APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2004; **18**: 662–669
Published online in Wiley InterScience (www.interscience.wiley.com). DOI:10.1002/aoc.732

Bioaccessibility of inorganic arsenic species in raw and cooked *Hizikia fusiforme* seaweed

J. M. Laparra[1], D. Vélez[1], R. Montoro[1]*, R. Barberá[2] and R. Farré[2]

[1]Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Apartado 73, 46100 Burjassot (Valencia), Spain
[2]Nutrition and Food Chemistry, Faculty of Pharmacy, University of Valencia, Avda. Vicente Andrés Estellés s/n, 46100 Burjassot, Spain

Received 24 April 2004; Accepted 19 June 2004

Samples of *Hizikia fusiforme* edible seaweed, a commercially available dried food with high concentrations of total arsenic (t-As) and inorganic arsenic (i-As), both raw and cooked (boiling at 100 °C, 20 min), were selected for the bioaccessibility study. Cooking caused a significant reduction in the concentrations of t-As (30–43%) and i-As (46–50%), despite which the i-As contents in the cooked product were high (42.7–44.6 μg g^{-1} seaweed). An *in vitro* gastrointestinal digestion (pepsin, pH 2, and pancreatin–bile extract, pH 7) was applied to the seaweed to estimate arsenic bioaccessibility (maximum soluble concentration in gastrointestinal medium) of t-As, i-As, arsenic(III) and arsenic(V). The influence of the gastric and intestinal stages of the *in vitro* digestion method was evaluated. The gastric stage is the key stage in the solubilization of both t-As and i-As. The bioaccessible i-As in raw seaweed (54.0–66.5%) increases after cooking (78.3–84.4%), a fact that is considered to be of interest because this is the usual form in which this seaweed is ingested. Speciation of the i-As in the bioaccessible fraction revealed a different arsenic(III)/arsenic(V) relationship in the product when raw or cooked. When raw, the majority species was arsenic(III) after either the gastric or the gastrointestinal stage, whereas in the cooked product it depended on the batch analysed, with bioaccessible arsenic(III) contents of 7.1–25.4 μg g^{-1} of dried seaweed, which represents 5–17% of the i-As tolerable daily intake.

KEYWORDS: arsenite; arsenate; inorganic arsenic; seaweed; cooking; bioaccessibility

INTRODUCTION

Of all the species of arsenic that humans can ingest from foods, inorganic arsenic(III) and arsenic(V) species are the most toxic. The sum of these two species, known as inorganic arsenic (i-As), has been classified as a human carcinogen by the International Agency for Research on Cancer,[1] and, on the basis of epidemiological data referring to i-As in drinking water, the WHO has established a provisional tolerable weekly intake (PTWI) for i-As of 15 μg $week^{-1}$ per kilogram body weight.[2]

Seafoods, including fish, molluscs, crustaceans and edible seaweed, are the foods in which the highest arsenic contents are found. The i-As contents in fish and fish products generally do not exceed 0.1 mg kg^{-1} wet weight,[3] so that at present it is considered that fish consumption does not represent a health risk. For edible seaweed the situation might well be different, as high contents of i-As have been quantified in species such as *Hizikia fusiforme*,[4–7] attaining 135 mg kg^{-1} dry weight (DW), 91% of the total arsenic (t-As).[8] These high contents do not seem to be attributable to growth of the seaweed in an environment contaminated by arsenic, but rather to its natural tendency to accumulate i-As. We are not aware of any studies that evaluate dietary exposure to i-As in seaweed, but an estimation performed by Almela *et al*.[6] on the basis of an analysis of various seaweeds showed that the risk of exceeding the PTWI value is a reality with a consumption of 3 g day^{-1} of *H. fusiforme*.

Any evaluation of the risk associated with the ingestion of i-As should consider not only the content in the product, but also the arsenic bioavailability for humans (fraction of arsenic absorbed which reaches the systemic circulation and is available to exercise its action in the receiving organism).

*Correspondence to: R. Montoro, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Apartado 73, 46100 Burjassot (Valencia), Spain.
E-mail: rmontoro@iata.csic.es
Contract/grant sponsor: Ministry of Science and Technology (MCyT).

Bioavailability depends largely on the ability to cross the intestinal barrier of the ultimate soluble physicochemical forms in which arsenic, after gastrointestinal digestion, reaches the absorption site, mainly the duodenum. After human gastrointestinal digestion the soluble arsenic species need not necessarily coincide with the species present in the raw product, as modifications of species may be brought about both by processing of the product[9] and by the digestive process.[10] The *in vitro* gastrointestinal models offer a simple and inexpensive approach to achieve information about bioavailability.[11] The results of these models must be taken as relative indexes for bioavailability, which means that the methods provide a good basis for establishing tendencies, making comparisons and determining effects caused by different factors.[12]

Various models of *in vitro* digestion have been used to estimate the bioaccessible fraction (maximum concentration soluble in simulated gastrointestinal media that is available for subsequent processes of absorption into the intestinal mucosa)[13] of arsenic in soil.[13,14] However, the bioaccessible fraction of arsenic in foods has only been studied in seaweed.[7,15] In the work carried out by Laparra *et al.*,[7] it was shown that over 40% of the i-As present in raw seaweed and over 70% of the content in cooked seaweed remains available for absorption into the intestinal mucosa.

The aim of the present work was to study the influence of the gastric and intestinal stages of a simulated human digestion method on the bioaccessibility of t-As, i-As, arsenic(III) and arsenic(V) in *H. fusiforme* edible seaweed. The effect of cooking of the seaweed on the bioaccessibility of these arsenic species was also studied.

MATERIALS AND METHODS

Instruments

For the separation of arsenic species the high-performance liquid chromatography (HPLC) system consisted of a Hewlett Packard Model 1100 (Barcelona, Spain) with a quaternary pump, an on-line degassing system, an automatic injector and a thermostatted column compartment. Separations were performed on a Hamilton PRP-X100 anion-exchange column (10 μm, 250 mm × 4.1 mm i.d., Teknokroma, Barcelona, Spain). A guard column packed with the same stationary phase (12–20 μm; 25 mm × 2.3 mm i.d.) preceded the analytical column. For quantification of arsenic species, the outlet of the HPLC column was directed to a hydride-generation system (PSA 10.004, Analytical, UK) coupled to an atomic fluorescence spectrometry (AFS) system (PSA 10.044 Excalibur PS, Analytical, UK) equipped with a boosted-discharge hollow cathode lamp (BDHCL, Photron, Super Lamp, Victoria, Australia). A Hewlett Packard Model 35 900 C digital–analog converter was used to acquire the AFS signal, which was processed by the chromatographic software.

For t-As determination, a Perkin Elmer (PE) model 3300 atomic absorption spectrometer equipped with an autosampler (PE AS-90), a flow-injection hydride-generation system (PE FIAS-400) and an electrothermally heated quartz cell was employed. Other equipment used included a lyophilizer equipped with a microprocessor controlling the lyophilization process (FTS Systems, New York, USA), 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, 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 glassware was treated with 10% (v/v) HNO_3 for 24 h and then rinsed three times with deionized water before being used.

For the *in vitro* gastrointestinal digestion, enzymes and bile salts were purchased from Sigma Chemical Co. (St Louis, MO): pepsin (Porcine: cat. no. P-7000), pancreatin (Porcine; cat. no. P-1750), and bile extract (Porcine; cat. no. B-8631). Standard solutions of arsenic(V) (Merck) and arsenic(III) [prepared by dissolving 1.320 g of arsenic trioxide (Riedel de Haën, Hanover, Germany) in 25 mL 20% (w/v) KOH solution, neutralized with 20% (v/v) H_2SO_4 and diluted to 1 l with 1% (v/v) H_2SO_4)] were employed.

Samples

H. fusiforme edible brown seaweed, sold as dry seaweed, cut and packed in plastic, was purchased in healthfood stores in the city of Valencia (Spain). Samples from three different batches (A, B, C) were analysed just as they were sold, which we have called the raw state, and after being cooked by applying the cooking treatment indicated on the product label: boil in water 100 °C/20 min (10 g of seaweed/167 ml of water). The samples (raw and cooked) were maintained at 4 °C until analysis.

In vitro gastrointestinal digestion[7]

Samples of each batch of *H. fusiforme* (5 g), raw or cooked, were weighed and cellular-grade water (90 ml) was added. 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 2.0. 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 with water, and incubated in a shaking water bath (stroke rate 120 min^{-1}) at 37 °C for 2 h. With the gastric digestion terminated at this point, 40 g aliquots were transferred from half the samples to polypropylene centrifuge tubes and centrifuged (15 000 rpm/30 min/4 °C) to separate the soluble fraction. The t-As, i-As and individual species arsenic(III) and arsenic(V) were determined in these gastric soluble fractions.

In the other half of the samples the digestion continued in the intestinal stage. For this purpose the pH value was raised to pH 5.0 by drop-wise 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 2 h. The pH was then adjusted to 7.2 by drop-wise addition of 0.5 mol l^{-1} NaOH. Aliquots of 40 g were transferred to polypropylene centrifuge tubes and centrifuged (15 000 rpm/30 min/4 °C) to separate the soluble fraction. The t-As, i-As and individual species arsenic(III) and arsenic(V) were determined in these gastrointestinal soluble fractions.

Determination of t-As

Analysis was performed by flow-injection hydride-generation atomic absorption spectrometry (FI-HG-AAS) after a dry ashing step.[6] Samples of seaweed and soluble fractions from the gastric and gastrointestinal stages obtained by *in vitro* digestion were analysed.

Raw and cooked seaweed (0.25 g) or soluble fraction (0.2 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 mixture was evaporated to dryness and mineralized at 450 °C with a gradual increase in temperature. The white ash was dissolved in 5 ml of 6 mol l^{-1} HCl and reduced with 5 ml of reducing solution (5% w/v KI and 5% w/v ascorbic acid). After 30 min this solution was filtered through Whatman No. 1 filter paper into a 25 ml volumetric flask and diluted to volume with 6 mol l^{-1} HCl. The arsenic was quantified by FI-HG-AAS using the following instrumental conditions: 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.

The accuracy of measurement throughout the experiment was checked by analysing a certified reference material with each batch of sample: BCR-279 sea lettuce *Ulva lactuca* (Institute for Reference Materials and Measurements, IRMM, Brussels, Belgium).

Determination of i-As

Analysis was performed by acid digestion, solvent extraction FI-HG-AAS.[6] Samples of seaweed and soluble fractions from the gastric and gastrointestinal stages obtained by *in vitro* digestion were analysed.

Deionized water (4.1 ml) and concentrated HCl (18.4 ml) were added to raw and cooked seaweed (0.5 g) or soluble fraction (0.2 g) and the mixture was left overnight. After reduction by HBr (2 ml) and hydrazine sulfate (1.5% w/v, 1 ml), the i-As was extracted into chloroform (3 × 10 ml) and back-extracted into 1 mol l^{-1} HCl (2 × 10 ml). For determination of i-As in the back-extraction phase, 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. The mixture was evaporated to dryness and then treated in the same way as for t-As (dry ashing FI-HG-AAS).

There are no reference materials with certified i-As content, so the quality criterion adopted was the overlapping between the ranges of i-As found in BCR-279 sea lettuce *U. lactuca* (IRMM) and those reported in this sample in a previous study (1.21–1.33 μg g^{-1} DW).[6]

Clean-up procedure

The soluble fractions (gastric and gastrointestinal stages) obtained after applying the *in vitro* digestion method to both raw and cooked *H. fusiforme* were subjected to a clean-up procedure prior to quantification of arsenic(III) and arsenic(V) by HPLC–HG-AFS. A strong cation-exchanger resin AG 50W-X8 H^+ (100–200 mesh, Bio Rad), bed height 30 mm, bed diameter 10 mm, was used.[16] The sample was eluted by gravity. Aliquots of soluble fraction (10 g) were adjusted to a pH less than 2 with 4 mol l^{-1} HCl. The acidified solution was passed through the resin, which was then washed with 20 ml of 0.01 mol l^{-1} HCl solution and 20 ml of deionized water. The two fractions were collected jointly and lyophilized. The dry residue was redissolved with deionized water (5 ml) and filtered through a Whatman 0.45 μm filter before HPLC injection.

Determination of arsenic(III) and arsenic(V) by HPLC–HG-AFS

Aliquots of 100 μl of the soluble fractions (gastric and gastrointestinal) treated by the clean-up procedure were injected into the PRP-X100 anion-exchange column. Separations were performed with a flow rate of 1 ml min^{-1} at 25 °C with a gradient of mobile phases (A: 5 mmol l^{-1} $(NH_4)H_2PO_4$, pH 5.75; B: 100 mmol l^{-1} $(NH_4)H_2PO_4$, pH 5.75; gradient programme 0–4 min: 100% A; 4.1–10 min: 50% A and 50% B; 10.1–15 min: 100% A). The outlet of the HPLC column was mixed with a continuous flow of HCl (1.5 mol l^{-1}, 6.0 ml min^{-1}) and $NaBH_4$ (1.5% w/v $NaBH_4$ in 0.7% w/v NaOH, 2.5 ml min^{-1}) using PTFE tubing and T-joints. Using a gas–liquid separator and a continuous flow of argon (300 ml min^{-1} flow rate), the arsines generated were introduced into the AFS system by means of a hygroscopic-membrane drying tube (Perma Pure). An additional flow of hydrogen gas (60 ml min^{-1}) permitted partial maintenance of the flame. The arsenic lamp operated at a primary current of 27.5 mA and a boost current of 35 mA.

Arsenic(III) and arsenic(V) were identified by matching the retention times of the peaks in the sample chromatograms with those obtained from standards, and were quantified with external calibration curves established with arsenic(III) and arsenic(V).

Statistical analysis

A paired-sample comparison by a Student's *t*-test was applied to evaluate differences in t-As and i-As contents.[17] 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

In the raw samples, t-As and i-As were analysed and were subjected to gastrointestinal digestion. The samples were cooked and the resulting wet product was analysed for t-As and i-As and subjected to gastrointestinal digestion. In order to compare the results obtained in the analysis of raw and cooked samples the results were expressed in the same units, as micrograms per gram of seaweed, dry weight. In doing so we took into account the residual moisture in the raw seaweed and the moisture of the cooked product.

The t-As and i-As contents in raw and cooked *H. fusiforme*

In the raw seaweed, the three manufactured batches analysed (A, B, C) all had very high arsenic contents: the t-As was 125.8–131.7 μg g^{-1} seaweed, DW, and the i-As was 79.7–87.7 μg g^{-1} seaweed, DW, (Table 1). It is worth stressing the high percentage of i-As with respect to t-As (62–70%), unlike the situation in other brown seaweeds, where the abundance of literature published, although not necessarily coinciding in terms of extraction methods, shows that arsenosugars are the majority species.

Boiling (100 °C/20 min) caused a significant reduction ($p < 0.05$) in the contents with respect to the raw product (Table 1): 30–43% for t-As and 46–50% for i-As. The losses of arsenic were caused by solubilization in the cooking water. Hanaoka *et al*.[8] have shown that treatments of washing and soaking with water applied before cooking reduce the t-As content of *H. fusiforme* in a range from 32% to 60%, the reduction being due mainly to loss of i-As. In our work, the results obtained after analysis of batch A, i.e. the mass balance between the t-As in the raw sample (128.7 ± 2.9 μg g^{-1} seaweed, DW), the t-As in the cooked sample (90.7 ± 7.1 μg g^{-1} seaweed, DW) and the t-As in the cooking water (41.9 ± 1.8 μg g^{-1} seaweed, DW), rule out the possibility of losses of arsenic by some other mechanism. In the cooking water there was a predominance of arsenic(V) (37.9 ± 1.8 μg g^{-1} seaweed, DW) over arsenic(III) (0.43 ± 0.11 μg g^{-1} seaweed, DW).

Despite the decrease in the i-As contents as a result of cooking, the final concentration in the cooked product was still high (42.7–44.6 μg g^{-1} seaweed), much higher than the concentrations found previously in any other seaweed or food consumed by humans. This particular food should, therefore, be the object of greater toxicological attention because of its high content of carcinogenic i-As. The results obtained also show how important it is in the evaluation of risk assessments to consider the effect that the treatments applied to seaweeds before consumption have on the i-As contents.

The t-As and i-As contents found in the three batches analysed were fairly homogeneous. The significant differences ($p < 0.05$) observed were between the t-As values of two of the batches of cooked seaweed analysed (A and B) and between the i-As values of raw batch A compared with the other two batches.

The t-As and i-As in soluble fractions

The t-As and i-As contents in the soluble fractions obtained after applying a gastric and a gastrointestinal stage to the various batches of raw and cooked *H. fusiforme* are shown in Table 2. In raw *H. fusiforme*, the solubilized t-As in the gastric stage decreased significantly ($p < 0.05$) during the intestinal stage in batches A and C, remaining unchanged in batch B. For i-As, the solubility only varied significantly ($p > 0.05$) between the gastric stage and the intestinal stage in batch C.

In cooked *H. fusiforme* the behaviour was different from that described for raw seaweed, as no statistically significant changes ($p > 0.05$) took place in t-As or i-As solubilized during the gastric and gastrointestinal stages.

The results obtained show that in both raw and cooked *H. fusiforme* the gastric stage limits the maximum content of t-As and solubilized i-As (Table 2). This was also shown by Hamel *et al*.[18] in a study on bioavailability of arsenic from soils, in which they indicated that the stomach is the region of the gastrointestinal tract that is considered to have the greatest influence on arsenic bioavailability. The low pH in the gastric stage seems to be a prerequisite for solubilizing arsenic.[14] In fact, in studies carried out with contaminated soils, a linear correlation ($r = 0.82$) was obtained between the arsenic solubilized in the gastric stage after *in vitro* gastrointestinal digestion and excretion of arsenic in the urine of immature

Table 1. Total and inorganic arsenic contents in raw and cooked *Hizikia fusiforme* (μg g^{-1}, dry weight)

	Total As		Inorganic As	
Batch	Raw	Cooked	Raw	Cooked
A	$128.7 \pm 2.9^{a,x}$	$90.7 \pm 7.1^{b,x}$	$79.7 \pm 1.01^{a,x}$	$42.8 \pm 2.1^{b,x}$
B	$131.7 \pm 0.5^{a,x}$	$75.3 \pm 2.3^{b,y}$	$86.0 \pm 4.3^{a,y}$	$42.7 \pm 2.6^{b,x}$
C	$125.8 \pm 5.9^{a,x}$	$83.7 \pm 4.8^{b,xy}$	$87.7 \pm 3.0^{a,y}$	$44.6 \pm 4.4^{b,x}$

Results expressed as mean values ± SD (n = 3).
[a,b] A difference in this superscript letter in the same row indicates significant differences ($p < 0.05$) for a given batch between raw and cooked values.
[x,y] A difference in this superscript letter in the same column indicates significant differences ($p < 0.05$) between batches.

Table 2. Total and inorganic soluble arsenic contents (μg g^{-1} seaweed, dry weight) and bioaccessibility (percentages of bioaccessible AsT and AsI with respect to the total AsT or AsI contents in seaweed) obtained after the gastric and gastrointestinal stages of an *in vitro* digestion method applied to raw and cooked *H. fusiforme*

		Raw				Cooked			
		t-As		i-As		t-As		i-As	
Batch	*In vitro* stage	μg g^{-1}	%	μg g^{-1}	%	μg g^{-1}	%	μg g^{-1}	%
A	G	$90.3 \pm 3.4^{a,x}$	$69.6 \pm 3.1^{a,x}$	$57.8 \pm 5.8^{a,x}$	$72.5 \pm 7.3^{a,x}$	$57.1 \pm 2.5^{a,x}$	$62.9 \pm 4.8^{a,x}$	$31.7 \pm 2.6^{a,x}$	$74.1 \pm 6.1^{a,x}$
	GI	$76.5 \pm 7.4^{b,x}$	$60.7 \pm 5.4^{b,x}$	$53.0 \pm 0.8^{a,x}$	$66.5 \pm 1.0^{a,x}$	$52.1 \pm 4.3^{a,x}$	$61.9 \pm 2.5^{a,x}$	$34.8 \pm 3.0^{a,x}$	$81.4 \pm 7.1^{a,x}$
B	G	$98.6 \pm 8.0^{a,xy}$	$74.3 \pm 5.4^{a,x}$	$53.8 \pm 0.8^{a,x}$	$62.5 \pm 1.0^{a,x}$	$51.1 \pm 3.1^{a,x}$	$67.8 \pm 4.1^{a,xy}$	$32.4 \pm 2.7^{a,x}$	$75.8 \pm 6.4^{a,x}$
	GI	$97.3 \pm 3.3^{a,y}$	$73.9 \pm 2.5^{a,y}$	$51.3 \pm 1.7^{a,xy}$	$59.7 \pm 2.0^{a,y}$	$55.7 \pm 1.5^{a,x}$	$74.0 \pm 1.9^{b,y}$	$36.1 \pm 2.3^{a,x}$	$84.4 \pm 5.5^{a,x}$
C	G	$106.0 \pm 5.4^{a,y}$	$84.3 \pm 4.3^{a,y}$	$57.7 \pm 0.5^{a,x}$	$64.5 \pm 2.3^{a,x}$	$54.9 \pm 3.9^{a,x}$	$72.9 \pm 5.2^{a,y}$	$32.0 \pm 2.1^{a,x}$	$71.7 \pm 4.7^{a,x}$
	GI	$69.9 \pm 3.2^{b,x}$	$55.6 \pm 2.6^{b,x}$	$48.4 \pm 0.9^{b,y}$	$54.0 \pm 2.0^{b,y}$	$52.0 \pm 4.2^{a,x}$	$68.9 \pm 5.5^{a,y}$	$35.7 \pm 2.3^{a,x}$	$78.3 \pm 1.2^{a,x}$

Results are expressed as mean ± standard deviation ($n = 3–4$).
G: gastric stage; GI: gastrointestinal stage.
[a,b] A difference in this superscript letter in a particular column indicates significant differences ($p < 0.05$) for a given batch in the soluble As contents in the gastric and gastrointestinal stages.
[x,y] A difference in this superscript letter in a particular column indicates significant differences ($p < 0.05$) between batches for the soluble As contents in the same stage of the digestion process.

swine exposed to soils treated with arsenic.[19] The solubility of arsenic in the acid environment of the stomach might, therefore, be predictive for the relative oral bioavailability of this element in animal models.[13] There are no similar studies for humans.

The bioaccessibility (percentage of t-As or i-As solubilized after the gastrointestinal stage with respect to the total t-As or i-As contents in seaweed) varied in raw seaweed in the range 55.6–73.9% for t-As and 54.0–66.5% for i-As (Table 2). After cooking, the bioaccessibility remained at similar percentages for t-As (61.9–74.6%), increasing for i-As (78.3–84.4%). Boiling may bring about a decrease in fibre content and a denaturation of protein, permitting greater accessibility of enzymes during proteolysis. Since arsenic(III) bonds to protein groups with sulfur moieties and cysteine,[1] boiling might facilitate the solubilization of arsenic(III) in cooked seaweed, and consequently the solubilization of i-As.

The mean bioaccessibility of i-As in the batches analysed (raw: 60.1 ± 6.3%; cooked: 81.4 ± 3.1%) is similar to the value obtained by us for this seaweed in a previous study (raw: 75%; cooked: 88%).[7] Studies of this nature could provide the basis for defining an i-As bioaccessibility range in *H. fusiforme* (60.1–88%), to be taken into account when establishing the maximum i-As contents permitted in this food.

The tolerable daily intake (TDI) of i-As established for an adult with a body weight of 70 kg is 150 μg day^{-1}.[2] For i-As it is usual to establish toxicological considerations on the basis of the content in the raw product, although this may not be the correct option. On this basis, assuming the mean i-As content in the raw samples analysed (Table 1; mean value: 83.2 μg g^{-1}), consumption of 3 g (minimum average daily consumption of brown seaweed by the Japanese)[20] of raw *H. fusiforme* would provide 250 μg of i-As, exceeding the TDI by 67%. However, a more realistic approximation can be obtained by including both the cooking of the product and the bioaccessibility of the i-As. Assuming the mean bioaccessible i-As content found in the cooked seaweed (35.5 μg g^{-1}, Table 2), after consumption of 3 g of seaweed 107 μg of i-As could remain available for absorption, which is 71% of the TDI. The difference between the percentage of TDI represented by ingestion of this seaweed in the two approximations indicates the need to take cooking and bioaccessibility into account when evaluating the food safety of *H. fusiforme* with respect to i-As.

Arsenic(III) and arsenic(V) in soluble fractions

HPLC–HG-AFS was the analytical method used for determination of arsenic(III) and arsenic(V). This technique only allows detection of the arsenic species capable of forming volatile hydrides, which have traditionally included arsenic(III), arsenic(V), monomethylarsonic acid, dimethylarsinic acid and trimethylarsine oxide, to which, after a recent study by Schmeisser *et al*.,[21] the dimethylarsinoylribosides (glycerol ribose, phosphate ribose, sulfonate ribose and sulfate ribose) have been added. These arsenosugars,

which attain high concentrations in seaweed and are solubilized during the gastrointestinal process,[15] give rise to problems of overlapping with arsenic(III) and arsenic(V) in a PRP-X100 column.[22] In the conditions used in the present study for HPLC–HG-AFS quantification, the injection of a brown algae extract (*Fucus serratus*), which contained only the four dimethylarsinoylribosides,[23] did not generate any signals. This indicates that our system is free from interference produced by volatile arsenosugars, perhaps because arsenosugar hydride-activity is strongly dependent on the type of hydride-generation system, the conditions employed, or both.[21]

In order to quantify arsenic(III) and arsenic(V), first the soluble fractions obtained from the gastric and gastrointestinal stages were injected directly into the chromatograph. The mass balance between the t-As present in the soluble fraction injected and the t-As eluted from the HPLC column showed a recovery rate of 90%. However, the quantification of arsenic(III) and arsenic(V) against the standard curve for each species showed that for some samples the sum of arsenic(III) and arsenic(V) was 60% of the i-As content detected in the extract to be injected, which was quantified by the solvent extraction flow injection-HG-AAS method described here (Table 2). In the samples in which this disparity was found, the addition of standards of arsenic(III) and arsenic(V) did not produce the expected increase in the chromatograph signal. Thus, there was an interference effect that could not be eliminated by dilution or by using the standard addition method. Seaweeds have high contents of minerals, proteins and fibre,[24] compounds that are highly solubilized after *in vitro* digestion,[25,26] and these might by responsible for the matrix interference effect.

In order to eliminate this interference, all the soluble fractions from raw and cooked seaweed, gastric and

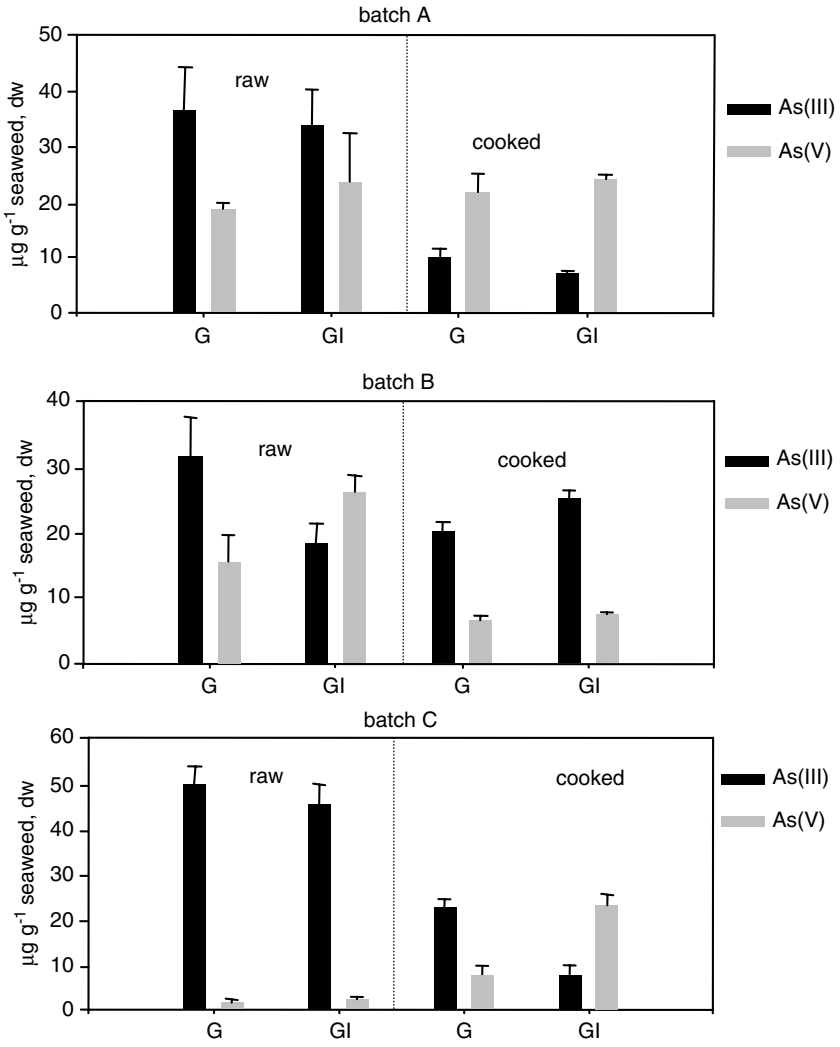

Figure 1. Soluble arsenic(III) and arsenic(V) contents obtained after the gastric (G) and gastrointestinal (GI) stages of an *in vitro* digestion method applied to three batches of raw and cooked *Hizikia fusiforme*. The error bars represent the standard deviation of independent replicates ($n = 4$).

gastrointestinal stages, were subjected to a clean-up procedure using a strong cation-exchanger resin. When the clean-up procedure was applied, only arsenic(III) and arsenic(V) appeared in the eluate, there was a recovery of arsenic(III) and arsenic(V) ranging from 80 to 100%, and the oxidation state of the species added did not change.

The arsenic(III) and arsenic(V) contents found in each of the stages of the *in vitro* digestion of the various batches of raw and cooked *H. fusiforme* are shown in Fig. 1. No general pattern was observed in the solubilization of either arsenic(III) or arsenic(V). In the gastric stage for raw seaweed, all the batches had the same majority species, with arsenic(III) representing 63–86% of the solubilized i-As. When the raw sample digestion process concluded (gastrointestinal stage, GI) the solubilized arsenic(III) and arsenic(V) were in the ranges 18.4–45.3 μg g^{-1} seaweed and 2.2–26.1 μg g^{-1} seaweed respectively, and the percentages of the solubilized i-As were in the ranges 36–94% and 5–51% respectively. In cooked samples, the arsenic(III) and arsenic(V) solubilized contents (GI stage) were in the ranges 7.1–25.4 μg g^{-1} seaweed and 7.5–23.8 μg g^{-1} seaweed respectively, and the percentages of the solubilized i-As were in the ranges 20–70% and 21–68% respectively. It must be emphasized that, in the cooked seaweed analysed, the solubilized arsenic(III) contents per gram of sample consumed represented 5–17% of the TDI of i-As. This is a very high contribution if one considers that it comes from only one type of food.

It is not possible to say with certainly whether the arsenic(III) and arsenic(V) contents quantified reflect the true nature of the species present in the sample or whether they are a consequence of the effect of cooking, the conditions of the *in vitro* digestion method, or the action that the solubilized macro- and micro-nutrients may have on the oxidation state of the inorganic species. However, it is possible that the factors mentioned may act in an *in vivo* human digestion. It has recently been shown that *H. fusiforme* is a natural source of soluble antioxidants in water and in fat.[27] Other compounds, such as ascorbic acid, amino acids (histidine, tryptophan, tyrosine, cysteine, glutathione) and numerous proteins, also have a reductive capacity. The effect of the reducing substances present in foods on the transformation of arsenic(V) into arsenic(III) has been reported by other workers.[28,29] Small variations in the solubilized oxidizing and/or reducing substances in each batch and even between the two digestions of a particular sample might be the cause of the different arsenic(III)/arsenic(V) relationships found, and of the relative standard deviation (up to 28%) obtained in some of the samples analysed.

CONCLUSIONS

All the batches of *H. fusiforme* edible seaweed analysed had high contents of t-As and i-As. Cooking decreased the i-As contents but increased the bioaccessibility of the toxic i-As, a fact that we consider to be of interest, as this cooking is customary for the consumption of *H. fusiforme*. The results obtained show that a more realistic estimation of the toxicological risk involved in consumption of *H. fusiforme* should take the bioaccessibility of arsenic(III) and arsenic(V) into account. The estimation made in the present study for the fraction of the element theoretically available for intestinal absorption (bioaccessible) is a first approach to evaluation of the toxicological risk. An improvement in these *in vitro* systems has been initiated by our work group with the introduction of cell cultures (Caco-2 cells), a model of the intestinal epithelium, which will make it possible to achieve a simulation closer to the *in vivo* situation.

Acknowledgements

This research was supported by project MCyT AGL2001-1789, for which the authors are deeply indebted. J. M. Laparra received a Personnel Training Grant from the project to carry out this study. *Fucus serratus* samples were kindly donated to us by Dr Kevin A. Francesconi (Karl-Franzens-University, Austria).

REFERENCES

1. Tsuda T, Babazono A, Ogawa T, Hamad H, Mino Y, Aoyama H, Kuramatani N, Nagira T, Hotta N, Harada M, Inomata S. *Appl. Organometal. Chem.* 1992; **6**: 309.
2. 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.
3. Muñoz O, Devesa V, Suñer MA, Vélez D, Montoro R, Urieta I, Macho ML, Jalón M. *J. Agric. Food Chem.* 2000; **48**: 4369.
4. Yasui A, Tsutsumi C, Toda S. *Agric. Biol. Chem.* 1978; **42**: 2139.
5. Shibata Y, Jin K, Morita M. *Appl. Organometal. Chem.* 1990; **4**: 255.
6. Almela C, Algora S, Benito V, Clemente MJ, Devesa V, Súñer MA, Vélez D, Montoro R. *J. Agric. Food Chem.* 2002; **50**: 918.
7. Laparra JM, Vélez D, Montoro R, Barberá R, Farré R. *J. Agric. Food Chem.* 2003; **51**: 6080.
8. Hanaoka K, Yosida K, Tamano M, Kuroiwa T, Kaise T, Maeda S. *Appl. Organometal. Chem.* 2001; **15**: 561.
9. Devesa V, Martínez A, Súñer MA, Vélez D, Almela C, Montoro R. *J. Agric. Food Chem.* 2001; **49**: 2272.
10. Gamble BM, Gallagher PA, Shoemaker JA, Weis X, Schwegel CA, Creed JT. *Analyst* 2002; **127**: 781.
11. Hocquellet P, L'Hotellier MD. *J. AOAC Int.* 1997; **80**: 920.
12. Azenha MAGO, Vascondecelos MTSD. *Food Chem. Toxicol.* 2000; **38**: 899.
13. Ruby MV, Schoof R, Brattin W, Goldade M, Post G, Harnois M, Mosby DE, Casteel SW, Berti W, Carpenter M, Edwards D, Cragin D, Chappell W. *Environ. Sci. Technol.* 1999; **33**: 3697.
14. Oomen AG, Hack A, Minekus M, Zeijdner E, Cornelis C, Schoeters G, Verstraete W, van de Wiele T, Wragg J, Rompelberg CJM, Adriëne JAM, Sips A, van Wijnen JH. *Environ. Sci. Technol.* 2002; **36**: 3326.
15. Van Hulle M, Zhang C, Schotte B, Mees L, Vanhaecke F, Vanholder R, Zhang XR, Cornelis R. *J. Anal. At. Spectrom.* 2004; **19**: 58.
16. De Blas OJ, Gonzalez V, Seisdedos R, Reañades J. *J. AOAC Int.* 1994; **2**: 441.

17. Box GEP, Hunter NG, Hunter JS. *Statistics for Experimenters. An Introduction to Design, Data Analysis and Model Building*. John Wiley: New York, USA, 1978.
18. Hamel SC, Ellickson CM, Lioy PJ. *Sci. Total Environ.* 1999; **243–244**: 273.
19. Rodriguez R, Basta NT, Casteel SW, Pace LW. *Environ. Sci. Technol.* 1999; **33**: 642.
20. Sakurai T, Kaise T, Ochi T, Saitoh T, Matsubara C. *Toxicology* 1997; **122**: 205.
21. Schmeisser E, Goessler W, Kienzl N, Francesconi KA. *Anal. Chem.* 2004; **76**: 418.
22. Raber G, Francesconi KA, Irgolic KJ, Goessler W. *Fresenius J. Anal. Chem.* 2000; **367**: 181.
23. Madsen AD, Goessler W, Pedersen SN, Francesconi KA. *J. Anal. At. Spectrom.* 2000; **15**: 657.
24. Mabeau S, Fleurence J. *Trends Food Sci. Technol.* 1993; **4**: 103.
25. Goñi I, Gudiel-Urbano M, Saura-Calizto F. *J. Sci. Food Agric.* 2002; **82**: 1850.
26. Rúperez P, Toledano G. *J. Sci. Food Agric.* 2003; **83**: 1267.
27. Siriwardhana N, Lee KW, Kim SH, Ha JW, Jeon YJ. *Food Sci. Technol. Int.* 2003; **5**: 339.
28. Muñoz O, Vélez D, Montoro R. *Analyst* 1999; **124**: 601.
29. Heitkemper DT, Vela NP, Stewart KR, Westphal CS. *J. Anal. At. Spectrom.* 2001; **16**: 299.

