Determination of Arsenic Species in Edible Seaweeds by HPLC–ICP-MS After Pressurized Hot Water Extraction

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INTRODUCTION

Seaweed consumption is an attractive alternative food source. Edible seaweeds are commonly commercialized and recommended as health food, diet pills, vitamin supplements, protein source, etc. The human intake of arsenic (As) and other toxic substances occurs mainly via seafood and seaweed ingestion (1). Thus, a careful nutritional and toxicological evaluation of these products is mandatory. Although arsenic concentration in open seawater is extremely low, marine organisms can bio-accumulate inorganic arsenic from the surrounding seawater (2). Toxic arsenic species (arsenite and arsenate) transform into less toxic organoarsenic compounds in seaweeds, mainly arsenobetaine (AB) and dimethylarsinic acid (DMA) in seafood and arsenosugars. The wide-ranging levels of toxicity exhibited by different arsenic species have led to the development of several analytical methods for their extraction and quantification. Arsenic speciation studies focusing mainly on different edible seaweeds include *Nori* (3–8), *Wakame* (4–9), *Hijiki* (4, 5, 10–12), *Fucus* (5, 13), *Sea Spaghetti* (5), *Kombu* (5, 7, 8), *Laminaria* (5, 6), *Sargaso* (5, 6), *Sea Lettuce* (7, 8, 13) or *Codium* (13). The arsenic content in other algae and seaweeds were reported by *Arame* (5), *Hypnea* (13), *Cladophora* and *Chara* (14), Turner

ABSTRACT

Aqueous soluble arsenic species in Atlantic edible seaweeds have been quantified by high performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC–ICP-MS). A pressurized high-temperature water extraction (PHWE) method has been developed for arsenic species. The significance of several parameters involved in the arsenic species extraction by PHWE has been evaluated using a Plackett–Burman experimental design. The extraction temperature and modifier amounts were optimized by using the central composite design. The extraction procedure was completed within 7 minutes. The analytical performance of the method, such as limit of quantification (in the range of 47 to 101 ng g⁻¹), repeatability (<18%), column recoveries (in the range of 44 to 99%), and extraction efficiency (in the range of 32 to 81%), was established. The developed method was applied to the arsenic speciation in several edible seaweeds such as *Dulse*, *Nori*, *Kombu*, *Wakame*, *Sea Spaghetti*, as well as canned seaweed, and verified by analyzing the certified reference material BCR-279 *Sea Lettuce*.

(15), and *Kelp* (16). In addition, bioaccessibility and bioavailability studies were developed to assess the bioavailability ratios of toxic and non-toxic arsenic species in edible seaweeds (4, 7, 8, 17).

Several “classic” pre-treatments based on solvents assisted by mechanical shaking (4, 13–15, 18) and ultrasonic (3, 4, 7–9, 11, 16) or microwave (5, 6, 12) energy have been applied to arsenic species extraction from seaweeds. The main drawback with most of these procedures is that they are very time-consuming. Typical extraction times reported are 15 minutes (3, 4, 16), 30 minutes (11), 60 minutes (7, 8), or 180 minutes (9) for ultrasound-assisted extraction (UAE); 5 minutes (12), 15 minutes (5), and 30 minutes (6) for microwave-assisted extraction (MAE); or 120 minutes (18), overnight (15), and 16 hours (13, 14) for shaking procedures. Also, the use of large amounts of solvents [de-ionized water (DIW) (5, 9, 11–14, 18), methanol (MeOH)/DIW mixtures (3, 4, 15, 16), ammonium carbonate (6) or ammonium acetate (7, 8) buffers] and extract handling (centrifugation steps are required) before analysis are other important disadvantages of these classical procedures. There is, therefore, an interest in developing automated, rapid extraction, and green methods that guarantee high recovery of the analytes.

Pressurized hot water extraction (PHWE) offers short extraction times compared to conventional sample pre-treatment. Also, extract handling is avoided, thus a filtration step is not required. Since no toxic solvents are used, PHWE pre-treatment is considered environmentally friendly. The addition of small amounts of modifiers [<10% (v/v)] to pressurized hot water has been

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usually applied to enhance the solubility of the analytes in water or to increase the interaction of water with the analytes. Several organic (mainly MeOH) and inorganic (mainly nitric acid and weak carboxylic acids) substances in the range of 1 to 10% (v/v) have been used as modifiers for arsenic and other organometallic compound extractions by PHWE (19, 20). Pressurized hot water, without addition of modifiers, has been successfully applied for arsenic species extraction in plants and vegetables (21, 22). The addition of small amounts of modifiers [EDTA 1.2% (w/v) (23) or acetic acid 1.0 % (v/v) (24)] to pressurized hot water has also been proposed for arsenic species extraction in particulate matter (23) and human hair (24). Finally, less "green" extraction methods by using pressurized methanol/water mixtures for the extraction of arsenic species in fish (25), seaweeds (26), seafood (27), and food of animal origin (28) are reported in the literature.

In this work, an automated and fast extraction procedure by using pressurized hot water, modified with small amounts of acetic acid, has been applied to several Atlantic edible seaweeds for aqueous soluble arsenic species extraction with good results.

EXPERIMENTAL

Instrumentation

An ASE-200 system (Dionex, Sunnyvale, CA, USA), equipped with 11-mL stainless steel cells and cellulose filters (D28, 1.983 cm diameter, Dionex), was used for PHWE. Arsenic species separation was performed on a Hamilton PRP-X 100 anion-exchange HPLC column (250 mm×4.1 mm id×10 µm particle size) (Hamilton Company, Reno, Nevada, USA) in a Dionex HPLC Ulti-MateO 3000 LC, equipped with a GP50 gradient pump (Dionex), an AS50 thermal compartment

(Dionex), and an AS-50 auto-sampler (Dionex). An ICP-MS Thermo Finnigan X Series (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for the quantification of the arsenic species. A Fritsch Planetary Micro Mill Pulverisette 7 ball mill (Fritsch Industriest, Idar-Oberstein, Germany), equipped with zircon cups and zirconium balls, was used to mill the seaweed. Particle-size distribution was obtained by use of a Laser Coulter Series LS200, Fraunhofer optical model particle sizer with an LS variable-speed fluid module plus (Coulter Electronics, Hialeah, FL, USA). A Multiwave® sample preparation system (PerkinElmer, Inc., Shelton, CT, USA), equipped with six Anton Paar (Graz, Austria) reactors programmable for time and microwave power, was used for digestion of the seaweed samples. The chemometrics package of Statgraphics® Plus V 5.0 for Windows®, 1994-1999 (Manugistics, Inc., Rockville, MD, USA) was used for the statistical, graphic, reporting, processing, and tabulating procedures.

Chemicals, Reagents, and Standard Solutions

All chemicals used were of ultrapure grade, and diluted using water of 18 MΩ·cm⁻¹ resistance obtained with a Milli-Q® purification system (Millipore Corporation, Bedford, MA, USA). Glacial acetic acid of 99.99% was used as the modifier and diatomaceous earth (DE) 95% SiO₂ as the dispersing agent (Aldrich, Milwaukee, WI, USA). Nitrogen Alphagaz™ (Air Liquide, Madrid, Spain) was used as a purge gas. Arsenite [As(III)] and arsenate [As(V)], (1000 mg L⁻¹) stock standard solutions were purchased from Panreac (Barcelona, Spain). Standard solutions of MMA(V), DMA(V) (1.000 g L⁻¹) were prepared by dissolving the appropriate amounts of MMA(V) [CH₃AsO(ONa₂)·6H₂O] (Carlo Erba, Milan, Italy) and DMA(V)

(C₂H₆AsNaO₂·3H₂O) (Merck, Poole, U.K.) in ultrapure water. Ammonium hydrogen carbonate (NH₄HCO₃) (Panreac) and methanol gradient grade for liquid chromatography (Merck) were used in the mobile phase preparations. Concentrated nitric acid (Merck) and hydrogen peroxide (Panreac) were used to perform the acid digestions. BCR 279 Sea Lettuce, *Ulva lactuca*, from the Community Bureau of Reference – Commission of the European Communities (Brussels, Belgium) was used for method validation. To avoid metal contamination, all glassware and plastic ware was washed and kept for 48 hours in 10% (v/v) nitric acid, then rinsed several times with ultra-pure water before use.

Edible Seaweed Samples and Sample Pretreatment

Seven different Atlantic seaweeds, harvested on the Galician coast (Northwestern Spain), were obtained from a local manufacturer. One of the samples is commercialized as cooked and canned in brine (around 25 g) and consists of a mixture of Sea Spaghetti (*Himanthalia elongata*) and Furbelows (*Saccorhiza polyschides*). This sample was freeze-dried before grinding. The remaining seaweed samples are commercialized as dehydrated products (approximately 100 g): Dulse (*Palmaria palmata*), Nori (*Porphyra umbilicalis*), Kombu (*Laminaria ochroleuca*), Wakame (*Undaria pinnatifida*), and Sea Spaghetti (*Himanthalia elongata*). The dehydrated samples were kept in an oven at 40 °C for 2 hours to eliminate water traces, then pulverized in a vibrating zircon ball mill for 45 minutes (power at 75%). The particle-size distribution was determined by laser diffraction spectrometry. The mean particle size after this treatment was approximately 250 µm. All samples were preserved in pre-cleaned polyethylene bottles.

Pressurized Liquid Extraction Procedure

Amounts of 0.5 g of the homogenized seaweeds were mixed thoroughly with 1.0 g of inert DE (DE mass / sample mass ratio = 2). The mixture was poured into Dionex standard 11-mL stainless steel extraction cells containing cellulose filters. After closing the cells, they were placed on the carousel of the ASE 200 system, and the samples extracted under the following PHWE conditions: 5.5 % (v/v) acetic acid, static extraction time of 5 minutes, one extraction cycle, flush volume at 60%, extraction temperature at 110 °C, and extraction pressure at 1500 psi. After the extraction process, the cells were purged for 60 seconds with N₂ gas, and the displaced solvent was collected in pre-cleaned glass vials. The total extraction time and the total water-modified volume used were 7 minutes and 20 mL, respectively. The extracts were placed into polyethylene bottles and kept at 4 °C until analysis. At least two different blanks were performed in each set of PHWE conditions. The extraction cells were cleaned between each run with ultra-pure water. Then, the cells were conditioned with methanol as the solvent using the optimized PHWE conditions (Table I).

TABLE I
Pressurized Liquid Extraction Conditions

Modifier concentration	5% (v/v)
Extraction temperature	110 °C
Static time	5 min
Extraction steps	1
Pressure	1500 psi
Flush volume solvent	60%
DE mass/sample mass ratio	2
N ₂ purge time	60 s
Cell size	1 mL ⁻¹

Microwave Acid Digestion Procedure

The powdered seaweed samples were subjected to an acid microwave-assisted extraction (MAE) to assess the total arsenic content in seaweeds (29). After acid extraction, the solutions were diluted to 25 mL with ultra-pure water and kept in polyethylene bottles at 4 °C before measurement.

ICP-MS Measurements

Total arsenic in the acid digests after microwave acid extraction and total arsenic in the aqueous extracts after PHWE were measured by ICP-MS (24) (Table II). The calibrations were based on

5.5% (v/v) acetic acid aqueous standard solutions covering arsenic concentrations from 0 to 1000 µg L⁻¹. Collision cell technology (H₂/He at 5.85 mL min⁻¹) was used to reduce the ArCl (m/z 75) polyatomic interference. Germanium (10.0 µg L⁻¹) was selected as the internal standard.

After performing different calibrations over six different days, good repeatability of the slopes of the calibrations was obtained. Table III lists the mean and standard deviation of the slopes of the calibration graphs for total arsenic quantification. The limit of detection (LOD) [3 SD criterion, standard deviation (SD) of 11 measurements

TABLE II
Operating HPLC–ICP-MS Conditions

ICP-MS	Radio frequency power	1400 W
	Peristaltic pump speed	2.5 rpm
	Nebulizer type	MicroMist
	Nebulizer chamber	Bead impact equipped with a Peltier cooling device (5°C)
Gas flows	Plasma	14.0 L min ⁻¹
	Auxiliary	0.8 Lmin ⁻¹
	Nebulizer	0.85 L min ⁻¹
Torch alignment	Horizontal	117 mm
	Vertical	317 mm
	Sampling depth	210 mm
Ion optics	Extraction	-102 V
	Focus	-7.8 V
	Pole bias	-20 V
	Hexapole bias	-17 V
CCT	H ₂ /He (8% H ₂ in He)	5.85 mL min ⁻¹
Mass-to-charge ratio	As	75
	Ge (internal standard),	72
	post-column addition at 5 µg L ⁻¹	
HPLC	Hamilton PRP-X100 (250 mm × 4.1 mm × 10 µm) anion-exchange column	
	Injection volume	50 µL
	Column temperature	25 °C
	Mobile phases flow rate	1.0 /mL min ⁻¹
Mobile phase	20 mM NH ₄ HCO ₃ (pH 9.0), 1% (v/v) MeOH	
Elution program	Isocratic	

of a reagent blank], expressed as $\mu\text{g g}^{-1}$, and the limit of quantification LOQ (10 SD criterion) were calculated (see Table III).

HPLC-ICP-MS Measurements

The anion-exchange HPLC conditions (summarized in Table II) were used to obtain separation of the arsenic species in a single chromatographic run using a Hamilton PRPX100 column and 20 mM NH_4HCO_3 (pH 9.0) containing 1.0% (v/v) MeOH as the mobile phase (30). The anion-exchange HPLC conditions, as well as the ICP-MS settings, are summarized in Table II. The aqueous calibrations covered arsenic concentrations of 0, 12.5, 50, 100, and 200 $\mu\text{g L}^{-1}$ (expressed as arsenic) for As(III), As(V), MMA, and DMA. Germanium (^{72}Ge) at a flow of 5 $\mu\text{g L}^{-1}$ was the internal standard continuously mixing the chromatographic eluate (1.0 mL min^{-1}) at an external flow of the internal standard (1.25 mL min^{-1}) via a T-junction after the column and just before the nebulizer. Since arsenosugar standards are not available, aqueous calibration for seaweed extracts was achieved by using DMA standards (in the range of 0 to 200 $\mu\text{g L}^{-1}$, expressed as arsenic) for quantification of Gly-Sugar (3-[5'-deoxy-5'-(dimethyl-

arsinoyl)- β -ribofuranosyloxy]-2-hydroxypropylene glycol), PO_4 -Sugar (3-[5'-deoxy-5'-(dimethylarsinoyl)- β -ribofuranosyloxy]-2-hydroxypropyl-2,3-hydroxypropyl phosphate), SO_3 -Sugar (3-[5'-deoxy-5'-(dimethylarsinoyl)- β -ribofuranosyloxy]-2-hydroxypropane sulfonic acid), and SO_4 -Sugar (3-[5'-deoxy-5'-(dimethylarsinoyl)- β -ribofuranosyloxy]-2-hydroxypropyl hydrogen sulfate) (4). Table III lists the mean and the standard deviation of the slopes of the calibration graphs for inorganic arsenic species and DMA and MMA. Good repeatability of the calibration curves was observed over six different days, with a %RSD of around 16% in all cases. Keeping in mind the sample weight, the LODs and LOQs, expressed as ng g^{-1} (Table III), are low enough to perform arsenic speciation in the seaweed samples (LOQs ranging from 46.9 to 269 ng g^{-1}).

Figure 1 (a and b) shows typical chromatograms for a mixture of arsenic species standards [As(III), DMA, MMA, and As(V)], and a pooled seaweed sample after applying the proposed extraction and quantification conditions. Identification of the arsenosugar, shown in Figure 1b, is based on previous works (7-8). By using the separation

conditions shown in Table II, arsenosugar retention times were assigned after analysis of a lyophilized algal extract of *Fucus serratus* (containing the four arsenosugars: Gly-Sugar, PO_4 -Sugar, SO_3 -Sugar, and SO_4 -Sugar) kindly donated by Professor Dr. K. A. Francesconi (Karl-Franzens University, Graz, Austria) (8). Also, arsenosugar identification was confirmed after electrospray ionization tandem mass spectrometry (ESI-MS/MS) with two-dimensional HPLC (size exclusion, followed by anion exchange) (7).

RESULTS AND DISCUSSION

Pressurized Hot Water Extraction (PHWE) Procedure Optimization

Different variables affecting the PHWE (pressure, temperature, static time, number of cycles, sample mass/dispersing agent mass ratio, and modifier amount) for the extraction of aqueous arsenic species in edible seaweeds were fully studied by using the Dionex ASE 200 system.

Selection of Dispersing Agent

A preliminary experiment was performed to determine the proper dispersing agent for PHWE. A homogenized pooled seaweed sample was selected as the dispersing agent. In order to establish the reference arsenic concentration, the pool was acid-digested four times, and the arsenic concentration measured twice by ICP-MS. The mean arsenic concentration after microwave-assisted acid digestion ($N=8$) was $20.1 \pm 0.7 \text{ mg kg}^{-1}$.

Different silica-based solid supports, such as diatomaceous earth (DE), sea sand (SS), octyl-bonded silica, C18, and silica, were tested. PHWE was performed with 0.50 g of a dry pooled seaweed sample and 1.0 g of supporting agent (dispersing mass-to-sample mass ratio of 2). Extractions were performed

TABLE III
Mean Slopes for Calibration Graphs and Detection and Quantification Limits

	Mean Calibration Slope $\pm$ S. D. ^a	LOD ^b (ng g^{-1})	LOQ ^b (ng g^{-1})
Total As	0.027 $\pm$ 0.01	1.5	6.3
Gly-Sugar	-	27.2 ^c	86.7 ^c
As(III)	970 $\pm$ 80	33.5	83.4
DMA	1530 $\pm$ 250	14.9	46.9
PO_4 -Sugar	-	22.3 ^c	72.4 ^c
SO_3 -Sugar	-	25.4 ^c	84.2 ^c
MMA	1980 $\pm$ 340	25.8	79.9
SO_4 -Sugar	-	31.7 ^c	101 ^c
As(V)	390 $\pm$ 60	88.6	269

^a $N=6$

^b $N=11$

^c Values achieved using slope from DMA calibration.

using an acetic acid concentration of 6.0% (v/v), an extraction temperature of 100 °C at a pressure of 1500 psi, during 5 minutes, and by using two extraction steps. Two different extractions were performed with each dispersing agent, and a blank was also prepared. The results are shown in Figure 2 for Gly-sugar, DMA, PO₄-Sugar, SO₃-Sugar, SO₄-Sugar, and total arsenic concentrations. It can be seen that the use of DE results in highest arsenic species concentrations.

On the other hand, for inorganic arsenic, high blank signals were found when using SS [150 and 10,200 counts for As(III) and As(V), respectively]. The low blank signals for DE were [110 and 3100 counts for As(III) and As(V), respectively],

and for C18 [130 and 1170 counts for As(III) and As(V), respectively]. It must be said that negligible blanks were found for the organic arsenic species by using all dispersing agents. Thus, DE was chosen as the dispersing agent for further experiments.

Factors/Variables for Screening Study -- Variables Affecting As Leaching by PHWE

A homogenized pooled seaweed sample (with a total arsenic concentration of $20.1 \pm 0.7 \text{ mg kg}^{-1}$) was also used for the screening variables study and for further significant variables optimization. For screening and optimization purposes, the response variables were the percentages of the released arsenic species according to the following equation:

$$\text{Recovery (\%)} = \frac{\text{CPHWE}}{\text{CMAE}} \times 100$$

where CPHWE is the total arsenic concentration obtained after PHWE and ICP-MS determination (total arsenic) or after PHWE and HPLC-ICP-MS determination (total arsenic as sum of arsenic species), and CMAE is the arsenic concentration after the microwave acid digestion procedure.

A $2^8 \times 3/32$ type III resolution Plackett-Burman design (PBD) for eight factors, four degrees of freedom, 12 runs and two replicates, was chosen to screen the variables affecting the PHWE of arsenic species from edible seaweeds. The variables studied and the field definition of the variables, e.g., the low (-) and high (+) levels assigned to each variable, are listed in Table V.

Fig. 1 (a&b). HPLC-ICP-MS chromatograms for arsenic species standards: $200 \mu\text{g L}^{-1}$ for As(III), DMA, MMA and As(V) (a); and PHWE extract from pooled seaweed sample using conditions shown in Section 2.7 (b). (1): Gly-Sugar, (2): As(III), (3): DMA, (4): PO₄-Sugar, (5): SO₃-Sugar, (6): UK, (7): MMA, (8): SO₄-Sugar and (9): As(V).

Fig. 2. Effect of the nature of the dispersing agent (diatomaceous earth, DE; sea sand, SS; octyl-bonded silica, C18 and silica gel, SG) for the PHWE of total arsenic, Gly-sugar, DMA, PO₄-Sugar, SO₃-Sugar, SO₄-Sugar and total arsenic as sum of arsenic species from a pooled seaweed sample ($N = 2$).

The PBD experimental conditions and the values for the response variables are listed in Table V. At a 95.0% confidence interval, a minimum t value of 3.16 was obtained after the iterative process by the Statigraphic routine. It is stated that variables with t values higher than ± 3.16 must be considered statistically significant factors. The main effect Pareto Chart (Figure 3) showed statistical significance for some factors, such as for the extrac-

tion temperature and the modifier concentration.

The statistically non-significant variables included the selection of lowest extraction time and extraction steps so that PHWE can be performed in the shortest time/sample pulverization to guarantee a certain particle size is not required (mean particle size was not statistically significant in the 100 – 250 μm range); a low DE mass/sample mass

ratio was chosen due to the use of 1.0 g of DE which facilitates dispersion of the sample and extraction cell filling. The dummy factor was not significant for any of the variables, meaning that there are no systematic errors nor any unknown variables affecting the system under study. For the next stage in the optimization process, extraction time, extraction steps, extraction pressure, and DE mass/sample mass ratio were fixed at 5 minutes, using one extraction step, 1500 psi and the ratio of 2, respectively.

TABLE IV
Experimental Field Definition for the Plackett–Burman (PBD) Designs^a

Variable	Symbol	Low Level (–)	High Level (+)	Unit
Acetic acid concentration	A	1.0	6.0	% (v/v)
Extraction temperature	T	25	100	°C
Extraction time	t	5	10	min
Extraction step	S	1	3	
Pressure	P	500	1500	psi
Particle size	s	125	200	μm
DE mass/sample mass ratio	R	2	5	
Dummy factor	D	– 1	+ 1	

^a 60% flush volume, 60 s N₂ purge time, and cell size of 11 mL.

TABLE V
2⁸ * 3/32 PBD for the Determination of Significant Parameters Involved With PHWE

Run	A	T	t	S	P	s	R	D	Mean Analytical Recovery(%) ^a	
									Total As ^b	Sum of As Species ^c
1	+	–	+	–	–	–	+	+	43.4	36.9
2	+	+	–	+	–	–	–	+	58.5	47.4
3	–	+	+	–	+	–	–	–	46.3	38.0
4	+	–	+	+	–	+	–	–	42.1	32.9
5	+	+	–	+	+	–	+	–	47.2	41.6
6	+	+	+	–	+	+	–	+	52.7	42.7
7	–	+	+	+	–	+	+	–	44.5	37.8
8	–	–	+	+	+	–	+	+	41.7	31.3
9	–	–	–	+	+	+	–	+	33.2	26.6
10	+	–	–	–	+	+	+	–	37.7	33.6
11	–	+	–	–	–	+	+	+	36.7	33.1
12	–	–	–	–	–	–	–	–	33.5	30.5

^a N=2. ^b After PHWE and ICP-MS. ^c After PHWE and HPLC-ICP-MS.

Optimization of Extraction Temperature and Modifier Concentration by Central Composite Designs

The optimization of acetic acid concentration (A) and extraction temperature (T) was accomplished by an orthogonal central composite design (2² + star) which studied the effects of these factors in 10 randomized runs with 5 degrees of freedom. The low and high values for each factor are shown in Table V. Table VI gives the central composite design matrix and the percentages of released arsenic for each experiment. The results of the experiments were analyzed by the response surface plot obtained for the percentages of total released arsenic (Figure 4a) and the percentages of total released arsenic as the sum of the arsenic species (Figure 4b). Careful study of these results leads to compromise values for acetic acid concentration and extraction temperature of 5.5% (v/v) and 110 °C, respectively. By using the above optimized conditions, the extraction procedure was completed within 7 minutes.

Determination of Total Arsenic and Arsenic Species in Atlantic Edible Seaweeds

Total arsenic concentrations in several Atlantic seaweeds and in the certified reference material BCR-279 Sea Lettuce (from the

Fig. 3. Main effect Pareto chart for total arsenic extraction (a) and total arsenic extraction as sum of arsenic species (b) by PHWE

Table VI
Central 2^2 + Star Orthogonal Composite Design Used for Acetic Acid Concentration (A) / Extraction Temperature (T)

Run No.	A	T	Mean Metal Recovery(%)	
			Total As ^b	Sum of As Species ^c
1	K ₀ ^d	K ₀ ^g	61.6	56.1
2	-	-	27.8	25.0
3	+	-	75.0	63.8
4	-	+	47.5	39.4
5	+	+	58.6	49.2
6	- α ^e	K ₀ ^g	26.2	23.8
7	+ α ^f	K ₀ ^g	50.2	45.2
8	K ₀ ^d	- α ^h	29.0	25.3
9	K ₀ ^d	+ α ⁱ	58.9	53.0
10	K ₀ ^d	K ₀ ^g	62.3	56.7

^a N=2. ^b After PHWE and ICP-MS.

^c After PHWE and HPLC-ICP-MS.

^d K₀ = 3.5 % (v/v). ^e - α = 0 % (v/v).

^f + α = 7.0 % (v/v). ^g K₀ = 110 °C.

^h - α = 25 °C. ⁱ + α = 200 °C.

Community Bureau of Reference, Commission of the European Communities) were assessed after a conventional acid digestion, followed by ICP-MS quantification (Table VII). Total arsenic concentrations and total arsenic concentrations (as sum of arsenic species) were also assessed using the pressurized hot water extraction procedure. The repeatability of the over-all procedure was studied by analyzing several seaweed samples for 11 times. Each aqueous extract was analyzed once and the RSD values were lower than 18% for all cases.

Fig. 4. Estimated response surface for total arsenic extraction (a) and total arsenic extraction as sum of arsenic species (b). Experiments were performed with a static time of 5 min, one extraction step, a pressure of 500 psi, DE mass / sample ratio of 2, N₂ purge time of 60 s, and a flush volume of 60% were used.

Determination of Total Arsenic After Acid Digestion

Table VII lists the results obtained for total arsenic determination in the cooked seaweed (mixture of Sea Spaghetti and Furbelows) and in five raw edible seaweeds (Dulse, Kombu, Nori, Wakame, and Sea Spaghetti). The total arsenic concentration (after MAE-ICP-MS) varied widely and was in the range of 7.8 to 55.6 $\mu\text{g g}^{-1}$. These variations are attributable to the species of algae analyzed and other factors, such as collection points, maturity degrees of seaweeds, light intensity, turbidity, temperature, depth, salinity, and nutrient uptake (4, 13, 31). High arsenic concentrations were obtained for the *Kombu* (55.6 $\mu\text{g g}^{-1}$) and *Nori* (39.4–54.4 $\mu\text{g g}^{-1}$) samples. Those levels are in agreement with reported data for Atlantic seaweeds (5, 6, 8, 32). The lowest arsenic value (7.8 $\mu\text{g g}^{-1}$)

was found in cooked and canned seaweed (Sea Spaghetti and Furbelows), which could be due to the fact that arsenic dissolves into the cooking water (8, 33).

Accuracy of the method was assessed by analyzing BCR-279 Sea Lettuce. The concentration found ($3.25 \pm 0.20 \text{ mg kg}^{-1}$) is in good agreement with the certified values ($3.09 \pm 0.21 \text{ mg kg}^{-1}$) and was obtained after statistical evaluation by applying the Student's *t*-test at a 95% confidence level for six degrees of freedom. The *t*cal value at 2.12 was found to be lower than the *t* tab value at 2.45.

Determination Total Arsenic Extractable iton Pressurized Hot Water

Table VII also shows the amount of total arsenic extracted into the pressurized hot water from the edible seaweeds studied, and also for

the BCR-279 Sea Lettuce. As commented above for total arsenic extraction by MAE, the total arsenic extractable into pressurized hot water varied widely ($5.0\text{--}44.1\text{ }\mu\text{g g}^{-1}$). High arsenic concentrations were obtained for the Kombu and Nori (41.7 and $28.4\text{--}44.1\text{ }\mu\text{g g}^{-1}$, respectively) samples. The pressurized hot water extraction efficiencies (calculated as the ratio of total arsenic in the water extract to total arsenic in the seaweed, after MAE digestion) were in the range of 65% (for canned seaweed) to 81% (for Nori). The extraction efficiencies achieved are in agreement with previously reported data (3–5, 15, 16, 26), except that the arsenic species are extracted in a very short period of time (extraction was completed within 7 minutes: 5 minutes of extraction time plus one minute of purge time and one minute of end relief). This extraction time is considerably shorter in comparison to classical extraction times as indicated above and reported in the literature. Also, the filtration step was avoided. An arsenic extraction efficiency of $76\pm 3\%$ (by using a mixture of MeOH:DIW and sonication) (16) and $67.9\text{--}73.3\%$ (by using MeOH:DIW and pressurization) (26) has been reported for kelp samples. Wei et al. (3), García-Salgado et al. (5), Terol et al. (15), and Raab et al. (18) reported high extraction efficiency for *Porphyra* sp. (3); *Sea Spaghetti*, *Kombu*, *Wakame*, and *Nori* (5); Turner (15); and Hijiki and Sea Lettuce (18) when using a mixture of MeOH:DIW plus sonication ($90\text{--}96\%$) (3) or shaking ($84\text{--}98\%$) (15), and by using DIW with shaking ($73\text{--}96\%$) (5,18). On contrary, Almela et al. reported low extraction efficiencies (in the range of 5 to 73%) for raw Kelp, *H. fusiforme*, *U. pinnatifida*, *Fucus* sp. and *Porphyra* sp. when mixtures of MeOH:DIW were used as the extraction solvent (4). Non-quantitative extraction efficiencies can be due to the presence of arsenic lipid-

soluble compounds and arsenic bound to proteins which is not extracted into the water (–). Recently, the content of lipophilic arsenic species in *Saccharina latisima* (35), Hijiki (37), and *Ectocarpus* (38) has been identified and quantified.

Table VII also lists the total arsenic concentration extracted into pressurized hot water for the BCR-279 sample. As can be seen, the hot water extraction efficiency (calculated as the ratio of total arsenic in the water extract to certified value) is 74.5%. This result is in agreement with previously reported data about arsenic extraction by using DIW water as the solvent extraction (14,18,39).

Determination of Arsenic Species Extractable in Pressurized Hot Water

The arsenic species concentrations obtained ranged from $1.2\text{--}9.7\text{ }\mu\text{g g}^{-1}$ for Gly-Sugar, $0.33\text{--}2.9\text{ }\mu\text{g g}^{-1}$ for DMA, $0.25\text{--}32.9\text{ }\mu\text{g g}^{-1}$ for PO_4 -Sugar, $<0.08\text{--}18.9\text{ }\mu\text{g g}^{-1}$ for SO_3 -Sugar, and $<0.10\text{--}7.0\text{ }\mu\text{g g}^{-1}$ for SO_4 -Sugar (Table VII). The MMA and As(III) levels were below the LOQs in all analyzed samples. The As(V) concentrations for these samples, except for Dulce-2, are also below the LOQ. As can be seen, the analyzed seaweeds showed a pattern of arsenic species similar to Gly-Sugar, DMA, and PO_4 -Sugar as major species. However, the Nori samples offer a high PO_4 -Sugar concentration, yet the SO_3 -Sugar concentration is below the LOQ, and the *Sea spaghetti* samples offer a high SO_3 -Sugar concentration. Finally, an unknown arsenic species was also found in both Dulce samples. Similar Gly- and PO_4 -Sugar values were reported by García-Salgado et al. ($1.6\text{--}14.3$ and $0.1\text{--}20.1\text{ }\mu\text{g g}^{-1}$, for Gly- and PO_4 -Sugar, respectively) in *Sea Spaghetti*, *Kombu*, *Wakame*, and *Nori* seaweeds (5) and by Hsieh and Jiang (in the range of 0.34 to $23.7\text{ }\mu\text{g g}^{-1}$

for Gly-Sugar and 0.834 to $27.3\text{ }\mu\text{g g}^{-1}$ for PO_4 -Sugar) in *Wakame*, *Kombu*, *Sargassum*, and *Porphyra dentada* (6). However, low values for SO_3 -Sugar ($<\text{LOQ}\text{--}4.2\text{ }\mu\text{g g}^{-1}$) and SO_4 -Sugar ($<\text{LOQ}$), and high values for As(V) ($<\text{LOQ}\text{--}24\text{ }\mu\text{g g}^{-1}$) were reported (5). Low DMA values ($<\text{LOQ}\text{--}0.4\text{ }\mu\text{g g}^{-1}$) (5) and $0.235\text{--}1.3\text{ }\mu\text{g g}^{-1}$ (6) were also published in these Atlantic seaweeds. As commented above, these variations could be attributed to the species of algae analyzed and other factors (collection points, maturity degrees, light intensity, turbidity, temperature, etc.) (4, 13, 40).

The efficiency of the arsenic extraction by using pressurized hot water was also evaluated as the percentage of total arsenic (as the sum of arsenic species after PHWE-HPLC-ICP-MS) with respect to total arsenic concentration in the sample (after MAE-ICP-MS). The efficiency of the extraction (Table VI) was very variable ($40\text{--}81\%$). *Dulse*, *Kombu* and canned seaweeds offer low extraction efficiencies (around 40 to 50%). The highest extraction efficiencies were achieved for *Nori* and *Wakame* (around 70–80%). Pell et al. (13, 14) reported low extraction efficiencies (as the sum of arsenic species with respect to total arsenic concentration in the sample) in the range of 5 to 69% in several *Cystoseira* species, *Codium*, *Ulva* species, *Cladophora* sp., and *Chara* sp when using DIW and mechanical shaking. Hsieh and Jiang (6) reported high extraction efficiencies in *Wakame* (96%), *Kombu* (96%), *Sargassum* (92%) and *Porphyra dentada* (89%) by using a mixture of MeOH/ammonium carbonate and microwave energy. The column efficiency, as the percentage of total arsenic (as the sum of arsenic species after PHWE-HPLC-ICP-MS) with respect to the total arsenic concentration in the aqueous extract injected into the column, was also calculated. The column recoveries ranged

TABLE VII
PHWE Efficiency and Analysis of Several Atlantic Seaweeds From the Galician Coast
After PHWE and HPLC-ICP-MS

Concentration (mean value $\pm$ SD) ^a Expressed in $\mu\text{g g}^{-1}$													
Sample	Total As ^b	Total As ^c	Gly-Sugar ^c	As(III)	DMA	PO ₄ -Sugar ^d	SO ₃ -Sugar ^d	Un-known ^d	SO ₄ -Sugar ^d	As(V)	Total As as Sum of As Species	Column Recovery (%) ^e	Extraction Efficiency (%) ^f
Dulse-1	16.8 $\pm$ 0.7	11.4 $\pm$ 0.5	4.9 $\pm$ 0.1	<0.08	1.4 $\pm$ 0.1	0.81 $\pm$ 0.02	0.31 $\pm$ 0.01	0.18 $\pm$ 0.02	<0.10	<0.27	7.7 $\pm$ 0.2	66.6 $\pm$ 0.5	45.8 $\pm$ 0.7
Dulse-2	14.9 $\pm$ 0.8	10.6 $\pm$ 0.4	3.5 $\pm$ 0.1	<0.08	0.83 $\pm$ 0.02	0.76 $\pm$ 0.01	0.46 $\pm$ 0.01	0.27 $\pm$ 0.01	0.17 $\pm$ 0.01	<0.27	6.0 $\pm$ 0.1	57.8 $\pm$ 0.4	40.4 $\pm$ 0.8
Kombu	55.6 $\pm$ 0.9	41.7 $\pm$ 0.8	7.4 $\pm$ 0.4	<0.08	0.33 $\pm$ 0.01	2.6 $\pm$ 0.2	18.9 $\pm$ 0.5	-	<0.10	<0.27	29.4 $\pm$ 0.7	70.2 $\pm$ 1.1	52.9 $\pm$ 1.1
Nori-1	39.4 $\pm$ 0.6	28.4 $\pm$ 0.3	9.4 $\pm$ 0.5	<0.08	2.9 $\pm$ 0.1	14.3 $\pm$ 0.5	<0.08	-	<0.10	<0.27	26.8 $\pm$ 0.7	93.7 $\pm$ 0.8	67.5 $\pm$ 0.9
Nori-2	54.4 $\pm$ 0.8	44.1 $\pm$ 0.4	9.7 $\pm$ 0.5	<0.08	0.83 $\pm$ 0.02	32.9 $\pm$ 0.5	<0.08	-	<0.10	0.38 $\pm$ 0.02	43.9 $\pm$ 0.7	99.5 $\pm$ 0.8	80.6 $\pm$ 1.1
Wakame-1	8.1 $\pm$ 0.6	5.8 $\pm$ 0.1	2.9 $\pm$ 0.1	<0.08	0.71 $\pm$ 0.02	0.45 $\pm$ 0.03	0.23 $\pm$ 0.01	-	0.11 $\pm$ 0.01	<0.27	4.4 $\pm$ 0.1	76.1 $\pm$ 0.1	54.8 $\pm$ 0.6
Wakame-2	19.3 $\pm$ 1.1	14.5 $\pm$ 0.6	3.1 $\pm$ 0.1	<0.08	1.0 $\pm$ 0.1	4.8 $\pm$ 0.1	5.3 $\pm$ 0.2	-	<0.10	<0.27	14.3 $\pm$ 0.3	98.0 $\pm$ 0.7	73.5 $\pm$ 1.1
Sea -1 spaghetti-1	21.8 $\pm$ 1.2	16.4 $\pm$ 0.6	4.2 $\pm$ 0.3	<0.08	0.97 $\pm$ 0.04	0.42 $\pm$ 0.01	4.3 $\pm$ 0.3	-	3.3 $\pm$ 0.1	<0.27	13.2 $\pm$ 0.7	80.5 $\pm$ 0.9	60.4 $\pm$ 1.4
Sea spaghetti-2	28.9 $\pm$ 0.8	20.8 $\pm$ 0.4	6.5 $\pm$ 0.1	<0.08	0.50 $\pm$ 0.1	0.25 $\pm$ 0.08	5.8 $\pm$ 0.8	-	7.0 $\pm$ 0.2	<0.27	20.2 $\pm$ 0.8	96.6 $\pm$ 0.9	69.8 $\pm$ 1.1
Canned seaweed-1	7.8 $\pm$ 0.1	5.0 $\pm$ 0.3	1.2 $\pm$ 0.1	<0.08	0.46 $\pm$ 0.01	0.91 $\pm$ 0.01	0.30 $\pm$ 0.01	-	0.38 $\pm$ 0.01	<0.27	3.3 $\pm$ 0.1	65.2 $\pm$ 0.3	42.4 $\pm$ 0.1
BCR-279	3.25 $\pm$ 0.2	2.30 $\pm$ 0.2	0.30 $\pm$ 0.04	0.11 $\pm$ 0.01	0.05 $\pm$ 0.002	0.08 $\pm$ 0.002	<0.08	-	<0.10	0.47 $\pm$ 0.03	1.0 $\pm$ 0.1	43.5 $\pm$ 0.2	32.4 $\pm$ 0.2 ^g

^a N=4. ^b Concentration after MAE-ICP-MS. ^c Concentration after PHWE-ICP-MS. ^d Values achieved using slope from DMA calibration. ^e Calculated as the ratio between the sum of arsenic species concentrations (after PHWE-HPLC-ICP-MS) and the total arsenic concentration in the extract (after PHWE-ICP-MS). ^f Calculated as the ratio between the sum of arsenic species (after PHWE-HPLC-ICP-MS) and the total arsenic concentration after (MAE-ICP-MS). ^g Calculated as the ratio between the sum of arsenic species (after PHWE-HPLC-ICP-MS) and the total arsenic concentration after (MAE-ICP-MS).

between 58 and 99% (Table VI). Canned seaweed offers the lowest column recoveries (58–67 %). The cooking process of canned seaweed might cause changes in the chemical structure of certain arsenic species, which could not be accurately quantified. These results were consistent with column efficiencies reported for several Atlantic seaweeds (5,13–15). The low column recoveries achieved for some samples could be due to (a) the presence of species at concentration levels lower than the LOQs, (b) arsenic species that are not able to elute from the analytical column, (c) the presence of several arsenic compounds that could not be quantified with the separation method used, or (d) the use of DMA stan-

dards for arsenosugar quantification (5,13).

Although the arsenic species concentrations in BCR-279 Sea Lettuce are not certified, this reference material was submitted to PHWE and the arsenic concentrations found (after HPLC-ICP-MS) were compared with the concentrations reported for this CRM by other authors. Table VII shows the arsenic species levels extracted in pressurized hot water for the BCR-279. Inorganic arsenic and Gly-Sugar were the major arsenic species found. DMA and PO₄-Sugar were also quantified at low concentrations; however, the MMA, SO₃-Sugar and SO₄-Sugar levels were below the LOQs. Good agree-

ment between the found inorganic arsenic concentrations and most of As(III) and As(V) concentrations reported in this CRM were achieved: 0.06 $\mu\text{g g}^{-1}$ for As(III) (14,18) and 0.46 $\mu\text{g g}^{-1}$ (18) and 0.53 $\pm$ 0.04 $\mu\text{g g}^{-1}$ (14) for As(V). Similarly, Gly-Sugar, PO₄-Sugar and DMA contents are not certified in the BCR-279, but some reported concentrations can be found in the literature. The content of Gly-Sugar varied in the range of 0.096 (14) to 0.21 (18) $\mu\text{g g}^{-1}$. DMA and PO₄-Sugar concentrations found are quite similar to those given by other authors: 0.06 $\pm$ 0.03 $\mu\text{g g}^{-1}$ (14) and 0.07 $\mu\text{g g}^{-1}$ (18) for DMA; and 0.08 $\pm$ 0.01 $\mu\text{g g}^{-1}$ (14) and 0.10 $\mu\text{g g}^{-1}$ (18) for PO₄-Sugar. Hsieh and Jiang (6) reported high aqueous

arsenic species concentrations: 1.07 ± 0.012 , 0.065 ± 0.003 , 0.200 ± 0.004 , 0.302 ± 0.008 , and $0.674 \pm 0.022 \mu\text{g g}^{-1}$ for Gly-Sugar, As(III), DMA, PO_4 -Sugar, and As(V) respectively, by MAE using a mixture of MeOH/ammonium carbonate. The hot water extraction efficiency and column recovery (Table VII) are close to previously reported data about arsenic extraction by using water as the solvent extraction (14,18). Raab et al. and Pell et al. reported a total arsenic concentration (as sum of arsenic species) of $1.076 \mu\text{g g}^{-1}$ (14) and $1.00 \mu\text{g g}^{-1}$ (18), respectively, in BCR-249. However, Hsieh and Jiang (6) reported high extraction efficiency (84%). The quantification of species such as arsenobetaine, MMA, and tetramethylarsonium ion in BCR-279 could explain the high extraction efficiency; a total arsenic concentration (as sum of arsenic species) of $2.55 \mu\text{g g}^{-1}$ was assessed (6).

CONCLUSION

A green, highly efficient, fast, and automated procedure for arsenic species (mainly arsenosugars) extraction from edible Galician seaweeds has been developed. Pressurized hot water modified with a small amount of acetic acid was used as the green solvent. The arsenic species efficiencies (in the range of 42 to 81%) obtained within 7 minutes (5 minutes of extraction time plus one minute of purge time and one minute of end relief) were achieved. This extraction time is considerably shorter when compared with classical extraction times proposed in the literature. The steps of extracting solvent addition and extract filtration are totally automated. After factor screening, the extraction temperature and acetic acid concentration are the most significant variables involved in the determination of arsenic species in edible seaweeds by PHWE. Inorganic arsenic (As(III)

and As(V)), as well as, MMA, although separated by the HPLC method, were not quantified in any seaweed sample. Gly-Sugar, PO_4 -Sugar, and SO_3 -Sugar were the main organic arsenic present in the samples.

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