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Arsenosugars in Raw and Cooked Edible Seaweed: Characterization and Bioaccessibility

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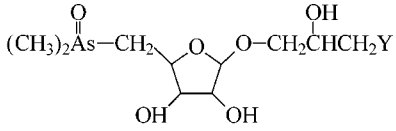
The aim of this study was to examine arsenic species contents in raw and cooked edible seaweed and the bioaccessibility (maximum soluble concentration in gastrointestinal medium) of arsenosugars (glycerol ribose, phosphate ribose, sulfonate ribose, and sulfate ribose). For the analysis, a new chromatographic separation was developed in anion exchange, coupled with thermooxidation−hydride generation−atomic fluorescence spectrometry. An in vitro digestion (pepsin, pH 2; pancreatin−bile extract, pH 7) was applied to estimate arsenosugar bioaccessibility. Cooking of *Undaria pinnatifida* and *Porphyra* sp. did not alter the arsenic species present in the methanol−water extract, but it produced a substantial increase (2 and 5 times) in the As(V) extracted from *Hizikia fusiforme*. In all of the seaweeds analyzed, arsenosugar bioaccessibility was high (>80%) and did not vary as a result of cooking. Arsenosugar degradation as a result of in vitro digestion was not observed.

KEYWORDS: Arsenic speciation; arsenosugars; seaweed; bioaccessibility; cooking

INTRODUCTION

Seaweed is a traditional food in the Far East, but its consumption in western countries is recent. Edible seaweeds are a good source of proteins, polysaccharides (*1*), and minerals (*2*), but they are not free of contaminants. In edible seaweed, arsenic (As) attains concentrations of up to 152 mg kg^{-1} of dry weight (dw) (*3*), much higher than those reported for Pb, Cd, and Hg (*4*). To assess the risk from arsenic ingested from edible seaweeds, speciation analysis is necessary.

The arsenic species usually identified in seaweed are As(III), As(V), dimethylarsinic acid (DMA), and four dimethylarsinoylriboside derivatives (**Figure 1**). Inorganic arsenic (i-As), a term that embraces the most toxic species so far detected in foods, [As(III) + As(V)], rarely exceeds 1 mg kg^{-1} of dw in seaweed (*4*), with the exception of the brown edible seaweed *Hizikia fusiforme*, in which up to 135 mg kg^{-1} of dw of i-As has been quantified (*5*). The dimethylarsinoylribosides are generally the species that reach the highest concentrations, sometimes exceeding 100 mg kg^{-1} of dw (*6*). Other species such as trimethylated arsenosugars have been found in some individual samples of seaweed (*7*), whereas arsenobetaine (AB) has not been reported (*8*). Although some authors have detected a species with the same retention time as monomethylarsonic acid (MMA) in seaweeds (*6*, *9*, *10*), structural verification of this compound has not been performed.



Name	Y
Glycerol ribose	-OH
Phosphate ribose	$-OPO_3HCH_2CH(OH)CH_2OH$
Sulfonate ribose	$-SO_3H$
Sulfate ribose	$-OSO_3H$

Figure 1. Chemical structures of the four major arsenosugars found in seaweed and investigated in this work.

High-performance liquid chromatography (HPLC) using an anion exchange column is the technique most commonly employed to separate arsenic species. In seaweed, the presence of arsenosugars complicates chromatographic separation in anion exchange columns (*11*, *12*). Optimization of this chromatographic separation and its coupling with hydride generation atomic fluorescence detection, an absorption technique with sensitivity, reproducibility, repeatability, and limits of detection similar to those of inductively coupled plasma mass spectrometry (ICP-MS) detection (*13*) at a much lower cost, would provide an alternative to the customary quantification by means of HPLC-ICP-MS.

On the other hand, one must bear in mind that the consumer may ingest seaweed raw or after various treatments (soaking, boiling, toasting, or baking). These treatments might change the arsenic species qualitatively or quantitatively, altering the

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toxicological risk with respect to the product as sold. Previous studies have shown that washing, soaking, and boiling usually decrease i-As contents in edible seaweeds (*5*, *14*). Arsenosugars seem to be stable during short-term heating (*15*), but there is a lack of studies about the possible influence of other cooking treatments.

The carcinogenic nature of i-As is well-known. The toxicity of arsenosugars, however, has not yet been studied in depth, and only the low cytotoxicity of glycerol ribose has been shown (*16*). The presence of arsenosugars in seaweed might represent a health risk, yet metabolic studies have shown that ingestion of arsenosugars entails urinary elimination of numerous arsenic metabolites, primarily DMA(V) (*15*, *17*, *18*). This implies an increase in the toxicity of arsenosugars, given a promotional and carcinogenic activity of DMA(V), which could be mediated by its reduced metabolite dimethylarsinous acid [DMA(III)] (*19*). How these arsenosugars are converted to DMA is still unclear. Gastrointestinal digestion might contribute to the appearance of DMA by degradation of arsenosugars, although the studies carried out so far show that in simulated gastric juice these species are transformed only into another arsenosugar (ion at *m*/*z* 254) and not into DMA (*20*, *21*).

The fact that qualitative changes in arsenic species take place during digestion might have toxicological repercussions. However, the quantity of arsenic solubilized in the gastrointestinal tract does not have to be equal to the quantity present in the food consumed. For all of these reasons, arsenic bioavailability studies (fraction of arsenic that reaches the systemic circulation) should be carried out. A first stage in the study of bioavailability is the study of bioaccessibility, which indicates the fraction of arsenic present in food that is soluble in gastrointestinal medium (bioaccessible fraction) and, consequently, available for subsequent absorption into the intestinal mucosa (*22*). To our knowledge, only three studies have advanced along this line: Gamble et al. (*20*) have studied the effect of simulated gastric juice on arsenosugars present in seaweeds, although the intestinal stage has not been evaluated; Laparra et al. (*14*, *23*) have studied the bioaccessibility of i-As from seaweeds.

To help to ascertain the risk to human health that the ingestion of seaweed implies, the present study focuses on three objectives: (1) to develop a new methodology using anion exchange chromatography to permit suitable separation of the four main arsenosugars and other arsenic species present in seaweed; (2) to evaluate the effect of various types of cooking on arsenosugars and other arsenic species present in raw seaweed; and (3) to estimate the bioaccessibility of arsenosugars in raw and cooked edible seaweed and their possible degradation during gastrointestinal digestion.

MATERIALS AND METHODS

Equipment. For arsenic speciation analysis, the HPLC system employed (Hewlett-Packard model 1100) was equipped with a quaternary pump, an on-line degassing system, an automatic injector, and a thermostated column compartment. Separations were performed on a Hamilton PRP-X100 anion exchange column (Teknokroma). A guard column packed with the same material preceded the analytical column. A heated bath (Julabo model HC, Merck) was used to thermooxidize the outlet from the HPLC column. The quantification of arsenic species was performed on a hydride generation (HG) system (PSA 10.004, Analytical) and an atomic fluorescence spectrometer (AFS) detector (PSA 10.044 Excalibur PS, Analytical) equipped with a boosted-discharge hollow cathode lamp (BDHCL, Photron, Super Lamp). The arsines generated were conveyed to the AFS detector by means of a semipermeable membrane dryer tube (Perma Pure). A Hewlett-Packard model 35900 C digital analogical converter was used to acquire the AFS signal, which was processed by the chromatographic software.

The determination of total arsenic was performed with an atomic absorption spectrometer (AAS) model 3300 (Perkin-Elmer) equipped with an autosampler (AS-90, Perkin-Elmer), a flow injection hydride generation (FI-HG) system (FIAS-400, Perkin-Elmer), and an electrothermally heated quartz cell.

Other equipment used included a lyophilizer equipped with a microprocessor controlling the lyophilization process (FTS Systems), a PL 5125 sand bath (Raypa, Scharlau S.L.), a K1253 muffle furnace equipped with a Eurotherm Controls 902 control program (Heraeus S.A.), a KS 125 basic mechanical shaker (IKA Labortechnik, Merck), a mechanical shaker (Rotabit, Selecta), an Eppendorf 5810 centrifuge (Merck), a Heraeus Biofuge Pico centrifuge (Merck), and a Sorvall RC-50B centrifuge (Sorvall Instrument, DuPont).

Reagents. All chemicals used were of pro analysi quality or better and included methanol (Merck), extra pure ammonium carbonate consisting of an equimolar mixture of anhydrous carbonate ($CH_6N_2O_2$) and bicarbonate (CH_5NO_3) (Merck), ammonia solution 25% (Merck), sodium tetrahydroborate(III) (Panreac), hydrochloric acid (Merck), potassium peroxodisulfate (Prolabo), nitric acid (Merck), magnesium oxide (Merck), magnesium nitrate (Prolabo), potassium iodide (Prolabo), and ascorbic acid (Prolabo). Fresh sodium tetrahydroborate(III) solution was prepared daily and was filtered through Whatman no. 42 paper before use. Deionized water (18.2 MΩ cm) was used for the preparation of reagents and standards. All material was treated with 10% v/v HNO_3 for 24 h and then rinsed three times with deionized water before being used.

Water of cellular grade (B. Braun Medical, S.A.) was used throughout the in vitro assay. Enzymes and bile salts employed in the in vitro digestion 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).

Arsenic Compounds. Standard solution of As(V) (1000 mg L^{-1}) was purchased from Merck. Standard solution of As(III) (1000 mg L^{-1}) was prepared by dissolving 1.320 g of arsenic trioxide (Riedel de Haën) in 25 mL of 20% (w/v) KOH, neutralizing with 20% (v/v) H_2SO_4, and diluting to 1 L with 1% (v/v) H_2SO_4. Standard solutions of MMA (Carlo Erba) and DMA (Fluka Chemika Biochemika) were prepared in deionized water.

For the identification and quantification of the four dimethylarsinoyl ribosides (**Figure 1**), a lyophilized algal extract of *Fucus serratus*, kindly supplied by Dr. Kevin Francesconi, was employed. This extract is a homogeneous supply of a material containing arsenosugars. Details of the *F. serratus* sample preparation and arsenosugar characterization procedure were given in a previous work (*24*).

Samples. Kelp powder sample, a commercially available algae product from eastern Canada, was purchased from a food store in Vancouver, BC. *Hizikia fusiforme*, *Undaria pinnatifida*, and *Porphyra* sp., each from two different suppliers (A and B), were purchased in health food stores in Valencia (Spain).

All samples were analyzed just as they were sold (dry, cut, and wrapped in plastic), which we have called the raw state. *H. fusiforme*, *U. pinnatifida*, and *Porphyra* sp. were also analyzed after application of the cooking treatment indicated on the product label. *H. fusiforme* was boiled (water, 100 °C, 20 min; 10 g of sample/250 mL of water) or soaked (cold water, 15 min; 10 g of sample/180 mL of water). *U. pinnatifida* was boiled (water, 100 °C, 20 min; 10 g of sample/250 mL of water) or soaked (15 min; 10 g of sample/180 mL of water) and then boiled (water, 100 °C, 2 min). *Porphyra* sp. was baked (200 °C, 5 min) or toasted over a flame to obtain a bright green color. After being cooked, the boiled or soaked samples were lyophilized. All samples, raw or cooked, were crushed to a fine powder in a mill and stored at 4 °C until analysis.

Total Arsenic Determination. Analysis was performed by FI-HG-AAS after a dry-ashing step (*4*). Triplicate analyses were performed for each sample. Throughout the experiment, the accuracy of measurement was checked by analyzing one certified reference material with each batch: *Fucus* sp. (IAEA-140/TM, International Atomic Energy Agency) or *Ulva lactuca* (BCR-279, Institute for Reference Materials and Measurements).

Extraction of Arsenic Species. Raw or cooked seaweed (1.00 ± 0.01 g) was weighed into a 50 mL centrifuge tube, and 20 mL of

Table 1. Instrumental and Analytical Conditions for HPLC–Thermooxidation–HG-AFS Analysis

High-Performance Liquid Chromatography	
column	Hamilton PRP X-100; 10 μm; 250 mm × 4.1 mm i.d.
guard column	Hamilton PRP X-100; 12–20 μm; 25 mm × 2.3 mm i.d.
mobile phase	A, 20 mmol L^{-1} NH_4CO_3, pH 9 B, deionized water C, 20 mmol L^{-1} NH_4CO_3, pH 10.3
gradient program	0–7 min, 5% A and 95% B 7–14 min, 100% A 14–20 min, 100% C 20–25 min, 5% A and 95% B
injection volume	100 μL
flow rate	1 mL min^{-1}
Thermooxidation	
oxidant	1.29% (w/v) $K_2S_2O_8$ in 2.5% (w/v) NaOH; 1 mL min^{-1} flow rate
reaction coil	3 m × 0.3 mm i.d.
bath temperature	155 °C
Hydride Generation	
reducing agent	1.5% (w/v) $NaBH_4$ in 0.7% (w/v) NaOH; 2.5 mL min^{-1} flow rate
HCl solution	1.5 mol L^{-1}; 6.0 mL min^{-1} flow rate
carrier gas	argon; 300 mL min^{-1} flow rate
dryer gas	air; 2.5 L min^{-1} flow rate
Atomic Fluorescence Spectrometry	
hydrogen flow rate	60 mL min^{-1}
resonance wavelength	193.7 nm
primary current	27.5 mA
boost current	35.0 mA

methanol/water (1+1 v/v) was added. The mixture was agitated for 15 min in a mechanical shaker and centrifuged at 2000 rpm for 10 min, and the supernatant was collected. The extraction process was repeated two more times. The supernatants were combined, evaporated to dryness (T < 50 °C), and stored at −18 °C until analysis. The dry residue was redissolved in 3 mL of water and centrifuged at 12000 rpm for 10 min, and the supernatant was filtered through a Whatman 0.45 μm nylon membrane filter prior to injection into the chromatographic column. Triplicate analyses were performed for each sample.

Arsenic Species Determination by Anion Exchange−Thermooxidation−HG-AFS. Details of the operating conditions are given in **Table 1**. Samples were injected into the PRP-X100 column and, using PTFE tubing and T-joints, the eluate from the column was mixed with the persulfate solution. The mixture was thermooxidized by being passed through a loop of Teflon tubing placed in a heated bath. After cooling in an ice bath, the eluate was mixed with a continuous flow of HCl and $NaBH_4$. Using a gas−liquid separator and a continuous flow of argon, the arsines generated were introduced into the AFS. The hygroscopic membrane drying tube used to transport the arsines allowed elimination of moisture by circulating a counterflow of air. An additional flow of hydrogen gas permitted partial maintenance of the flame.

Arsenic compounds were identified by matching the retention times of the peaks in the sample chromatograms with those obtained from standards and were quantified with a calibration curve for DMA.

In Vitro Gastrointestinal Digestion. The method developed by Laparra et al. (*14*) was employed. To 5 g of seaweed, raw or cooked, was added 90 mL of cellular grade water. 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 of seaweed. The sample was made up to 100 g 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 of seaweed and 0.015 g of bile extract/5 g of seaweed, and the incubation at 37 °C was 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 were transferred to polypropylene centrifuge tubes and centrifuged (15000 rpm/30 min/4 °C) to separate soluble fractions (bioaccessible fraction). Total arsenic and arsenosugars were determined in these fractions.

RESULTS AND DISCUSSION

Arsenic Extraction Efficiencies. Arsenic species in the seaweed have been extracted using water (*6*), phosphoric acid (*12*), methanol (*8*), and especially methanol/water mixtures (*11*, *25*, *26*). Methanol/water in the proportions 9+1 and 1+1 and other extraction variables (sonication or mechanical agitation; number of extraction cycles) were assayed in the present work.

The extraction was optimized on kelp powder, settling on 1 g of sample and 10 mL of MeOH/H_2O in each extraction cycle. The efficiency of the extraction with MeOH/H_2O (9+1), evaluated as the percentage of total arsenic in the extract with respect to total arsenic in the sample, was 25% less than that obtained with MeOH/H_2O (1+1), and for this reason MeOH/H_2O (1+1) was chosen. The efficiency of the extraction did not differ between the use of an ultrasonic bath or a mechanical shaker, and a shaker was employed in this study because it made working arrangements easier for our laboratory. With regard to the number of extraction cycles, with three cycles (total volume = 30 mL) it was possible to extract 63% of the arsenic present in kelp powder. A fourth extraction cycle increased the efficiency of the process by only 1%. This extraction process was applied to the other seaweed samples (**Table 2**).

In raw seaweed the extraction efficiency was very variable (5−73%), as has been reported in the literature for other raw seaweeds (14−85%) (*27*, *28*). The extraction percentages were low for raw *H. fusiforme* (14 and 25%), but after cooking the extraction efficiency increased 2 and 4 times. In the two samples of raw *U. pinnatifida* there were large differences in the extractable arsenic (5 and 49%), and these values did not change substantially after cooking. Raw *Porphyra* sp. had the highest extraction efficiency (65−73%), which did not vary after cooking. The variations in the extraction efficiencies might be attributable to the species of alga analyzed. Other factors may also have an influence. Extraction efficiencies varying between 6 and 98% have been reported for a single species (*Fucus gardiner*) collected at different points in time and with different degrees of maturity (*29*). Probably a high proportion of the arsenic not extracted with methanoll/water mixtures is present as lipid-soluble compounds, which can represent up to 50% of total arsenic in algae (*30*).

Chromatographic Separation of Arsenic Compounds. The chromatographic separation of As(III), As(V), MMA, DMA, and four dimethylarsinoyl ribosides (**Figure 1**) was investigated in extracts of *F. serratus* and kelp powder. In the literature, using an anion exchange column, there are reports of overlapping of As(III)−glycerol ribose (*11*, *12*, *24*, *26*, *27*), As(III)−glycerol ribose−DMA (*12*, *27*), and MMA−sulfate ribose (*26*). Gallagher et al. (*9*) show the best separation between the species of interest in this work, although without attaining the baseline between As(III)−glycerol ribose.

One of the aims of this study was to improve separation of the species cited in a PRP-X100 column. In the literature, two kinds of mobile phases are used for separation of the arsenic species in seaweed: phosphate and carbonate. We began the studies by using NH_2HPO_4 as the mobile phase, at pH 5.75, with a gradient of 5−50 mM. With these conditions the separation between the arsenosugars present in *F. serratus* was

Table 2. Concentrations of Arsenic in Edible Seaweed and in the Methanol–Water Extract (Mean ± Standard Deviation; $n = 3$) and Extraction Efficiency

sample			total arsenic (μg g^{-1} of dry wt)	extractable arsenic (μg g^{-1} of dry wt)	extraction efficiency (%)
kelp			27.65 ± 0.38	17.41 ± 0.39	63
H. fusiforme	A[a]	raw	103.73 ± 7.41	26.34 ± 2.26	25
		boiled	56.24 ± 3.51	32.44 ± 1.79	58
		CL[b]	50.23 ± 0.66		
	B[a]	raw	131.61 ± 4.72	18.57 ± 1.06	14
		soaked	94.42 ± 0.86	53.70 ± 0.25	57
		CL	31.84 ± 0.25		
U. pinnatifida	A	raw	49.34 ± 0.48	2.51 ± 0.15	5
		boiled	49.51 ± 0.70	2.27 ± 0.06	5
		CL	0.71 ± 0.03		
	B	raw	47.42 ± 0.02	23.07 ± 0.21	49
		soaked and boiled	34.04 ± 0.30	11.76 ± 042	35
		CL	14.71 ± 0.50		
Porphyra sp.	A	raw	33.60 ± 0.68	21.96 ± 0.30	65
		baked	33.28 ± 0.79	19.49 ± 0.74	59
	B	raw	27.19 ± 1.58	19.73 ± 1.27	73
		toasted over a flame	26.43 ± 0.29	19.25 ± 0.97	73

[a] A and B, different manufacturers. [b] CL, cooking liquid obtained after cooking of the seaweed.

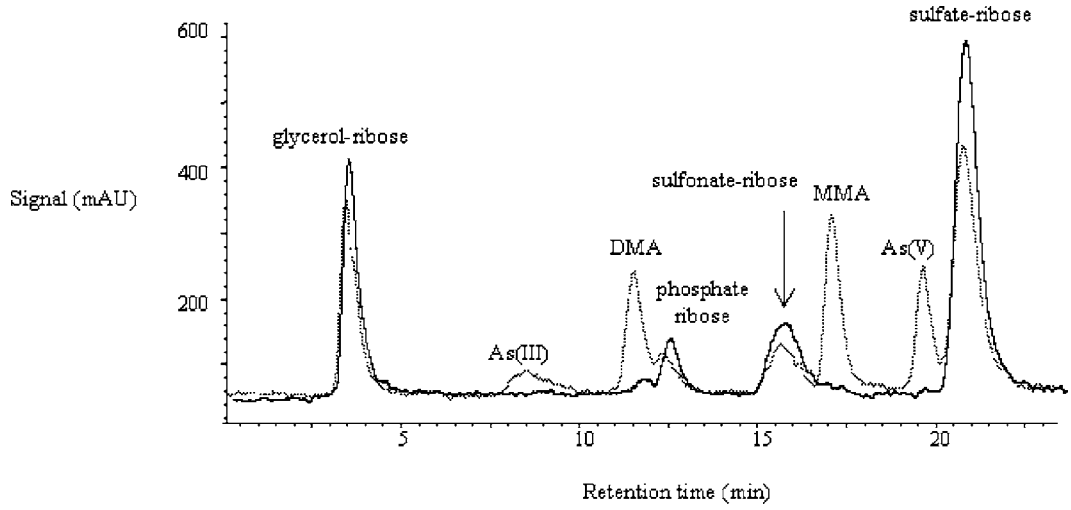

Figure 2. Overlaid HPLC–thermooxidation–HG-AFS chromatograms of kelp powder sample (solid line) and kelp powder spiked with standards of As(III), DMA, MMA, and As(V), 0.5 μg of As mL^{-1} of each species (dotted line). Analytical conditions are shown in **Table 1**.

adequate, and it was possible to identify the t_R of each arsenosugar by comparison with the data available in the literature (*24*). In view of the low availability of *F. serratus*, we then decided to continue the optimization with a sample of kelp powder in which four arsenosugars and DMA had been identified in a previous study (*31*). After injection of the methanol/water extract of kelp powder and kelp powder spiked with 0.5 μg mL^{-1} of As(III), DMA, MMA, and As(V), we observed an overlap of DMA and phosphate ribose. We therefore decided to change the mobile phase.

As an alternative, we tried replacing the $(NH_4)H_2PO_4$ mobile phase with extra-pure ammonium carbonate. Working at pH 9 and l mM, we achieved an optimum separation between glycerol ribose ($t_R = 3.1$ min) and As(III) ($t_R = 8$ min). In the literature there are previous reports concerning the suitability of this pH, in 20 mM ammonium phosphate or carbonate mobile phase, for the separation of these two species (*9*, *11*), but in no case was the separation between peaks so wide. By maintaining pH 9 and increasing the concentration of the eluent to 20 mM, we achieved suitable DMA−phosphate ribose and MMA−sulfonate ribose resolutions. However, there was an As(V)−sulfate ribose overlap, which could be resolved only by increasing the pH to 10.3.

After these assays, we selected a gradient of pH and concentrations of ammonium carbonate as the mobile phase, in the conditions described in **Table 1**. **Figure 2** shows the chromatogram obtained in these conditions for the methanol/water extract of kelp powder sample spiked with As(III), As(V), DMA, and MMA.

Quantification of Arsenic Species by Anion Exchange−Thermooxidation−HG-AFS. There are reports in the literature which suggest that in the HPLC-ICP-MS hyphenated system it is possible to use the calibration curve of As(V) (*24*) or DMA (*11*, *27*, *32*) to quantify arsenosugars.

In the HPLC−thermooxidation−HG-AFS conditions in our study (**Table 1**), the responses for As(V), As(III), MMA, and DMA standards were very similar, with maximum differences of 7%. As important sensitivity differences were not observed, any of these species could be used for calibration. We decided to use DMA because its signal is most conditioned by the thermooxidation and hydride generation stages, and therefore

Table 3. Concentrations of Arsenosugars in *F. serratus* Extract Found in This Study and Comparison with Reported Data

	mean As contents in μg (RSD%)		
arsenosugar	HPLC–thermo-oxidation–HG-AFS[a]	HPLC-ICPMS[b]	HPLC-HG-ICPMS[c]
glycerol ribose	0.098 (1.6)	0.1 (4.8)	0.098 (4.1)
phosphate ribose	0.084 (0.4)	0.086 (2.9)	0.082 (6.5)
sulfonate ribose	0.65 (3.4)	0.62 (3.8)	0.58 (5.3)
sulfate ribose	0.39 (2.5)	0.40 (3.1)	0.39 (5.9)

[a] This work; three independent analyses. [b] Madsen et al. (*24*). [c] Schmeisser et al. (*6*).

Table 4. Analytical Characteristics of HPLC–Thermooxidation–HG-AFS Methodology

	limit of detection		RSD[a] (%)	
arsenic species	instrumental (μg mL^{-1} As)	sample (μg g^{-1} As, dw)	kelp powder	*Fucus* sp.[b]
glycerol ribose	0.026	0.078	5 (0.14)	1 (0.067)
DMA	0.011	0.033	8 (0.012)	5 (0.056)
phosphate ribose	0.012	0.036	5 (0.035)	6 (0.043)
sulfonate ribose	0.028	0.084	5 (0.072)	4 (0.33)
As(V)	0.023	0.069		
sulfate ribose	0.024	0.072	7 (0.38)	3 (0.35)

[a] Relative standard deviation from six independent analyses. Values in parentheses are the species average concentration in μg g^{-1} As, dry weight (dw) for the samples analyzed. [b] *Fucus* sp., certified reference material (IAEA-140/TM).

it permits detection of variations in working conditions throughout an analysis session. The calibration range was 0.05−0.5 μg g^{-1}. The *F. serratus* sample, the arsenosugar content of which is known (*24*), was used to check the validity of quantification with the DMA calibration curve. The results obtained for each arsenosugar (**Table 3**) are very close to those reported in the literature (*6*, *24*).

The analytical methodology is characterized in terms of limits of detection and precision for arsenosugars, DMA, and As(V) (**Table 4**). The instrumental limits of detection (LOD) were calculated by dividing 3 times the standard deviation of the absorbance area of 10 reagent blanks, determined at the retention time of each arsenic species, by the slope of the standard calibration curve. The sample mass (1 g) was taken into account to calculate the sample LODs. The lowest sample LOD was obtained for DMA (0.033 μg g^{-1}, dw), whereas the arsenosugar sulfate ribose was the arsenic species with the highest LOD (0.084 μg g^{-1}, dw). The precision of the method, expressed as the relative standard deviation evaluated by analyzing six subsamples, ranged between 1 and 8%.

Arsenic Species in Raw and Cooked Seaweed Samples. Contents of arsenic species found in *Fucus* sp. (CRM), kelp powder, and raw and cooked edible seaweeds are shown in **Table 5**. We did not detect As(III) or MMA in any of the samples.

The absence of commercial standards of arsenosugars makes it difficult for research groups to undertake analysis of these arsenic species. In this study we characterized the arsenosugars present in the CRM *Fucus* sp., a commercially available homogeneous material that could subsequently be used for quality assurance−quality control purposes in the analysis of arsenosugars. For this purpose, the extraction procedure employed in this work must be used. In *Fucus* sp., sulfate, sulfonate, and glycerol ribose appear in higher concentrations than phosphate ribose and DMA (**Table 5**). The distribution of these species follows the pattern reported for other *Fucus* sp. samples (*6*, *8*, *11*, *24*, *29*). Only Lai et al. (*29*) did not detect phosphate ribose in *F. gardneri*, suggesting that phosphate ribose can be decomposed to glycerol ribose and DMA. It was not possible to verify this hypothesis by the arsenic biotransformation studies carried out by Geiszinger et al. (*33*).

The gradation of species observed in kelp powder (**Table 5**) was sulfate ribose > sulfonate ribose > glycerol ribose > phosphate ribose > DMA. The arsenosugar pattern coincides with those reported in the literature for kelp powder (*25*, *28*, *34*, *35*). With regard to DMA, its presence in kelp powder seems to be random, as it was not detected in some earlier works (*25*, *35*), possibly owing to incorrect chromatographic separation.

The two raw *H. fusiforme* samples (batches A and B) showed the same pattern of arsenic species (**Table 5**), with As(V) and sulfate ribose as the major species. Earlier studies showed the same distribution pattern (*6*, *12*). After cooking, an increase in the As(V) content was observed in the methanol/water extract (2 and 5 times with respect to the raw product), with minimal variations for the other arsenic species. The higher As(V) content in cooked *H. fusiforme* might be due to greater efficiency in the extraction of this arsenic species from the cooked product. In fact, in the two samples analyzed the increase in total extractable arsenic between the raw and cooked product (**Table 2**) (sample A, 6.1 μg/g of dry weight; sample B, 35.13 μg/g of dry weight) is close to the increase in the concentration of As-(V) (**Table 5**) (sample A, 10 μg/g of dry weight; sample B, 33.05 μg/g of dry weight). It is also worth noting that there was a considerable solubilization of arsenic toward the cooking water (**Table 2**), mainly in the form of As(V).

In *U. pinnatifida* the arsenic species detected varied between the two batches analyzed (**Table 5**), perhaps as a result of the substantial difference in the extraction efficiency obtained for each batch (5 and 49%, **Table 2**). In sample A it was possible to quantify only glycerol ribose, whereas signals below the limit of quantification appeared at the retention times of DMA and phosphate ribose. In sample B the major species were phosphate and sulfonate ribose, and DMA and glycerol ribose were found in lower concentrations. The few previous studies concerning *U. pinnatifida* report the presence of glycerol, phosphate, and sulfonate ribose (*29*), agreeing with what was detected in the present study. The presence of an arsenolipid has also been reported (*36*). After cooking, the species detected in the methanol/water extract did not vary. The lower organoarsenical species concentrations in cooked sample B might be the result of the lower efficiency of the extraction of arsenic from the cooked seaweed (35%) with respect to the raw seaweed (49%) (**Table 2**).

In raw *Porphyra* sp., the main species were phosphate and glycerol ribose, and DMA was also detected. The differences in the efficiency of extraction from sample A, raw and cooked (65 and 59%, respectively, **Table 2**), might justify the lower concentrations of arsenosugars found in the methanol/water extract obtained in the baked product (**Table 5**). In sample B, with an arsenic extraction efficiency of the same order in the sample raw and cooked, we did not observe substantial variations in the arsenosugar concentrations of the methanol/water extract after cooking (**Table 5**). The results for sample B support the stability of arsenosugars after short-term heating (100 °C; 10 min) of methanol/water extracts of *Porphyra* reported by Wei et al. (*15*), although the treatment applied by those authors is not representative of the cooking procedure used by the consumer.

Table 5. Concentrations of Arsenic Species in the Seaweeds Analyzed (Mean ± Standard Deviation; $n = 3$)

sample		U[a] ($t = 1.9'$)	glycerol ribose (μg g^{-1} of dry wt as As)	DMA (μg g^{-1} of dry wt as As)	phosphate ribose (μg g^{-1} of dry wt as As)	sulfonate ribose (μg g^{-1} of dry wt as As)	As(V) (μg g^{-1} of dry wt as As)	sulfate ribose (μg g^{-1} of dry wt as As)
Fucus sp. (IAEA-140/TM)			7.67 ± 0.07	1.09 ± 0.06	0.75 ± 0.04	8.76 ± 0.33		10.7 ± 0.4
kelp powder			3.13 ± 0.14	0.18 ± 0.01	0.71 ± 0.04	1.60 ± 0.07		5.97 ± 0.38
H. fusiforme								
A	raw		1.24 ± 0.001	1.81 ± 0.06	0.79 ± 0.05	1.23 ± 0.02	13.2 ± 0.7	5.67 ± 0.34
	boiled	0.25 ± 0.03	1.06 ± 0.03	1.45 ± 0.04	0.79 ± 0.07	1.64 ± 0.07	23.2 ± 0.2	3.54 ± 0.10
B	raw		1.75 ± 0.07	1.97 ± 0.17	1.32 ± 0.02	1.94 ± 0.12	8.65 ± 0.43	2.77 ± 0.27
	soaked		2.13 ± 0.17	1.74 ± 0.16	1.18 ± 0.13	2.13 ± 0.03	41.7 ± 0.1	2.47 ± 0.04
U. pinnatifida								
A	raw	0.98 ± 0.14	0.19 ± 0.01	<LQ[b]	<LQ			
	boiled	1.35 ± 0.01	0.21 ± 0.03	<LQ	<LQ	<LQ		
B	raw		1.79 ± 0.09	0.71 ± 0.04	11.5 ± 0.6	8.09 ± 0.47		
	soaked and boiled	0.43 ± 0.06	0.88 ± 0.05	0.49 ± 0.03	4.50 ± 0.03	3.43 ± 0.08		
Porphyra sp.								
A	raw		10.5 ± 0.2	0.44 ± 0.03	10.8 ± 0.2			
	baked		7.04 ± 0.09	0.69 ± 0.07	7.16 ± 0.23			
B	raw		5.29 ± 0.4	1.45 ± 0.09	15.9 ± 0.3			
	toasted over a flame		4.86 ± 0.78	0.76 ± 0.02	16.7 ± 0.5			

[a] Unknown signal. [b] LQ, limit of quantification. Calculated by dividing 10 times the standard deviation of the absorbance area of 10 reagent blanks by the slope of the standard calibration curve.

Table 6. Bioaccessible Total As and Arsenosugars (Both Expressed as μg g^{-1} of Seaweed Dry Weight as As; Mean ± Standard Deviation; $n =$ 3–4) and Bioaccessibility (%)[a] in Raw and Cooked Seaweed

			bioaccessible fraction				
sample	treatment	bioaccessibilty	total As	glycerol ribose	phosphate ribose	sulfonate ribose	sulfate ribose
kelp powder	raw	(μg g^{-1})	12.5 ± 0.7	3.07 ± 0.37	0.81 ± 0.04	1.30 ± 0.11	5.00 ± 0.44
		%	45	98	114	82	84
H. fusiforme (sample B)	raw	(μg g^{-1})	69.9 ± 3.2	1.86 ± 0.08	1.59 ± 0.18	2.32 ± 0.18	3.51 ± 0.32
		%	53	106	120	120	127
	boiled	(μg g^{-1})	53.8 ± 2.3	1.73 ± 0.14	1.19 ± 0.26	2.43 ± 0.30	2.70 ± 0.30
		%	57	81	101	114	109
U. pinnatifida (sample B)	raw	(μg g^{-1})	18.3 ± 1.2	1.99 ± 0.27	10.20 ± 0.22	6.57 ± 0.41	nd[b]
		%	38	112	89	81	
Porphyra sp. (sample B)	raw	(μg g^{-1})	23.7 ± 0.3	6.28 ± 0.4	14.03 ± 1.95	nd	nd
		%	87	119	89		
	baked	(μg g^{-1})	27.8 ± 0.7	3.94 ± 1.05	15.58 ± 2.61	nd	nd
		%	106	81	93		

[a] Bioaccessibility of total As = [(total As in bioaccessible fraction)/(total As in seaweed)] × 100. Bioaccessibility of arsenosugars = [(arsenosugars in bioaccessible fraction)/(arsenosugar As in methanol–water extract)] × 100. [b] Not detected.

In *Porphyra* sp., the species detected and their distribution pattern are in good agreement with the data reported in the literature (*15*, *25*, *37*). The phosphate ribose−glycerol ribose relationship, although very variable (sample A, 1:1; sample B, 3:1), is also in agreement with what has been reported in most previous studies, where phosphate ribose > glycerol ribose (*15*, *28*, *37*). A concentration ratio favoring glycerol ribose has been reported in only three samples (*25*, *37*). The presence of a third arsenosugar, sulfonate ribose, has been shown only by Van Hulle et al. (*28*) in *Porphyra crispata*. With regard to DMA, its presence in *Porphyra* sp. does not seem to be customary, as it is only reported in two of the nine samples described in the literature (*25*, *28*).

It should be noted that in some of the samples of *H. fusiforme* and *U. pinnatifida* (**Table 5**) a chromatographic signal appeared at a retention time of 1.9 min, which does not correspond to any of the arsenic species identified in this study (**Figure 2**). With respect to the food safety of the contents of arsenic species found in seaweed, it can only be estimated for the inorganic arsenic contents, as these are the only species for which the WHO establishes a toxicological reference value, 15 μg/week/kg of body weight. There are no known data concerning the consumption of seaweed in Western countries, which makes it difficult to calculate intake levels, and consequently most estimates are based on the consumption of the population of Japan, with a daily average consumption of brown algae of 2−3 g and a maximum consumption of 12 g, dry weight (*16*). The risk of exceeding the WHO reference value is a reality for an adult with a consumption of 10 g/day for three of the four samples of *H. fusiforme* analyzed.

Bioaccessibility of Dimethylarsinoyl Ribosides. After food ingestion, only the fraction of arsenic species ingested solubles in gastrointestinal medium (bioaccessible fraction) can be available for subsequent absorption by intestinal mucosa. **Table 6** shows the bioaccessible content (μg g^{-1}) and the bioaccessibility (percentage of the arsenic in the sample that becomes soluble after in vitro digestion) of total arsenic and arsenosugars in one sample of each species of seaweed (raw or cooked).

The concentration of total arsenic in the bioaccessible fraction varied between 12.5 and 69.9 μg g^{-1} of seaweed (dw) (**Table 6**). For the raw samples, the bioaccessibility varied between 38 and 87%, with the gradation *Porphyra* sp. > *U. Pinnatifida* > *H. fusiforme*. In *H. fusiforme*, cooking did not alter the total arsenic bioaccessibility, which agrees with the results of a

previous study (*14*). However, after cooking of *Porphyra* sp. there was a marked increase in total arsenic bioaccessibility (from 87 to 106%).

The concentration of arsenosugars in the bioaccessible fraction (**Table 6**) was similar to the value quantified in the methanol/water extract (**Table 5**). Arsenosugar bioaccessibility was >80% in the raw and cooked samples (**Table 6**), indicating that they are species that easily become available for subsequent absorption by the gastrointestinal epithelium, exceeding the inorganic arsenic bioaccessibility reported in edible seaweeds (49−89%) (*14*).

In the bioaccessible fraction we did not detect peaks corresponding to unidentified species, which seems to indicate that during the in vitro digestion applied there was no degradation of the arsenosugars released. In the literature, using simulated gastric juice (pepsin and HCl) degradation of glycerol, phosphate, sulfate, and sulfonate ribose to a similar product, the free dimethylarsinoylribose (As254), has been shown in purified arsenosugars (*20*) and *Laminaria* sp. (*21*). The degradation was slow for arsenosugar standards (1.5% h^{-1}) (*20*) and much greater for the arsenosugars present in boiled *Laminaria* (changes in relative arsenosugar concentration between 32 and 86% after 4 h of incubation) (*21*). The studies cited noted that gastric pepsin does not play a significant role in this degradation. They did not evaluate the intestinal digestion. As gastrointestinal digestion does not seem to contribute to the generation of DMA either in our study or in the two works mentioned (*20*, *21*), it remains to be seen in what stage of metabolism this urinary metabolite is generated.

The arsenosugar bioaccessibility data presented in our study represent an advance on existing information (*20*, *21*) in working with foods prepared as ingested by the consumer and in considering the overall gastrointestinal digestion process. These data make it possible to estimate the maximum concentration of soluble species that would be available for absorption, and they are useful for establishing comparisons between different edible seaweeds. Bioaccessibility has been proposed as a value for predicting the relative bioavailability of arsenic in animal models (*22*), providing a good correlation with in vivo studies performed with pigs (*38*). However, oral bioavailability can be reduced subsequently by partial uptake or biotransformation during absorption on intestinal epithelium. An improvement of these in vitro gastrointestinal systems has been initiated by our work group with the introduction of cell cultures (Caco-2 cells) as a model of the intestinal epithelium, which will make it possible to achieve a more reliable approximation, closer to the in vivo situation, to estimate the bioavailability of arsenosugars.

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