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Estimation of Arsenic Bioaccessibility in Edible Seaweed by an in Vitro Digestion Method

J. M. LAPARRA,† D. VÉLEZ,† R. MONTORO,*,† R. BARBERÁ,‡ AND R. FARRÉ‡

Institute of Agrochemistry and Food Technology (CSIC), Aptdo 73, 46100, Burjassot (Valencia), Spain, and Nutrition and Food Chemistry, Faculty of Pharmacy, University of Valencia, Avda. Vicente Andrés Estellés s/n. 46100 Burjassot, Spain

The aim of this study was to examine the bioaccessibility (maximum soluble concentration in gastrointestinal medium) of total (AsT) and inorganic (AsI) arsenic contents and the effect on them of cooking edible seaweed, a food of great interest because of its high As content. An in vitro gastrointestinal digestion (pepsin, pH 2, and pancreatin−bile extract, pH 7) was applied to obtain the mineral soluble fraction of three seaweeds (*Hizikia fusiforme*, *Porphyra* sp., and *Enteromorpha* sp.). AsT was determined by dry-ashing flow injection hydride generation atomic absorption spectrometry. AsI was determined by acid digestion, solvent extraction, and flow injection hydride generation atomic absorption spectrometry. The bioaccessibility of AsI increased significantly after cooking, attaining 73% in *Porphyra* sp. and 88% in *H. fusiforme*. For cooked *H. fusiforme*, the AsI attained in the bioaccessible fraction was 26 μg g^{-1} seaweed, a concentration that is a warning of the toxicological risk of this food.

KEYWORDS: Bioaccessibility; total arsenic; inorganic arsenic; seaweed; cooking

INTRODUCTION

In western countries, seaweed has been used mainly as a source of colloids, thickeners, and gelling agents, with a wide range and variety of applications in the food industry. In Asian countries, however, seaweed forms part of the customary diet, and among the main consumers, we must mention the Japanese, with an average intake of 1.6 kg/year (*1*).

From a nutritional viewpoint, edible seaweed is of interest because of its high content of dietary fiber (33−50%), greater than that of most of the higher plants. Seaweed fiber is rich in soluble fractions, with hypocholesterolemic and hypoglycemic effects (*2*). It is also well-known that seaweed is rich in minerals (8−40%) (*3*), with high contents of Ca, Mg, P, K, Na, Fe, and I (*2*). Edible seaweed is also a source of food proteins, with an amino acid composition of nutritional interest (*1*) but with significant amounts of fermentable and unavailable protein (*4*).

Their richness in dietary fiber, minerals, and proteins, together with their low lipid content (*2*), has caused the consumption of some kinds of seaweed to be considered among certain population groups in Europe as being of interest or at least a good dietary supplement, although the European Union has not yet proposed specific regulations concerning their utilization for human consumption (*3*).

In addition to the interesting nutrients mentioned, seaweed can have a high content of heavy metals and arsenic. In some species of seaweed, such as *Hizikia fusiforme*, the inorganic arsenic (AsI) content is greater than 50 μg g^{-1} dry weight (dw) (*5*). AsI, which includes As(III) and As(V), is a recognized carcinogen for which some countries establish maximum concentrations in edible seaweed, above which the seaweed cannot be sold, e.g., 1 μg g^{-1} in Australia and New Zealand (*6*) and 3 μg g^{-1} in France and the U.S.A. (*2*).

In recent decades, the study of the arsenic present in foods has been approached from various viewpoints: the characterization of arsenic species (*7*−*9*), the effect of cooking treatments on transformation of arsenic species (*10*, *11*), estimation of arsenic intake (*12*−*15*), studies of human metabolism (*16*), and evaluation of the toxicity of arsenic species in tests with animals and in cell cultures (*17*). However, there is one aspect that has been indicated as being of interest (*18*−*20*) but on which there are no known studies: the bioavailability (BA) for human beings of the arsenic species present in food.

What one understands by the BA of a food is the fraction of it that is absorbed and used by the organism. For an element to be absorbed, it is necessary that it should be soluble in the gastrointestinal tract. This depends, among other things, on the ability of the digestive enzymes to release the element in the intestinal gut, as well as on its solubility and behavior in the gastrointestinal tract, which in turn is a function of the chemical form released from the food (*21*, *22*).

Both in vitro and in vivo methods for evaluating BA have been proposed. The in vitro methods provide an effective approximation to in vivo situations and offer the advantage of good reproducibility, as it is possible to control conditions better than in in vivo tests.

* To whom correspondence should be addressed. Tel: (+34)963 900 022. Fax: (+34)963 636 301. E-mail: rmontoro@iata.csic.es.
† Institute of Agrochemistry and Food Technology (CSIC).
‡ University of Valencia.

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In the in vitro methods, a process of gastrointestinal digestion is simulated, followed by determination of the soluble fraction of the element or of the fraction capable of dialyzing through a semipermeable membrane with a specified pore size (*23*). In this way, the so-called bioaccessible fraction is determined, which is the maximum concentration soluble in simulated gastrointestinal media that is available for subsequent processes of absorption into the intestinal mucosa (*24*). Knowledge of the concentration of the element in the bioaccessible fraction is an indispensable requisite for the evaluation of BA. The existing studies of BA for toxic trace elements are relatively scarce. There are studies of the BA of Pb and Cd in foods (*25*), but the BA of As has only been studied in soils (*24*, *26*).

In most cases, seaweed products are cooked prior to consumption. During thermal processing, the application of heat enhances loss of water and other soluble constituents such as proteins and vitamins. Chemical contaminants may also be affected by the heat applied, and as the literature shows (*11*), arsenic is no exception.

For all of these reasons, the aim set for the present study was the evaluation of the bioaccessible fraction of total arsenic (AsT) and AsI in edible seaweed. A further aim was to study the influence of the cooking of seaweed on the bioaccessible fraction of this metalloid.

MATERIALS AND METHODS

Instruments. The quantification of AsT and AsI was performed with an atomic absorption spectrometer (AAS) model 3300 Perkin-Elmer (PE) equipped with an autosampler (PE AS-90) and a flow injection hydride generation system (PE FIAS-400) (FI-HG-AAS). Other equipment used included a lyophilizer equipped with a microprocessor controlling the lyophilization process (FTS Systems, New York), a PL 5125 sand bath (Raypa Scharlau S. L., Barcelona, Spain), a K1253 muffle furnace equipped with a Eurotherm Controls 902 control program (Heraeus S. A., Madrid, Spain), a KS 125 Basic mechanical shaker (IKA Labortechnik, Merck Farma y Química, S. A., Barcelona, Spain), an Eppendorf 5810 centrifuge (Merck), and a Sorvall RC-50B centrifuge.

Reagents. Deionized water (18.2 MΩ cm), obtained with a Milli-Q water system (Millipore Inc., Millipore Ibérica, Madrid, Spain) was used for the preparation of reagents and standards. Water of cellular grade (B. Braun Medical, S. A., Barcelona, Spain) was used throughout the in vitro digestion assay. All chemicals were pro analysis quality or better. All glassware was treated with 10% (v/v) HNO_3 for 24 h and then rinsed three times with deionized water before it was used.

Enzymes and bile salts were purchased from Sigma Chemical Co. (St. Louis, MO): pepsin (Porcine: catalog no. P-7000), pancreatin (Porcine; catalog no. P-1750), and bile extract (Porcine; catalog no. B-8631). Calibration standard solutions of As(III) were prepared from a reduced commercial standard solution (1000 mg L^{-1}) of As(V) (Merck) by taking appropriate amounts of commercial standard solutions. As the reducing solution, a mixture containing 5% (w/v) KI and 5% (w/v) ascorbic acid was employed.

Samples. Three edible seaweeds were analyzed as follows: brown seaweed, *H. fusiforme* (hijiki); red seaweed, *Porphyra* sp. (nori); and green seaweed, *Enteromorpha* sp. (green nori flakes). They were all acquired in health food stores in the city of Valencia (Spain). All analyzed samples of the same type came from the same manufacturing batch.

The samples were analyzed just as they were sold, which we have called the raw state. *H. fusiforme* and *Porphyra* sp. were also analyzed after they were cooked by applying the cooking treatment indicated on the product label. The treatment used for *H. fusiforme* was boiling in water (100 °C) for 20 min (30 g of seaweed/500 mL of water), and for *Porphyra* sp., the treatment was baking (200 °C) for 5 min. For *Enteromorpha* sp., the recommendation was that it should be eaten raw; therefore, it was not subjected to a cooking treatment. Both the raw and the cooked samples were maintained at 4° C until analysis.

In Vitro Gastrointestinal Digestion Method. The in vitro digestion method used was based on the method of ref *27*, modified and adapted for the seaweed being studied. To 5 g of seaweed, raw or cooked, cellular grade water was added, 90 mL of water for *H. fusiforme* and *Porphyra* sp. and 160 mL for *Enteromorpha* sp. The pH was adjusted to 2.0 with 6 mol L^{-1} HCl. After 15 min, the pH value was checked and if necessary readjusted to pH 2. Then, freshly prepared pepsin solution (1 g of pepsin in 10 mL of 0.1 mol L^{-1} HCl) was added to provide 0.01 g of pepsin/5 g seaweed. The sample was made up to 100 g (*H. fusiforme* and *Porphyra* sp.) or 170 g (*Enteromorpha* sp.) with water and incubated in a shaking water bath (120 strokes min^{-1}) at 37 °C for 2 h.

Prior to the intestinal digestion step, the pH of the gastric digests was raised to pH 5 by dropwise addition of 1 mol L^{-1} $NaHCO_3$. Then, the pancreatin−bile extract mixture (0.2 g of pancreatin and 1.25 g of bile extract in 50 mL of 0.1 mol L^{-1} $NaHCO_3$) was added to provide 0.0025 g of pancreatin/5 g seaweed and 0.015 g of bile extract/5 g seaweed, and the incubation at 37 °C continued for an additional 2 h. The pH was then adjusted to 7.2 by dropwise addition of 0.5 mol L^{-1} NaOH.

Aliquots of 40 g of the digests were transferred to polypropylene centrifuge tubes and centrifuged at 15 000 rpm for 30 min at 4 °C to separate soluble and precipitate. AsT and AsI were analyzed in soluble and in precipitate. Both soluble and precipitate were lyophilized before the analysis of AsI.

The soluble AsT and AsI contents ($\mu g\ g^{-1}$ seaweed, raw or cooked) constitute the bioaccessible fraction. The bioaccessibility of these contents, for raw and cooked seaweed, is defined as the proportion of total or AsI in seaweed available for absorption, and it was calculated as

$$\text{bioaccessibility} = \frac{(\text{AsT or AsI in bioaccessible fraction})}{(\text{AsT or AsI in seaweed})} \times 100$$

Determination of AsT (*5*). Raw seaweed (0.25 g) was treated with 2.5 mL of ashing aid suspension (20% w/v $MgNO_3$ + 2% w/v MgO) and 5 mL of nitric acid 50% (v/v). The same procedure was used with lyophilized soluble (0.2 g for *H. fusiforme*, 1 g for *Porphyra* sp., and 3 g for *Enteromorpha* sp.) and with lyophilized precipitate of in vitro digestion (0.25 g). The mixture was evaporated to dryness and mineralized at 450 °C with a gradual increase in temperature. The white ash was dissolved in 6 mol L^{-1} HCl, reduced with 5 mL of reducing solution (5% w/v KI and 5% w/v ascorbic acid), and brought to 25 mL with 6 mol L^{-1} HCl. The analytical conditions used for arsenic determination by FI-HG-AAS were the following: loop sample, 0.5 mL; reducing agent, 0.2% (w/v) $NaBH_4$ in 0.05% (w/v) NaOH, 5 mL min^{-1}, flow rate; HCl solution 10% (v/v), 10 mL min^{-1}, flow rate; carrier gas, argon; 100 mL min^{-1}, flow rate; wavelength, 193.7 nm; spectral band pass, 0.7 nm; electrodeless discharge lamp system 2; lamp current setting, 400 mA; cell temperature, 900 °C.

Determination of AsI (*5*). Deionized water (4.1 mL) and concentrated HCl (18.4 mL; d = 1.19 g mL^{-1}) were added to raw seaweed (0.5 g). The same procedure was used with lyophilized soluble (0.2 g for *H. fusiforme*, 5 g for *Porphyra* sp., and 15 g for *Enteromorpha* sp.) and with lyophilized precipitate of in vitro digestion (0.5 g). The mixture was left overnight. The reducing agent was then added (1 mL of 1.5% (w/v) hydrazine sulfate solution and 2 mL of HBr), and the sample was agitated for 30 s. Then, 10 mL of $CHCl_3$ was added, and after 3 min of shaking and 5 min of centrifuging (2000 rpm), the chloroform phase was separated. The extraction process was repeated two more times, and the chloroform phases were combined and filtered. The AsI in the chloroform phase was back-extracted by shaking for 10 min with 10 mL of 1 mol L^{-1} HCl. The phases were separated by centrifuging at 2000 rpm, and the aqueous phase was then aspirated and poured into a beaker. This stage was repeated once again, and the back extraction phases were combined. The AsI in the back extraction phase was determined by means of the following procedure: 2.5 mL of ashing aid suspension (20% w/v $MgNO_3$ + 2% w/v MgO) and 10 mL of concentrated HNO_3 were added to the combined back extraction phases, dry-ashed, and quantified by FI-HG-AAS in the conditions described previously for quantification of AsT.

Table 1. Accuracy of the Methods Employed for AsT and AsI Determination in Seaweed[a]

	total As (μg g^{-1}, dw)		inorganic As (μg g^{-1}, dw)	
samples	certified	found	previous date[b]	found
Fucus sp.[d]	42.2–46.4	42.3–46.4	1.21–1.33	1.22–1.29
L. major[d]	8.00[c]	7.2–7.7	4.50–4.72	4.49–4.70
U. lactuca[d]	2.89–3.29	2.92–2.97	1.27–1.37	1.26–1.34

[a] Confidence interval at the 95% confidence level for six independent analyses. [b] Almela et al. (2002) (5). [c] Indicative values. [d] *Fucus* sp., IAEA-140/TM (International Atomic Energy Agency); *L. major*, BCR 60 aquatic plant (Institute for Reference Materials and Measurements, IRMM); and *U. lactuca*, BCR-279 sea lettuce (IRMM).

Table 2. AsT and AsI Contents in Seaweed (μg g^{-1}, dw)[a]

	total As		inorganic As	
species (type)	raw	cooked	raw	cooked
Enteromorpha sp. (green)	2.9 ± 0.1^{a}		0.590 ± 0.022^{a}	
Porphyra sp.[b] (red)	$33.8 \pm 2.9^{b,x}$	$29.4 \pm 2.3^{d,x}$	$0.134 \pm 0.013^{b,x}$	$0.127 \pm 0.006^{d,x}$
Hzikia fusiforme[c] (brown)	$99.4 \pm 4.0^{c,x}$	$65.3 \pm 2.6^{e,y}$	$54.3 \pm 2.9^{c,x}$	$30.6 \pm 0.5^{e,y}$

[a] Results expressed as mean values ± SD ($n = 3$). Different superscript letters within a column indicate significant differences ($p < 0.05$) between the three raw seaweeds (a, b, c) and between the two cooked seaweeds (d, e). Different superscript letters within a row indicate significant differences between the same seaweed raw and cooked (x, y). [b] Baked seaweed (200 °C/5 min). [c] Boiled seaweed (100 °C/20 min).

Quality Assurance−Quality Control. The suitability of analytical methods employed for AsT and AsI determination has been checked previously by evaluating their analytical characteristics (limit of detection, precision, and accuracy) (*5*). In the present work, three certified reference materials (CRMs) were employed for quality assurance−quality control of the methodologies used, IAEA-140/TM *Fucus* sp. (International Atomic Energy Agency, Vienna, Austria), BCR 60 aquatic plant *Lagarosiphon major* (Institute for Reference Materials and Measurements, IRMM, Brussels, Belgium), and BCR-279 sea lettuce *Ulva lactuca* (IRMM).

These CRMs have a certified AsT content. There are no CRMs available for which the AsI content is given. The quality criterion adopted, therefore, was to overlap between the ranges of AsI found in these samples and those reported in a previous study carried out by our laboratory (*5*). The results obtained for the accuracy of the methods are summarized in **Table 1**.

Statistical Analysis (*28*). A Student's *t*-test (unpaired design) was applied to determine/evaluate the effects of cooking treatment on the total, inorganic, bioaccessible arsenic content and on bioaccessibility. A one factor analysis of variance and the Tukey test were applied to (i) determine the influence of sample weight on bioaccessible AsT content and bioaccessible AsI content and (ii) detect possible differences in the total, inorganic, bioaccessible arsenic content and on bioaccessibility between the three raw edible seaweeds analyzed. A significance level of $p < 0.05$ was adopted for all comparisons. Statgraphics Plus version 4.0 (Statistical Graphics) was used for the statistical analysis.

RESULTS AND DISCUSSION

Total and AsI Contents in Raw Edible Seaweed. Table 2 shows the AsT and AsI contents in raw edible seaweed. In raw seaweed, the AsT contents varied between 2.9 and 99.4 μg g^{-1} dw, with significant differences in concentration ($p < 0.05$) depending on the type of seaweed analyzed (brown seaweed > red seaweed > green seaweed). *H. fusiforme* had the highest AsT content, which was within the range found by other authors, 19−152 μg g^{-1} dw (*5*, *29*−*34*). For *Porphyra* sp. and *Enteromorpha* sp., the AsT contents found were similar to those described in earlier work carried out by our group (*Porphyra* sp., 24−29 μg g^{-1} dw; *Enteromorpha* sp., 2.3 μg g^{-1} dw (*5*)) and also in work carried out by other authors (*Enteromorpha* sp., 7.2 μg g^{-1} dw (*35*); *Porphyra* sp: 13−24 μg g^{-1} dw (*33*, *35*, *36*)).

With respect to AsI, the content varied between 0.590 and 54.3 μg g^{-1} dw, with a gradation in the concentrations (brown seaweed > green seaweed > red seaweed) that was different from the order observed for AsT. Previous references for AsI contents in edible seaweed are scarce, but the values reported for these three types of seaweed are close to those found in the present study: *Enteromorpha* sp., 0.37 μg g^{-1} dw (*5*); *Porphyra* sp., 0.19−0.57 μg g^{-1} dw (*5*); *H. fusiforme*, 37−88 μg g^{-1} dw (*5*, *29*, *31*).

The very high AsI content in *H. fusiforme* (54.3 μg g^{-1}) exceeds the maximum AsI limit in seaweed products set by France and the U.S.A. at 3 μg g^{-1} (*2*), a limit that in Australia and New Zealand is reduced to 1 μg g^{-1} (*6*). It must be noted that the consumption of just 3 g/day of *H. fusiforme* would imply an AsI intake equal to the toxicological reference value that the WHO has established as the Provisional Tolerable Weekly Intake (15 μg AsI/week/kg of body weight) (*37*). This is a product, therefore, for which suitable measures should be taken in order to provide chemical monitoring of the risks deriving from its consumption.

The percentage of AsI in relation to AsT in raw seaweed was 0.4% for *Porphyra* sp., 20% for *Enteromorpha* sp., and 55% for *H. fusiforme*. This very wide range of percentages includes values characteristic of seafood products of animal origin, where AsI generally represents less than 11% of AsT (*8*), and values characteristic of vegetables, where AsI may represent almost 100% of the AsT of the product (*7*, *38*). Thus, we are dealing here with foods that have a special behavior with respect to the AsI/AsT relation, more dependent on the species of seaweed analyzed than on its classification within a food group (meat, fish, cereals, etc.).

Total and AsI Contents in Cooked Edible Seaweed. Table 2 shows the AsT and AsI contents in the cooked edible seaweed. We did not find previous data for AsT or AsI contents in seaweed subjected to a cooking treatment, so that the results obtained in this study cannot be compared.

The effect of cooking on the AsT and AsI contents was different for each of the seaweeds assayed. In *Porphyra* sp., baking did not produce significant differences ($p > 0.05$) with respect to the AsT and AsI contents of the raw product. For *H. fusiforme*, however, the cooking treatment brought about a statistically significant ($p < 0.05$) reduction in the AsT and AsI concentrations in the resulting product (1.5 and 1.8 times, respectively). The loss of arsenic took place mostly through solubilization of AsI in the cooking water, as shown by the concentrations of AsT (4.6 ± 0.2 μg mL^{-1}) and AsI (3.7 ± 0.1 μg mL^{-1}) found in the water. The losses referred to seaweed were 45.6 ± 1.6 μg g^{-1} seaweed for AsT and 32.0 ± 0.5 μg g^{-1} seaweed for AsI. The result of this loss of AsI was that the percentage of AsI with respect to AsT in cooked *H. fusiforme* was 47%, as compared with 55% obtained in the raw seaweed. Despite the decrease in AsI after cooking, the concentration in cooked *H. fusiforme* considerably exceeded the limits established by the regulations existing in France, the U.S.A., Australia, and New Zealand, and the product continues to constitute a risk (*2*, *6*).

Improvement of in Vitro Gastrointestinal Digestion. The contribution of enzymes and reagents to the AsT content in the bioaccessible fractions was evaluated. To do so, digestion process blanks were analyzed, with the weight of seaweed in

Table 3. Effect of Sample Weight of *H. fusiforme* on the Bioaccessible AsT and AsI Contents (μg g^{-1} Seaweed, dw)[a]

sample weight (g)	total As	inorganic As
2	83.6 ± 2.0[a]	48.2 ± 2.6[a]
	n = 11	n = 7
5	61.9 ± 2.0[b]	40.6 ± 2.0[b]
	n = 9	n = 4
10	32.8 ± 2.5[c]	20.3 ± 1.2[c]
	n = 6	n = 6

[a] Results expressed as mean values ± SD; n = number of replicates. Different superscript letters within a column indicate significant differences ($p < 0.05$) between sample weights.

the in vitro process replaced by water of cellular grade (5 g). The average AsT content found after digestion and analysis by FI-HG-AAS was 0.29 ± 0.045 ng mL^{-1} (n = 14), equivalent to 6.1 ng g^{-1} of seaweed. As the contribution of the blanks was low and the in vitro digestion blank was not totally comparable to the sample, the As content contributed by the digestion blank was not taken into account.

The effect of the centrifugation speed was also studied. The most suitable centrifugation speed for separation of the bioaccessible fraction was optimized in *H. fusiforme* by testing two speeds, 4000 and 15 000 rpm. The results obtained did not show significant ($p < 0.05$) differences for AsT (4000 rpm = 7.1 ± 0.2 μg mL^{-1}; 15 000 rpm = 7.3 ± 0.3 μg mL^{-1}) or for AsI (4000 rpm = 4.5 ± 0.2 μg mL^{-1}; 15 000 rpm = 4.5 ± 0.2 μg mL^{-1}). We selected 15 000 rpm as the optimum speed for the subsequent analyses because it improved the separation between bioaccessible fraction and precipitate.

Finally, to evaluate the influence of the weight of seaweed used in the in vitro digestion process on the bioaccessible AsT and AsI contents, we assayed three weights of *H. fusiforme* sample (2, 5, and 10 g), in all cases keeping the seaweed/enzyme relation constant. The results obtained (**Table 3**) show that a decrease in sample weight brought about a significant ($p < 0.05$) increase in the bioaccessible AsT and AsI contents. This is probably due to the fact that the increase in the weight of sample gives rise to an unusable "bed" of sample during the enzymatic incubation stage, which might make it more difficult for the proteolytic enzymes to act and might lead to a lower solubilization of As. The AsT/AsI relation was of the same order for all of the sample weights (1.5, 1.6, and 1.7). A sample weight of 5 g was selected for the subsequent studies. This weight is within the range of daily seaweed consumption described for the Japanese population (2−12 g seaweed dw day^{-1}), the only consumption data that has come to our notice (*39*).

Analytical Validation of Arsenic Determinations. Matrix interference in the determination of AsT and AsI was checked by the method of standard additions, which was applied to the bioaccessible fraction of *H. fusiforme*. No interference was observed.

The precision of the method was determined from the relative standard deviation (RSD). This was calculated from the analysis, in six different sessions, of three homogeneous samples of bioaccessible fraction of *H. fusiforme* not subjected to cooking treatment (**Table 4**). The RSDs obtained for AsT (12%) and AsI (12%) in the bioaccessible fraction were acceptable, considering the number of stages that make up the process.

Bioaccessible Contents and Bioaccessibility of AsT and AsI from Raw Edible Seaweed. The AsT and AsI contents in the bioaccessible fraction expressed in μg g^{-1} seaweed (**Table 5**) and as bioaccessibility (percentages of bioaccessible AsT and AsI with respect to the total AsT or AsI contents in seaweed (**Table 6**)), obtained by applying the in vitro method previously described, can be considered as pioneering. We do not know of the existence of other studies in which the BA of arsenic species in seaweed is evaluated using in vitro methods that involve a simulated gastrointestinal digestion. However, we must mention the application of in vitro methods that combine a digestion process/mineral uptake with human intestinal Caco-2 cells in order to estimate the BA of iron (*40*) or magnesium (*41*) from mineral-fortified spirulina.

When studying bioaccessibility from the viewpoint of speciation, one should not rule out the possibility that the in vitro digestion method employed may bring about some transformation of the arsenic species present in the initial product. For example, the arsenosugars and DMA, organoarsenical species generally present in seaweed, might generate AsI. However, the references in the literature indicate that it is unlikely that these transformations could take place. In a study on the stability of four arsenosugars at pH 1.1, Gamble et al. (*42*) showed a slow hydrolytic degradation (1.5% h^{-1} at 38 °C), with the generation of another arsenosugar. As far as DMA is concerned,

Table 4. Precision of the in Vitro Digestion Method: Bioaccessible AsT and AsI Contents in *H. fusiforme* (μg g^{-1} Seaweed, dw; Mean ± SD; n = 3)

session	total As	inorganic As
1	85.7 ± 2.3	52.6 ± 1.3
2	66.5 ± 1.6	40.9 ± 0.4
3	76.9 ± 2.2	46.6 ± 1.8
4	88.5 ± 1.3	54.3 ± 0.1
5	74.2 ± 2.5	46.6 ± 1.3
6	67.2 ± 4.6	39.9 ± 0.9
mean concn	75.9 ± 8.9	46.3 ± 5.7
RSD (%)	12	12

Table 5. Bioaccessible AsT and AsI Contents (μg g^{-1} Seaweed, dw) in Raw and Cooked Seaweed[a]

	total As		inorganic As	
species	raw	cooked	raw	cooked
Enteromorpha sp. (green)	0.928 ± 0.07[a]		0.455 ± 0.023[a]	
Porphyra sp.[b] (red)	22.7 ± 1.3[b,x]	25.4 ± 0.9[d,y]	0.060 ± 0.008[b,x]	0.086 ± 0.01[d,y]
H. fusiforme[c] (brown)	61.9 ± 2.3[c,x]	42.9 ± 2.4[e,y]	40.6 ± 2.0[c,x]	26.1 ± 2.0[e,y]

[a] Results expressed as mean values ± SD (n = 6–10). Different superscript letters within a column indicate significant differences ($p < 0.05$) between the three raw seaweeds (a, b, c) and between the two cooked seaweeds (d, e). Different superscript letters within a row indicate significant differences between the same seaweed raw and cooked (x, y). [b] Baked seaweed (200 °C/5 min). [c] Boiled seaweed (100 °C/20 min).

Table 6. Bioaccessibility of AsT and AsI (AsI) in Raw and Cooked Seaweed[a]

	total As		inorganic As	
species	raw	cooked	raw	cooked
Enteromorpha sp. (green)	32.0 ± 2.4[a]		77.2 ± 3.9[a]	
Porphyra sp.[b] (red)	67.2 ± 3.8[b,x]	79.9 ± 2.7[d,y]	48.6 ± 4.1[b,x]	72.6 ± 4.7[d,y]
H. fusiforme[c] (brown)	62.3 ± 2.3[c,x]	65.7 ± 3.9[e,x]	74.7 ± 3.8[c,x]	87.9 ± 4.8[e,y]

[a] Bioaccessibility = [(AsT or AsI in bioaccessible fraction)/(AsT or AsI in seaweed)] × 100. Results expressed as mean values ± SD (n = 6–10). Different superscript letters within a column indicate significant differences ($p < 0.05$) between the three raw seaweeds (a, b, c) and between the two cooked seaweeds (d, e). Different superscript letters within a row indicate significant differences between the same seaweed raw and cooked (x, y). [b] Baked seaweed (200 °C/5 min). [c] Boiled seaweed (100 °C/20 min).

Devesa et al. (*43*) did not observe degradation in standards at pH 4.5−8.0 subjected to 120 °C for 44 min. The conditions employed in both of the studies cited are more drastic than those that we employed; therefore, we considered that the AsI present in the bioaccessible fraction came solely from the AsI existing in the undigested seaweed. Nevertheless, additional studies would be useful to evaluate the possible formation of AsI during the gastrointestinal digestion process.

In the three seaweed samples (raw and cooked), a mass balance was performed after the application of the in vitro digestion method, analyzing the soluble fraction and precipitate resulting from application of the in vitro digestion method. The results of mass balance for AsT were as follows: *Enteromorpha* sp., raw = 90%; *Porphyra* sp., raw = 82% and cooked = 95%; and *H. fusiforme*, raw = 102% and cooked = 112%. For AsI, the results were as follows: *Enteromorpha* sp., raw = 100%; *Porphyra* sp., raw = 97% and cooked = 110%; *H. fusiforme*, raw = 82% and cooked = 112%.

The AsT content in the bioaccessible fraction varied between 0.928 and 61.9 μg g^{-1} dw, with significant ($p < 0.05$) differences between the different seaweeds, presenting the following gradation: brown seaweed > red seaweed > green seaweed (**Table 5**). For AsI, the content varied between 0.060 and 40.6 μg g^{-1} dw, the differences between the seaweeds were significant ($p < 0.05$), and the gradation was different from that of AsT (brown seaweed > green seaweed > red seaweed) (**Table 5**). The very high bioaccessible AsI content in *H. fusiforme* (40.6 μg g^{-1} dw) is a further reminder of the health risk of this product.

When the results are expressed as percentages, i.e., as bioaccessibility (**Table 6**), it can be seen that the bioaccessibility of AsT in the red seaweed (67.2%) was slightly higher ($p < 0.05$) than that of brown seaweed (62.3%), and in both cases, the value was double that of the bioaccessibility in green seaweed (32%). Thus, green seaweed had the lowest AsT content and the lowest bioaccessibility. This might be due to a different capability of the enzymes in the in vitro method for releasing the As existing in each of the samples or to differences in the composition of the seaweeds, which might affect the solubility of the As. We did not observe the same tendency for the AsI in raw seaweed, where the bioaccessibility was higher in brown seaweed (74.7%) and green seaweed (77.2%) than in red seaweed (48.6%).

Bioaccessible Contents and Bioaccessibility of AsT and AsI from Cooked Edible Seaweed. In the bioaccessible fraction of the cooked seaweed (**Table 5**), the AsT varied between 25.4 and 42.9 μg g^{-1} seaweed dw, and the AsI was between 0.086 and 26.1 μg g^{-1} seaweed dw. In *Porphyra* sp., there was a significant increase ($p < 0.05$) in the AsT and AsI contents in the bioaccessible fraction of the cooked seaweed with respect to the raw seaweed, with a greater increase in AsI (43% higher than the value for raw seaweed). For *H. fusiforme*, the AsT and AsI contents in the bioaccessible fraction of the cooked seaweed were significantly lower ($p < 0.05$) than those existing in the bioaccessible fraction obtained from the raw seaweed, as was to be expected because of the difference in the contents in the initial sample (raw and cooked).

An interesting aspect is the proportion of toxic species existing in the seaweed and in the digest. For *H. fusiforme* cooked but not subjected to the in vitro digestion method (**Table 2**), AsI represented 46.7% of the AsT present in the sample. However, when the in vitro digestion method was applied to this seaweed, AsI represented 60.9% of the AsT in the resulting bioaccessible fraction (**Table 5**). It seems, therefore, that in vitro digestion of *H. fusiforme* favors the solubilization of AsI rather than other arsenic species, such as arsenosugars, present in the seaweed.

With regard to bioaccessibility (**Table 6**), a significant ($p < 0.05$) increase in AsI was observed after cooking, both in *Porphyra* sp. and in *H. fusiforme*, with a greater increase in *Porphyra* sp. With respect to AsT, a significant increase was only observed in *Porphyra* sp. after cooking. The differences between these two seaweeds might be due to the different thermal treatment used, baking in the case of *Porphyra* sp. and boiling for *H. fusiforme*. The effect that each of these treatments has on proteins, to whose sulfhydryl groups AsI bonds predominantly, might affect the efficiency with which the digestive enzymes employed release the As in each of the samples. It is noteworthy that in both of these seaweeds over 70% of the AsI existing in the cooked product was bioaccessible.

CONCLUSIONS

Of the three edible seaweeds analyzed, only *H. fusiforme*, both raw and cooked, presented toxicologically alarming levels of AsI. The influence of cooking on the AsT and AsI contents differs depending on the type of seaweed and the treatment applied. In *H. fusiforme*, the high levels of AsI in the bioaccessible fraction of the cooked seaweed (26.1 μg g^{-1} seaweed) once again call attention to the possible risk presented by this food. The bioaccessibility of AsI in raw seaweed was over 40% in all of the samples and over 70% for cooked seaweed, indicating that the toxic AsI remains available in these foods for absorption into the intestinal mucosa.

LITERATURE CITED

(1) Fleurence J. Seaweed proteins: biochemical, nutritional aspects and potential uses. *Trends Food Sci. Technol.* **1999**, *10*, 25−28.
(2) Mabeau, S.; Fleurence, J. Seaweed in food products: biochemical and nutritional aspects. *Trends Food Sci. Technol.* **1993**, *4*, 103−107.
(3) Rupérez, P. Mineral content of edible marine seaweeds. *Food Chem.* **2002**, *79*, 23−26.
(4) Goñi, I.; Gudiel-Urbano, M.; Saura-Calixto, F. In vitro determination of digestible and unavailable protein in edible seaweed. *J. Sci. Food Agric.* **2002**, *82*, 1850−1854.
(5) Almela, C.; Algora, S.; Benito, V.; Clemente, M. J.; Devesa, V.; Súñer, M. A.; Vélez, D.; Montoro, R. Heavy metals, total arsenic and inorganic arsenic contents of algae food products. *J. Agric. Food Chem.* **2002**, *50*, 918−923.
(6) ANZFA, Australian New Zealand Food Authority. Food Standards Code, Issue 41, 1997.
(7) Schoof, R. A.; Yost, L. J.; Eickhoff, J.; Crecelius, E. A.; Cragin, D. W.; Meacher, D. M.; Menzel, D. B. A market basket survey of inorganic arsenic in food. *Food Chem. Toxicol.* **1999**, *37*, 839−846.
(8) Muñoz, O.; Devesa, V.; Suñer, M. A.; Vélez, D.; Montoro, R.; Urieta, I.; Macho, M.; Jalón, M. Total and inorganic arsenic in fresh and processed fish products. *J. Agric. Food Chem.* **2000**, *48*, 4369−4376.
(9) Súñer, M. A.; Devesa, V.; Clemente, M. J.; Vélez, D.; Montoro, R.; Urieta, I.; Jalón, M.; Macho, M. L. Organoarsenical species contents in fresh and processed seafood products. *J. Agric. Food Chem.* **2002**, *50*, 924−932.
(10) Devesa, V.; Martínez, A.; Súñer, M. A.; Vélez, D.; Almela, C.; Montoro, R. Effect of cooking temperatures on chemical changes in species of organic arsenic in seafood. *J. Agric. Food Chem.* **2001**, *49*, 2272−2276.
(11) Hanaoka, K.; Goessler, W.; Ohno, H.; Irgolic, K. J.; Kaise, T. Formation of toxic arsenical in roasted muscles of marine animals. *Appl. Organomet. Chem.* **2001**, *15*, 61−66.
(12) Yost, L. J.; Schoof, R. A.; Aucoin, R. Intake of inorganic arsenic in the North American diet. *Hum. Ecol. Risk Assess.* **1998**, *4*, 137−152.

(13) Ysart, G.; Miller, P.; Crews, H.; Robb, P.; Baxter, M.; De l'Argy, C.; Lofthouse, S.; Sargent, C.; Harrison, N. Dietary exposure estimates of 30 elements from UK Total Diet study. *Food Addit. Contam.* **1999**, *16*, 391−403.

(14) Del Razo, L. M.; Garcia-Vargas, G. C.; Garcia-Salcedo, J.; Sanmiguel, M. F.; Rivera, M.; Hernández, M. C.; Cebrian, M. E. Arsenic levels in cooked food and assessment of adult dietary intake of arsenic in the Region Lagunera, Mexico. *Food Chem. Toxicol.* **2002**, *40*, 1423−1431.

(15) Llobet, J. M.; Falco, G.; Casas, C.; Teixido, A.; Domingo, J. L. Concentrations of arsenic, cadmium, mercury, and lead in common foods and estimated daily intake by children, adolescents, adults, and seniors of Catalonia Spain. *J. Agric. Food Chem.* **2003**, *51*, 838−842.

(16) Vahter, M. Mechanisms of arsenic biotransformation. *Toxicology* **2002**, *181*−*182*, 211−217.

(17) Kitchin, K. T. Recent advances in arsenic carcinogenesis: modes of action, animal model systems, and methylated arsenic metabolites. *Toxicol. Appl. Pharmacol.* **2001**, *172*, 249−261.

(18) Sanz-Medel, A. Trace elements analytical speciation in biological systems: importance, challenges and trends. *Spectrochim. Acta B* **1998**, *53*, 197−211.

(19) Ebdon, L.; Pitts, L. An overview. In *Trace Element Speciation for Environment, Food and Health*; Ebdon, L., Pitts, L., Cornelis, R., Crews, H., Donard, O. F. X., Quevauviller, Ph., Eds.; The Royal Society of Chemistry: Cambridge, U.K., 2001; pp 375−384.

(20) DRI, Dietary Reference Intakes for calcium, phosphorus, magnesium, vitamin D and fluoride. http://www.nap.edu.openbook/0309063507/html/21.html.copyright. The National Academy Press, 2000.

(21) Hocquellet, P.; L'Hotellier, M.-D. Bioavailability and speciation of mineral micronutrients: The enzymolysis approach. *J. AOAC Int.* **1997**, *80*, 920−927.

(22) Ekmekcioglu, C. A physiological approach for preparing and conducting intestinal bioavailability studies using experimental systems. *Food Chem.* **2002**, *76*, 225−230.

(23) Van Campen, D. R.; Glahn, R. P. Micronutrient bioavailability techniques: Accuracy, problems and limitations. *Field Crops Res.* **1999**, *60*, 93−113.

(24) Ruby, M. V.; Schoof, R.; Brattin, W.; Goldade, M.; Post, G.; Harnois, M.; Mosby, D. E.; Casteel, S. W.; Berti, W.; Carpenter, M.; Edwards, D.; Cragin, D.; Chappell, W. Advances in evaluating the oral bioavailability of inorganics in soil for use in human health risk assessment. *Environ. Sci. Technol.* **1999**, *33*, 3697−3705.

(25) Crews, H. M.; Burrell, J. A.; McWeeny, D. J. Trace element solubility from food following enzymolysis. *Z. Lebensm. Unters Forsch.* **1985**, *180*, 221−226.

(26) Hamel, S. C.; Ellickson, K. M.; Lioy, P. J. The estimation of the bioaccessibility of heavy metals in soils using artificial biofluids by two novel methods: mass-balance and soil recapture. *Sci. Total Environ.* **1999**, *243*, 273−283.

(27) Jovaní, M.; Barberá, R.; Farré, R.; Martín de Aguilera, E. Calcium, Iron, and Zinc uptake from digests of infant formulas by Caco-2 cells. *J. Agric. Food Chem.* **2001**, *49*, 3480−3485.

(28) Box, G. E. P.; Hunter, N. G.; Hunter, J. S. Statistics for Experimenters. *An Introduction to Design, Data Analysis and Model Building*; John Wiley & Sons: New York, 1978.

(29) Yasui, A.; Tsutsumi, C.; Toda, S. Selective determination of inorganic arsenic (III), (V) and organic arsenic in biological materials by solvents extraction, atomic absorption spectrophotometry. *Agric. Biol. Chem.* **1978**, *42*, 2139−2145.

(30) Watanabe, T.; Hirayama, T.; Takahashi, T.; Kokubo, T.; Ikeda, M. Toxicological evaluation of arsenic in edible seaweed, *Hizikia* species. *Toxicology* **1979**, *14*, 1−22.

(31) Phillips, D. J. H. The chemical forms of arsenic in aquatic organisms and their interrelationships. In *Arsenic in the Environment. Part I: Cycling and Characterization*; Nriagu, J. O., Ed.; John Wiley & Sons: New York, 1994; pp 26, 263−288.

(32) Munilla, M. A.; Gomez-Pinilla, I.; Rodenas, S.; Larrea, M. T. Determination of metals in seaweeds used as food by inductively coupled plasma atomic-emission spectrometry. *Analusis* **1995**, *23*, 463−466.

(33) van Netten, C.; Hoption Cann, S. A.; Morley, D. R.; van Netten, J. P. Elemental and radioactive analysis of commercially available seaweed. *Sci. Total Environ.* **2000**, *255*, 169−175.

(34) McSheehy, S.; Szpunar, J. Speciation of arsenic in edible seaweed by bi-dimensional size-exclusion anion exchange HPLC with dual ICP-MS and electrospray MS/MS detection. *J. Anal. At. Spectrom.* **2000**, *15*, 79−87.

(35) Phaneuf, D.; Cote, I.; Dumas, P.; Ferron, L. A.; LeBlanc, A. Evaluation of the contamination of marine algae (Seaweed) from the St. Lawrence river and likely to be consumed by humans. *Environ. Res. Sect. A* **1999**, *80*, S175−S182.

(36) Shibata, Y.; Jin, K.; Morita, M. Arsenic compounds in the edible red algae, *Porphyra tenera* and in *nori* and *yakinori*, food items produced from red algae. *Appl. Organomet. Chem.* **1990**, *4*, 255−260.

(37) WHO. Evaluation of Certain Food Additives and Contaminants; *33rd Report of the Joint FAO/WHO Expert Committee on Food Additives*; WHO Technical Report Series 759; WHO: Geneva, Switzerland, 1989.

(38) Muñoz, O.; Diaz, O. P.; Leyton, I.; Nuñez, N, Devesa, V.; Súñer, M. A.; Vélez, D.; Montoro, R. Vegetables collected in the cultivated Andean area of northern Chile: total and inorganic arsenic contents in raw vegetables. *J. Agric. Food Chem.* **2002**, *50*, 642−647.

(39) Sakurai, T.; Kaise, T.; Ochi, T.; Saitoh, T.; Matsubara, C. Study of in vitro cytotoxicity of a water soluble organic arsenic compound, arsenosugar, in seaweed. *Toxicology* **1997**, *122*, 205−212.

(40) Puyfoulhoux, G.; Rouanet, J.-M.; Besançon, P.; Baroux, B.; Baccou, J.-C.; Caporiccio, B. Iron availability from iron-fortified spirulina by an in vitro digestion/Caco-2 cell culture model. *J. Agric. Food Chem.* **2001**, *49*, 1625−1629.

(41) Planes, P.; Rouanet, J.-M.; Laurent, C.; Baccou, J. C.; Besançon, P.; Caporiccio, B. Magnesium bioavailability from magnesium-fortified spirulina in cultured human intestinal Caco-2 cells. *Food Chem.* **2002**, *77*, 213−218.

(42) Gamble, B. M.; Gallagher, P. A.; Shoemaker, J. A.; Weis, X.; Schwegel, C. A.; Creed, J. T. An investigation of the chemical stability of arsenosugars in simulated gastric juice and acidic environments using IC-ICP-MS and IC-ESI-MS/MS. *Analyst* **2002**, *127*, 781−785.

(43) Devesa, V.; Martínez, A.; Súñer, M. A.; Benito, V.; Vélez, D.; Montoro, R. Kinetic study of transformations of arsenic species during heat treatment. *J. Agric. Food Chem.* **2001**, *49*, 2267−2271.

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