only quantifiable species. In the case of the garlic sample, inorganic arsenic was also present in the bioaccessible fraction, but it did not correspond to the total arsenic quantified, indicating the presence of other arsenic species. These species, which generated a signal in the quantification by HG-CT-AAS (Figure 1), and which had a retention time that did not coincide with the retention times of the standards available in the laboratory, were not identified. The inorganic arsenic in these soluble fractions appeared in both oxidation states, As(V) and As(III). The As(III) concentrations varied with the vegetable analyzed (Table 1). In terms of percentage of As(III) in relation to solubilized inorganic arsenic, the highest values were found in broccoli (19−20%), followed by spinach (7−11%), asparagus (4−7%), and garlic (0.1−0.2%).

In the vegetables boiled with As(V), the gastrointestinal digestion process also produced a high solubilization of arsenic for the samples of broccoli, asparagus, and spinach (84−106%). In the garlic samples the bioaccessibility was lower (56−60%). The arsenic species detected in the soluble fractions followed the same pattern as for the soaked samples. Inorganic arsenic appeared in the bioaccessible fraction of boiled garlic, together with signals that did not correspond to the standards available in the laboratory, with the same retention times as those shown in Figure 1; in the other vegetables, only inorganic arsenic was detected. As(III) was also detected in the soluble fractions of these vegetables boiled with As(V) (Table 1), and the percentages in relation to solubilized inorganic arsenic (1−22%) were similar to those found in the soaked samples, although the order of the percentages in the samples

Table 2. Total Arsenic in Vegetables Treated with MMA(V) or DMA(V) (Soaked or Boiled), and in the Soluble Fraction Obtained after Gastrointestinal Digestion[a]

food and treatment			total As (ng/g ww)	bioaccessible fraction				
				total As (ng/g ww)	bioaccessibility (%)	MMA(III)/MMAS (ng/g ww)	DMA(III)/DMAS (ng/g ww)	transformation (%)
garlic	soaked	MMA(V)	1801 ± 112	1240 ± 31	69 ± 2	96 ± 5		8 ± 1
		DMA(V)	1405 ± 149	1064 ± 59	76 ± 4		182 ± 2	17 ± 3
	boiled	MMA(V)	1791 ± 91	226 ± 32	13 ± 2	181 ± 2		4 ± 1
		DMA(V)	2760 ± 141	245 ± 17	9 ± 1		128 ± 8	5 ± 1
broccoli	soaked	MMA(V)	1146 ± 23	1173 ± 25	102 ± 2	152 ± 3		13 ± 2
		DMA(V)	1359 ± 111	1060 ± 101	78 ± 7		200 ± 12	19 ± 3
	boiled	MMA(V)	1302 ± 45	257 ± 22	20 ± 2	57 ± 6		22 ± 3
		DMA(V)	1489 ± 79	204 ± 7	14 ± 1	−	44 ± 5	20 ± 1
asparagus	soaked	MMA(V)	1308 ± 131	1108 ± 66	85 ± 5	419 ± 35		35 ± 3
		DMA(V)	1139 ± 69	1091 ± 57	96 ± 5		288 ± 12	26 ± 1
	boiled	MMA(V)	1217 ± 47	139 ± 12	11 ± 2	48 ± 4		35 ± 3
		DMA(V)	1319 ± 99	104 ± 9	8 ± 1		25 ± 4	24 ± 4

[a]The table also shows the arsenic bioaccessibility, the concentrations of MMA(III)/MMAS and DMA(III)/DMAS in the soluble fraction, and the percentage of transformation of the methylated species. Data are expressed as mean ± standard deviation (n = 3).

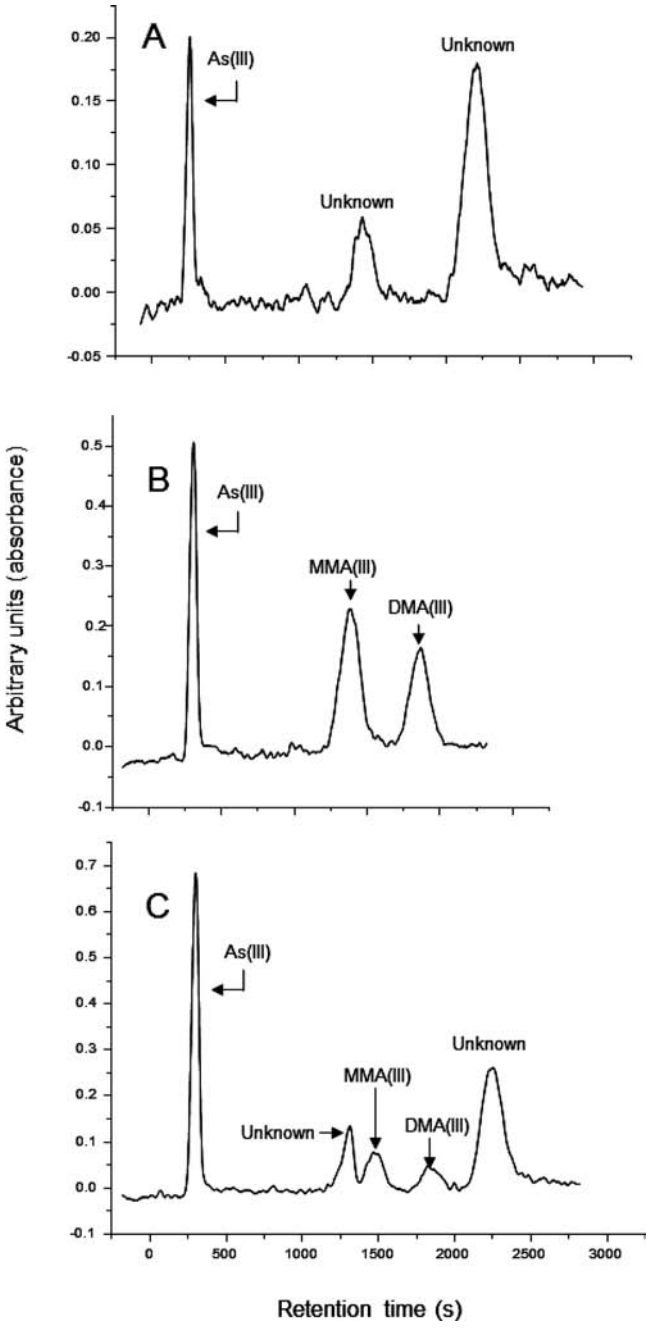

Figure 1. Chromatograms obtained in quantification by hydride-generation cryotrapping gas chromatography coupled to atomic absorption spectrometry (HG-CT-AAS) (pH 6) of (A) soluble fraction of garlic soaked with As(V); (B) standards of As(III), MMA(III), and DMA(III), 30 ng of each arsenic species; (C) soluble fraction of garlic soaked with As(V) spiked with As(III), MMA(III), and DMA(III).

was different [garlic (9−22%) ≈ asparagus (14−22%) > spinach(2−6%) > broccoli (1−2%)].

Samples Treated with MMA(V) and DMA(V). After soaking in the presence of MMA(V) or DMA(V), the arsenic bioaccessibility ranged between 69 and 102%, with the garlic sample having the lowest bioaccessibility (Table 2). In the boiled samples, the solubilization percentages (8−20%) were much lower than those found after soaking. The mass balance for the gastrointestinal process was adequate for all the combinations of vegetable and arsenic species (80−112%; data not shown), indicating that there were no losses during the experimental process or during quantification, and therefore the differences in bioaccessibility can only be attributed to the effects of the food matrix.

The analysis of the bioaccessible fraction showed that there was a transformation of MMA(V) and DMA(V) into MMA(III)/MMAS and DMA(III)/DMAS, respectively, in percentages that varied according to the food product (Table 2). Quantitatively, it is noteworthy that up to 35% of the arsenic in the bioaccessible fraction appeared in a form different from that added to the sample. The foods assayed had different transformation percentages (asparagus > broccoli > garlic), and no clear differences were observed in relation to species (mono- or dimethylated) or treatment (soaking/boiling).

Reducing Ability of the Soluble Fraction. *Antioxidant Capacity.* Figure 2 shows the $FeSO_4$ mM/100 g equivalents of

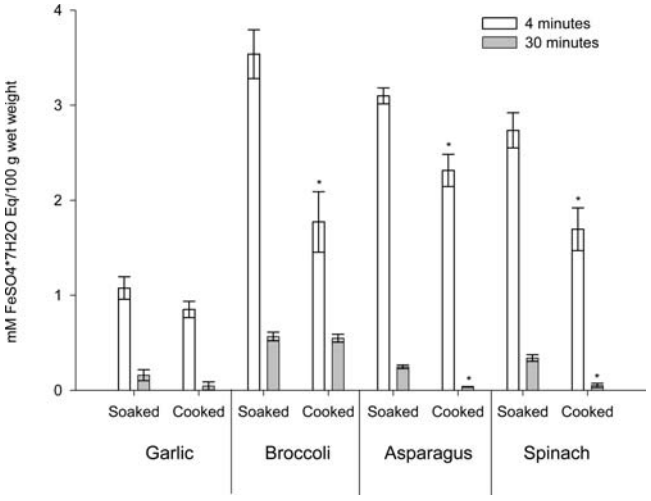

Figure 2. Antioxidant capacity of the soluble fraction of soaked or boiled samples, evaluated as equivalent concentration of $FeSO_4$ (mM/100 g) at 4 and 30 min (mean ± standard deviation; $n = 3$). Statistically significant differences ($p < 0.05$) between raw and boiled samples are marked with an asterisk.

the soluble fraction of the vegetables subjected to soaking or boiling. The antioxidant capacity in the soaked samples followed the order broccoli > asparagus > spinach > garlic, with no difference in the order for the two times analyzed (4 and 30 min). After boiling, the value of $FeSO_4$ equivalents decreased significantly in all the samples except garlic, indicating that the temperatures applied during boiling affected the compounds with reducing ability.

The As(III) contents found in the solubilized fraction after gastrointestinal digestion of the vegetables boiled with As(V) followed a linear correlation with the antioxidant capacity, evaluated as $FeSO_4$ mM/100 g equivalents ($R^2 = 0.82$). No correlation was observed between antioxidant capacity and As(III) contents in the solubilized fraction of the As(V)-soaked samples. Also, no correlation was found with the other arsenic species.

Oxidized and Reduced Glutathione. Figure 3 shows the GSH and GSSG contents in the soluble fraction obtained after gastrointestinal digestion of the soaked samples and the boiled samples. The highest GSH levels were detected in the asparagus sample, followed by spinach and broccoli. Glutathione was not detected in reduced form in garlic. After boiling

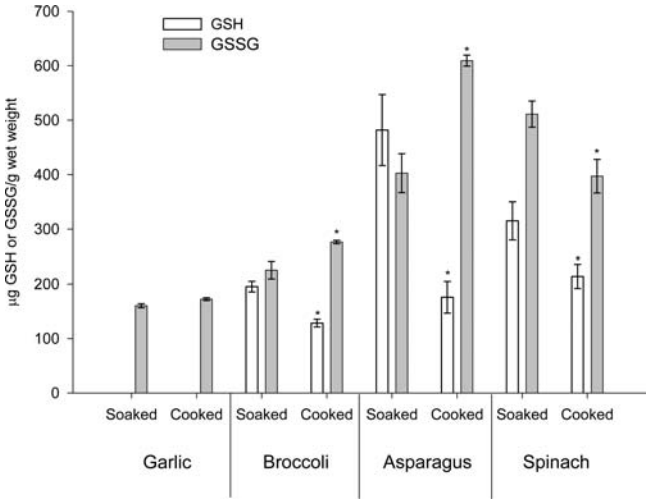

Figure 3. GSH (white bar) and GSSG (gray bar) concentrations in the soluble fraction of soaked or boiled samples (μg/g wet weight; mean ± standard deviation; n = 3). Statistically significant differences (p < 0.05) between GSH and GSSG levels in soaked and boiled samples are marked with an asterisk.

of the samples, a significant reduction in GSH levels was observed.

In the soluble fraction of the boiled vegetables, there was a linear correlation between MMA(III)/MMAS contents and GSH concentrations (R^2 = 0.8132). This correlation did not appear for the other arsenic species. Also, no correlation was observed between GSH and the species present in the soluble fraction of the soaked vegetables.

■ DISCUSSION

The greatest contribution of arsenic to the organism comes from consumption of drinking water and food. The toxicity of arsenic species differs, and therefore it is especially important to determine the chemical form of arsenic in food intake, focusing on inorganic arsenic because of its carcinogenic nature. Transformations of arsenic species could take place after ingestion and before it reaches the bloodstream. There are reports of modifications of inorganic arsenic by colonic microbiota[9,10] and by intestinal epithelium cells.[4] However, no studies have been conducted to evaluate these transformations that result from interaction between arsenic and customary components of the diet during the gastrointestinal digestion process.

The vegetable products used in this work had low levels of inorganic arsenic (≤14 ng/g wet weight). However, in arsenic endemic areas, the concentration of this element in the food consumed by the population is generally much higher because of the use of water contaminated with As(V) for cooking.[19,20] To emulate the situation in those areas the vegetables were soaked or boiled in the presence of As(V), and the resulting samples were then used to evaluate the effect of gastrointestinal digestion on the oxidation state of this pentavalent species. The same kind of study was conducted for the methylated species MMA(V) and DMA(V). These are minor species in drinking water, and they were used in the soaking and boiling of the vegetables in order to evaluate whether these arsenic forms, which can be found in foods such as seaweed, rice, and vegetables,[6,21,22] could appear in the intestinal lumen after digestion as different species from those ingested with the food.

The results showed that the bioaccessibility of inorganic arsenic after gastrointestinal digestion of the vegetables was high in both the soaked and the boiled vegetables (56−116%). However, for the methylated forms there were great differences between the bioaccessibility in the soaked samples (69−102%) and the boiled samples (8−20%). This might be because of the formation of stable complexes during boiling which keep the methylated forms adsorbed by the matrix and do not allow their solubilization. Moreover, it is also possible that boiling could solubilize substances that have the effect of precipitating methylated arsenic species. There are no previous studies on the bioaccessibility of methylated species from cooked products, and therefore it would be advisable to conduct further studies to corroborate whether the difference in bioaccessibility of MMA(V) and DMA(V) after boiling of food is an effect that appears in other vegetables or other kinds of food.

The chemical form of the arsenic species in the bioaccessible fraction was analyzed by HG-CT-AAS, a method based on selective generation of arsines from inorganic As, MMA, and DMA. Originally, it was thought that the specificity in the generation of arsines in relation to the working pH made it possible to differentiate the oxidation states;[7] at pH 1, both the trivalent and the pentavalent forms generated arsines, whereas at pH 6, only the trivalent forms were capable of generating arsines. However, the field of arsenic speciation is complex and is constantly changing as a result of the identification of new species. In this connection, new inorganic, monomethylated, and dimethylated arsenic species bound to thiol groups have been identified. Regmi et al.[16] showed that the thiolated pentavalent arsenic forms DMAS and MMAS are also capable of generating hydrides at pH 7, and in a cold trap system present chromatographic behavior similar to that observed for trivalent methylated forms. Therefore at pH 6 there might be an overlapping of trivalent methylated and pentavalent thioarsenical species, which in turn may cause an error in the quantification. These thiolated species might be present in the bioaccessible fractions analyzed, as a result of the complexation of the standards added for boiling or soaking and the thiol groups in the matrix. It should be taken into account that the vegetables analyzed are rich in thiol groups.[23,24] Raab et al.[25] reported the formation of a dimethylarsinothioyl glutathione complex (DMAS-GS) in plants rich in sulfur compounds, such as *Brassica oleracea*. For the reason given above, in this study the results at pH 6 of methylated species are considered as a sum of trivalent [MMA(III), DMA(III)] and pentavalent thiolated [MMAS, DMAS] species, without knowing with certainty the chemical structure of them.

After gastrointestinal digestion of the samples, the soluble fraction was found to contain arsenic species that were different from the ones used during soaking and boiling. The percentages of transformations varied according to the food sample; in the most extreme cases, in the bioaccessible fraction 22% of the inorganic arsenic, 35% of the MMA, and 26% of the DMA was a different species from what had been added to the sample. Only Laparra et al.[8] have previously reported reduction to As(III) after gastrointestinal digestion of rice samples cooked in the presence of As(V). However, there are no previous reports of changes in pentavalent methylated species after gastrointestinal solubilization. As this study shows, the process of gastrointestinal digestion of pentavalent arsenic standards that was applied does not produce changes in their chemical form, so the changes observed in the vegetables could only be due to effects of the food components on the arsenic species.

In addition, the presence in the soluble fraction of different species from what had been added to the sample might not be only the result of transformations during the gastrointestinal process but might take place previously, during the soaking or boiling of the food. This transformation might be due to the action of hydrosoluble compounds with antioxidant capacity in the vegetables, such as vitamin C, glutathione, uric acid, or phenolic compounds,[26−30] or of hydrosoluble sulfur groups. These compounds might solubilize in the water used for soaking or boiling, causing the reduction or thiolation of pentavalent species. In view of the greater toxicity of these species, it will be of interest to study the transformation of pentavalent forms due to the contact with the food matrix.

Food samples have a broad range of components with antioxidant ability, such as polyphenols or vitamins.[31] Among them we selected GSH as a key point in arsenic transformations because there have been many reports of its reducing activity in the reaction that converts pentavalent arsenic to trivalent arsenic in chemically defined systems and in cellular systems.[32] Asparagus was the sample with the highest GSH content and the highest percentage of reduction of As species, indicating the importance of this compound in the transformation of As during gastrointestinal digestion. GSH was not detected in the garlic samples, but there were several organosulfur compounds with antioxidant activity.[33] Nonenzymatic antioxidant activity of four organosulfur compounds derived from garlic[33] might be involved in the different responses observed for the various arsenical species in the presence of this vegetable.

However, the reduction ability of these vegetables does not correlate with the vegetable treatment (soaking or boiling), especially for inorganic arsenic. In the case of asparagus, for example, there was an increase in the As(III) levels of the bioaccessible fraction after the boiling procedure (4% soaked, 17% boiled), indicating a strong effect of vegetable processing on arsenic bioaccessibility. On the other hand, there was different behavior for the methylated species in general, with a high transformation ability of soaked samples in comparison with boiled ones. This might indicate inactivation of other redox-active compounds, such as vitamin C, or polyphenols involved in arsenic reduction processes. It would be necessary to evaluate a larger range of food samples and characterize other reducing compounds to approximate the complexity of the processes involved in the arsenic transformations observed during in vitro gastrointestinal digestion.

Trivalent species or thiolated pentavalent species have greater cytotoxicity than the pentavalent forms,[34−37] so the presence of these species in the soluble fraction resulting from gastrointestinal digestion might have toxicological consequences. However, the toxicological importance of these changes should be confirmed by in vivo assays. In this regard, in vivo studies have shown that the consumption of vegetables has a beneficial effect in relation to the toxicity of arsenic. Islam et al.[37] observed that in rats which had been given arsenic together with spinach extract the levels of arsenic in various organs decreased in comparison with animals that had been given arsenic without spinach. Zablotska et al.[38] also observed that consumption of food rich in vitamins and folic acid reduced the risk of suffering skin lesions associated with exposure to arsenic through drinking water. These effects might be the result of reduced absorption of arsenic forms solubilized after digestion of food. In this regard, Yang et al.[39] observed a reduction in absorption of arsenic and other metals in the presence of spinach, possibly resulting from chelation processes. This work shows the need for further study of transformations of arsenic species during their transit through the gastrointestinal tract and of the influence of food ingested with the contaminant on the absorption of arsenic.

AUTHOR INFORMATION

Corresponding Author

*E-mail: deni@iata.csic.es; telephone: (+34) 963 900 022; fax (+34) 963 636 301.

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