

Aqueous Extraction of Water-soluble Inorganic Arsenic in Marine Algae for Speciation Analysis

Tomohiro NARUKAWA,[†] Akiharu HIOKI, and Koichi CHIBA

National Metrology Institute of Japan (NMIJ), National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 3, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8563, Japan

An aqueous extraction of inorganic As species, such as arsenite (As(III)) and arsenate (As(V)), was developed for monitoring inorganic As in the edible brown alga *Hizikia fusiforme* (hijiki). The ultrasonic extraction with water, even without heating, was found to be an acceptable monitoring method for an evaluation of water-soluble inorganic As, since it could extract about 80% of total As. Such an extraction efficiency was almost the same as those of enzyme assisted extraction methods. The developed extraction procedure was applied to 15 hijiki samples that had been collected at different coasts in Japan. All samples contained a substantial proportion of As as arsenosugars; the relative amounts of the different As species extracted were dependent on the sample. The percentages of extractable As species in the hijiki samples were in the range from 70 to 90%, and the sums of the concentrations of As(III) and As(V), which was defined as i-As, were in the range from 36 to 79% of the total As concentration in each sample. The proposed method is appropriate for environmental monitoring for inorganic As speciation in algae.

(Received March 30, 2012; Accepted June 5, 2012; Published August 10, 2012)

Introduction

Marine algae are very important components of the Japanese diet because they contain quantities of the minerals necessary for human health. In particular, hijiki (*Hizikia fusiforme*, the edible marine brown alga) is one of the most popular food substances among marine algae in Japan. However, it is well known that hijiki contains the largest amount of inorganic arsenic (As) among marine algae;^{1,2} it has also been established that inorganic As, especially As(III), is much more toxic than organoarsenics, although the toxicity of As greatly varies from species to species.³⁻⁶ Toxic elements such as As in the human diet pose an obvious health risk. The Food and Agriculture Organisation/World Health Organisation (FAO/WHO) recommends that a provisional tolerable weekly intake (PTWI) of inorganic As be not more than 15 µg/kg body weight.⁷ Hence, it is important to determine which As species are present in foodstuffs along with determination of the total As concentration in order to make an implementation of the risk assessment and the safety evaluation. In addition, marine food analyses show pollution levels of the marine production system.⁸ In particular, the analysis of macroalgae can provide information on local pollution conditions, since they grow up at the same location.

The As species in hijiki were identified: about 50% of As is As(V) and the other 50% organic compounds, chiefly arsenosugars.⁹ A cancer risk assessment of cooked hijiki was undertaken,¹⁰ and the intake of inorganic As through hijiki consumption was discussed in relation to the PTWI.^{11,12} A lot of studies on As species in marine algae concerned with environmental monitoring and food safety have been

published.¹³⁻¹⁹ In addition, many techniques for As speciation in seafood were reported.²⁰⁻²⁵ Nowadays, we must develop a simple monitoring technique to determine inorganic As in algae, since a large amount of samples should be treated at one time to estimate various risks posed by ingestion inorganic As (i-As: As(III) and As(V)).

In this study, we investigated a simple extraction technique of As species for monitoring i-As in algae. Hijiki was used as a sample for development of the extraction conditions for i-As, because hijiki contains the largest amount of i-As among algae. The proposed technique was applied to 15 hijiki samples. In addition, three more kinds of edible marine algae: kombu (*Laminaria japonica*), wakame (*Undaria pinnatifida*) and tengusa (*Gelidium crinale*) were analyzed by the proposed method. They were collected at the same location as one of the 15 hijiki samples. The As species extracted were determined by high performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS).

Experimental

Apparatus

An ICP-MS (Agilent 7500ce, Agilent, Tokyo, Japan) equipped with a micromist nebulizer (100 µL type) and a Scott spray chamber (2°C) was used. The normal operating parameters for the ICP-MS were as follows: incident rf power was 1500 W, outer Ar gas flow rate 15 L min⁻¹, intermediate Ar gas flow rate 0.9 L min⁻¹, carrier Ar gas flow rate 0.8 L min⁻¹ and make-up Ar gas flow rate 0.4 mL min⁻¹. The ICP-MS was operated with a collision cell, flowing He as collision gas (3 mL min⁻¹) to reduce ⁴⁰Ar³⁵Cl⁺, which interferes with ⁷⁵As. The ion count was monitored at *m/z* = 75. Arsenic species were separated by HPLC (L-6000 pump, Hitachi High Technologies Co. Ltd.,

[†] To whom correspondence should be addressed.
E-mail: tomohiro-narukawa@aist.go.jp

Tokyo, Japan) with an automatic sample injector (Nanospece SI-2, Shiseido Co. Ltd., Tokyo, Japan) and introduced into ICP-MS directly. A CAPCELL PAK C₁₈ MG column (150 mm × i.d. 4.6 mm, Shiseido Ltd., Japan) was used with the mobile phase containing 10 mM sodium 1-butanedisulfonate/4 mM malonic acid/4 mM tetramethylammonium hydroxide/0.05% methanol (pH 3.0) at a flow rate of 0.5 mL min⁻¹. The exit of the HPLC column was connected to the nebulizer of the ICP-MS with PEEK tubing.

An ultrasonic bath was used to accelerate extraction of As species from hijiki samples. A Mar-X microwave system (CEM, Matthews, NC) was used for microwave-assisted extraction, and a heating block (Digi PREP, SCP Science Inc., Canada) was used for heat-assisted extraction.

An MSL 1200-mega and STRAT D (Milestone MLS, Leutkirch, Germany) were used for microwave-assisted digestion to decompose the hijiki samples.

Reagents and chemicals

Arsenic species standards. The Japan Calibration Service System (JCSS) arsenic standard solution (ca. 1000 mg L⁻¹ As, made from high purity As₂O₃) was used as the As(III) source standard solution (Kanto Chemical Industries Ltd., Tokyo, Japan). As(V) certified reference material (NMIJ CRM 7912-a) and dimethylarsinic acid (DMAA) certified reference material (NMIJ CRM 7913-a), issued by the National Metrology Institute of Japan/National Institute of Advanced Industrial Science and Technology (NMIJ/AIST, Ibaraki, Japan), were used as source standard solutions. They are guaranteed to be traceable to SI. Other methylated As species standard solutions, namely methylarsonic acid (MMAA), trimethylarsine oxide (TMAO), tetramethylarsonium iodide (TeMA), arsenobetaine (AsB), and arsenocholine bromide (AsC), were prepared from commercially available reagents (Tri-Chemical, Yamanashi, Japan) after their purities had been evaluated. Each of them was dissolved in water to prepare the stock standard solution containing 1000 mg As kg⁻¹. Working mixed standard solutions were prepared daily by mixing the stock solutions and diluting with water properly. Also, four kinds of arsenosugar (-OH, -OPO₃CH₂CHOHCH₂OH, -SO₃ and -OSO₃ type) standard solutions were prepared from our stock solutions to confirm the retention time of each peak in the HPLC-ICP-MS system.

Other chemicals. All the acids and alkaline used were of ultrapure grade (Kanto Chemical Industries, Ltd.). High purity enzymes of pepsin (from porcine gastric mucosa) and α-amylase (heat-stable) were obtained from Sigma (Sigma-Aldrich, St. Louis, MO). Water purified with a Milli Q-Labo filter (resistivity 18 MΩ cm, Nippon Millipore, Ltd., Tokyo, Japan) was used throughout the experiment.

Certified reference material (CRM). The seaweed (hijiki) CRM analyzed was NMIJ CRM 7405-a Seaweed (National Metrology Institute of Japan/National Institute of Advanced Industrial Science and Technology, Ibaraki, Japan). The certified value of the total As in NMIJ CRM 7405-a is 35.8 ± 0.9 mg kg⁻¹ (*k* = 2).

Seaweed samples. Fifteen samples of hijiki were collected from different coasts around Japan. Five samples were collected in eastern Japan, and 4 samples and 6 samples were collected in central and western Japan, respectively. One hijiki sample (sample ID 1) was used to investigate extraction conditions and behaviors, and all hijiki samples were analyzed by the proposed monitoring method.

Wakame, kombu (all brown algae), and tengusa (a red alga) were collected at the same coast where one of the 15 hijiki samples was collected, in order to compare the distribution of As species among different marine algae.

Procedure

Preparation of hijiki samples. A quantity of hijiki was washed, dried and pulverized in a food processor and the resulting powder was sieved using a 250-μm nylon mesh. The extracted samples were analyzed by HPLC-ICP-MS. The total As concentrations in the samples were determined by ICP-MS following microwave-assisted digestion with HNO₃-H₂O₂-HF mixed acid.

A gravimetric preparation method was employed in all preparations in this study. Blank tests were performed to investigate possible As contamination; none was detected.

Determination of total arsenic. Microwave-assisted digestion with HNO₃-H₂O₂-HF was used to digest the hijiki samples. A 0.50-g aliquot of hijiki was precisely weighed and put into a vessel, to which 5 mL of HNO₃, 2 mL of H₂O₂ and 1 mL of HF were then added. In the microwave system, the samples were heated at 250 W for 5 min, 250 W for 10 min, 400 W for 10 min, 650 W for 5 min and 250 W for 10 min, and then cooled to the room temperature. After cooling, the vessel of the dissolved sample was placed on a hot plate to evaporate the sample to dryness. The residue was dissolved with 1(w/w)% HNO₃ to make up 50 mL of a sample solution. After proper dilution, the resulting solution was used for the determination of total As by ICP-MS.

Extraction procedure for arsenic species. The extraction was carried out under the condition of a liquid/solid ratio of 20 (extraction solvent 10 g/sample 0.5 g). A proper amount of each hijiki sample was placed in a 50-mL polypropylene centrifuge tube or a container of the microwave-assisted extraction system and 10 g of extraction solvent was added to it. The extraction solvents investigated here were water, methanol, 1.0(w/w)% HNO₃, 2.0(w/w)% tetramethylammonium hydroxide (TMAH), 10(w/w)% pepsin and 10(w/w)% α-amylase solutions. The extraction was carried out through the three different extraction methods mentioned below. After extraction, the sample-solvent suspension was centrifuged at 4000 rpm for 5 min and the supernatant was passed through a 0.45-μm syringe-type PVDF membrane filter and the filtrate was analyzed as a test solution.

Ultrasonic extraction. A capped 50-mL polypropylene tube containing the hijiki sample and the extraction solvent was placed in an ultrasonic bath and ultrasonicated for 2 h.

Heat-assisted extraction. A capped 50-mL polypropylene tube containing the hijiki sample and the extraction solvent was placed in a heating block at temperatures ranging from 37°C (as the room temperature) to 100°C for 2 to 24 h.

Microwave-assisted extraction. A microwave container was screwed into a proper position in the microwave instrument. The operating program of the system was as follows: the temperature was raised from the room temperature to 60 or 80°C for 5 min, held at 60 or 80°C for 30 min, and then cooled to the room temperature over 10 min. One container was used to monitor the solution temperature and the others were used for extraction.

Results and Discussion

Concentration of total arsenic

The total As in the hijiki samples was determined by ICP-MS following microwave-assisted digestion of the samples. The analytical results (*n* = 3) are shown in Table 1. The concentration of As in the samples ranged from 46.4 to 147.1 mg kg⁻¹. The analytical precisions expressed as relative standard deviation (RSD) were less than 1.5% including sample digestion process.

To validate the method for total As, we analyzed the NMIJ CRM 7405-a. The result ($n = 6$) for total As in the CRM was $35.85 \pm 0.03 \text{ mg kg}^{-1}$ (mean \pm SD (RSD 0.1%)). Results for total As in the certified reference material were in good agreement with the certified value.

Extraction of arsenic species with water

Arsenic species were extracted under several different conditions, followed by measuring with HPLC-ICP-MS. When an As speciation standard solution containing As(III), As(V), MMAA, DMAA, TMAO, TeMA, AsB and AsC was measured with HPLC-ICP-MS, the chromatogram obtained is shown in Fig. 1. These 8 As species were well separated using the above instrumentation with an ODS column. The limits of detection (LOD, $\sigma = 3$) of each As species were 0.1 ng g^{-1} .

However, hijiki is known to contain substantial amounts of arsenosugars, some of which can overlap with some of the methylated species such as AsB under these separation conditions (Fig. 1). Therefore, the present measurement system has a potential to give higher results for the quantities of methylated species. Inorganic As species in hijiki have attracted more attention than organic ones from the viewpoint of epidemiology and public health, since As(III) is the most toxic among the As species. Therefore, we focused on developing a method to monitor the i-As in hijiki.

The ultrasonic extraction, the heat-assisted extraction and the microwave-assisted extraction with water were carried out at a range of temperatures. Results are shown in Table 2. When extraction was employed at the temperature range from room temperature to 80°C , the extracted amounts of As(V) and

DMAA ranged from 56.27 to 57.40 mg kg^{-1} (average $56.7 \pm 0.6 \text{ mg kg}^{-1}$ (RSD 1.1%)) and 1.83 to 2.02 mg kg^{-1} (average $1.9 \pm 0.1 \text{ mg kg}^{-1}$ (RSD 4.9%)), respectively. The extraction rate of As, that was calculated from the total extraction of As species and total As concentration, ranged from 77.1 to 79.3% (average $77.8 \pm 0.9\%$ (RSD, 1.2%)). Although the extraction rate was approximately 78%, the extraction ratio of each As species were almost the same regardless of the extraction procedure. As(III) and MMAA were not detected by any extraction procedure.

On the other hand, when the extraction temperature increased to 90 or 100°C , the extraction rate increased to approximately 86%, and the amount of the extracted As species increased. The high temperature may accelerate the extraction of As species from hijiki. But, the high temperature also has a potential to decompose some arsenic-containing components (arsenosugars, for example) during extraction.¹⁰ In such a case, the arsenical fragments produced were extracted. Even in this experiment, it was found that an arsenosugar was decomposed at over 80°C and the As species derived were shown in the chromatogram. Therefore, there were some concerns that small parts of As(V) and DMAA did detected were not originally existing species in hijiki but was produced as fragments through decomposition of arsenosugars when the extraction was carried out at rather high temperature.

Extraction of arsenic species with acid and alkaline solvents

To complete the extraction of all As species in hijiki, the samples were subjected to the heat-assisted extraction with $1.0(\text{w/w})\%$ HNO_3 solution or $2.0(\text{w/w})\%$ TMAH solution at the

Table 1 Concentrations of total As in 15 hijiki samples

Sample ID	Total As/ mg kg^{-1}
1	83.96 ± 0.59
2	86.77 ± 0.87
3	147.10 ± 1.18
4	137.37 ± 0.69
5	49.05 ± 0.29
6	124.91 ± 0.62
7	114.13 ± 0.46
8	72.55 ± 0.58
9	65.07 ± 0.65
10	73.13 ± 0.80
11	85.30 ± 0.77
12	96.15 ± 0.77
13	102.55 ± 1.03
14	113.15 ± 1.70
15	46.44 ± 0.03

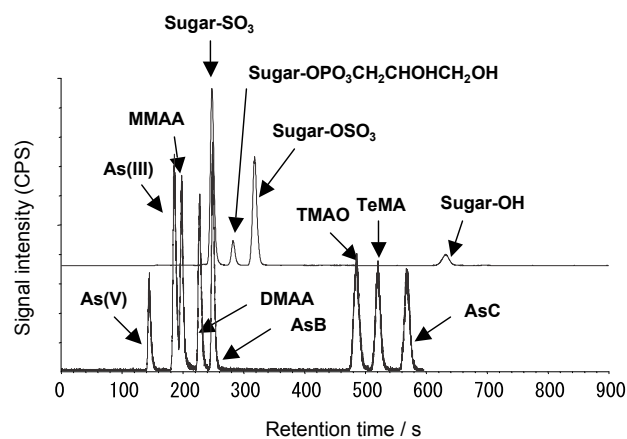


Fig. 1 HPLC-ICP-MS chromatograms of As species standards.

Table 2 Analytical results by water extraction

Condition	As(III)/ mg kg^{-1}	As(V)/ mg kg^{-1}	MMAA/ mg kg^{-1}	DMAA/ mg kg^{-1}	Other type/ mg kg^{-1}	Sum/ mg kg^{-1}	Ext. rate, %
Ultrasonic (RT)/2h	ND	56.42	ND	1.83	6.71	64.96	77.4
Heat-assisted $37^\circ\text{C}/24 \text{ h}$	ND	56.08	ND	1.91	8.63	66.62	79.3
Heat-assisted $80^\circ\text{C}/2 \text{ h}$	ND	57.40	ND	1.92	5.48	64.81	77.2
Heat-assisted $90^\circ\text{C}/2 \text{ h}$	ND	61.04	ND	2.41	8.52	71.97	85.7
Heat-assisted $100^\circ\text{C}/2 \text{ h}$	ND	62.34	ND	2.41	7.06	71.81	85.5
Microwave-assisted $60^\circ\text{C}/1 \text{ h}$	ND	56.27	ND	1.83	6.65	64.75	77.1
Microwave-assisted $80^\circ\text{C}/1 \text{ h}$	ND	57.39	ND	2.02	5.98	65.39	77.9

ND, not detected; RT, room temperature; Sum, sum of all As species. Ext. rate, extracted total As/total As concentration with acid digestion (83.96 mg kg^{-1}).

Table 3 Analytical results by heat-assisted extraction with HNO₃ and TMAH

Condition	As(III)/ mg kg ⁻¹	As(V)/ mg kg ⁻¹	MMAA/ mg kg ⁻¹	DMAA/ mg kg ⁻¹	Other type/ mg kg ⁻¹	Sum/ mg kg ⁻¹	Ext. rate, %
1.0 (w/w)% HNO ₃ /37°C	0.82	61.21	ND	1.89	16.35	80.27	95.6
1.0 (w/w)% HNO ₃ /100°C	ND	60.96	ND	4.92	18.21	84.09	100.2
2.0 (w/w)% TMAH/37°C	ND	37.29	ND	3.13	13.24	53.66	63.9
2.0 (w/w)% TMAH/100°C	ND	45.33	ND	5.46	9.28	60.07	71.5

Extraction time: 37°C for 24 h, 100°C for 2 h. ND, not detected; Sum, sum of all As species. Ext. rate: extracted total As/total As concentration with acid digestion (83.96 mg kg⁻¹).

Table 4 analytical results of combination (double) extraction: 2nd extraction after ultrasonic extraction with water

Condition	Extraction step	As(III)/ mg kg ⁻¹	As(V)/ mg kg ⁻¹	MMAA/ mg kg ⁻¹	DMAA/ mg kg ⁻¹	Other type/ mg kg ⁻¹	Sum/ mg kg ⁻¹	Total Ext. rate, %	2nd Ext. rate, %
Ultrasonic/water/2 h	1st	ND	56.42	ND	1.83	6.71	64.96	77.4	
Ultrasonic/water/2 h	2nd	ND (ND)	ND (ND)	ND (ND)	ND (1.83)	ND (6.71)	0.00 (64.96)		0.0
Ultrasonic/methanol/2 h	2nd	1.51 (1.51)	0.34 (56.76)	ND (ND)	0.04 (1.87)	1.25 (7.96)	3.15 (68.10)		3.7
Heat-assisted/TMAH/2 h	2nd	ND (ND)	3.24 (59.66)	ND (ND)	1.86 (3.69)	2.99 (9.70)	8.09 (73.04)		9.6
Heat-assisted/HNO ₃ /2 h	2nd	ND (ND)	4.90 (61.32)	ND (ND)	0.29 (2.12)	11.80 (18.51)	17.00 (81.96)		20.2

ND, not detected; Sum, sum of all As species. Ext. rate: extracted total As/total As concentration with acid digestion (83.96 mg kg⁻¹).
(): Sum of 1st extraction and 2nd extraction.

temperature of the room temperature (37°C) and 100°C. The results are shown in Table 3. When 1.0(w/w)% HNO₃ was used, the hijiki sample was visibly decomposed. The extracted amount of As(V) was almost the same as those extracted by the heat-assisted extraction with water at 90 or 100°C. The extracted amount of DMAA at 100°C increased was 2.0 – 2.5 times more than these obtained by the ultrasonication and the heat-assisted extraction with water, although it was almost the same as those at 37°C. The extracted amount of other As species including arsenosugars increased more than double, comparing to those obtained by the ultrasonication and the heat-assisted extraction with water. When 1.0(w/w)% HNO₃ was used as solvent, approximately 100% of As was extracted. However, new peaks appeared on chromatogram and some peaks decreased. Such changes showed that some chemical species were degraded and new chemical species were generated during the extraction process. The severe extraction conditions may be useful for the determination of total As but are not suitable for speciation analysis.

In contrast, when 2.0(w/w)% TMAH was used as solvent, the amount of extracted As(V) was much less than those with 1.0(w/w)% HNO₃ and those with the ultrasonication and the heat-assisted extraction with water, although the amount of extracted DMAA was the largest among the solvents examined here. TMAH was not useful as an extraction solvent for i-As species because the amount of extracted As(V) was extremely low.

Extraction of arsenic species using combined techniques

Water appeared to be the best solvent to extract i-As, although it could not extract all As species; especially, arsenosugars and other organoarsenic species were not extracted efficiently. The severe extraction conditions such as the use of HNO₃ and the application of higher temperature could extract all As species, but tended to decompose native As species to produce fragment species.

Therefore, a combination of extraction techniques was investigated, where the ultrasonic extraction with water was carried out first and then the filtrated residue was extracted with another solvent by using ultrasonication and a heat-assisted extraction system. Methanol (ultrasonication, room temperature), 1.0(w/w)% HNO₃ (heat-assisted extraction, 100°C) and 2.0(w/w)% TMAH (heat-assisted extraction, 100°C) were tested as the second solvent. The results are shown in Table 4. The total extraction ratio increased by 3.7 and 9.6% with methanol and TMAH, respectively. In particular, when 1.0(w/w)% HNO₃ was used, the total extraction rate reached almost 100%. Furthermore, another 5 mg kg⁻¹ of As(V) was extracted by the second extraction and the total amount of the extracted As(V) was the same as that obtained by the 1.0(w/w)% HNO₃ extraction at 100°C. These results suggested that it is necessary to destroy body tissue of hijiki to extract As completely. However, some As species is likely to be decomposed under such conditions, and this makes it difficult to evaluate the natural concentration levels of each As species, because, when arsenosugars were spiked to the sample, several unknown peaks were observed.

On the other hand, even if the complete extraction of i-As is not achieved by the ultrasonic extraction with water, it was considered that the residual i-As is expected to be less than 5% of the total As and about 8% of total As(V) in hijiki samples. Therefore, we can evaluate to i-As within 8% error at a maximum by using the ultrasonic extraction with water.

Extraction of arsenic species using enzymes

One of the most important purpose for As speciation in hijiki is the risk assessment for human bodies. Therefore, the extraction efficiency of i-As by the enzymes was considered at the same time. Pepsin and α -amylase were used as the extraction solvent to extract As species in hijiki, because pepsin is a typical enzyme released from the chief cells in the stomach and

Table 5 Analytical results by heat-assisted extraction with enzymes

Condition	As(III)/ mg kg ⁻¹	As(V)/ mg kg ⁻¹	MMAA/ mg kg ⁻¹	DMAA/ mg kg ⁻¹	Other type/ mg kg ⁻¹	Sum/ mg kg ⁻¹	Ext. rate, %
Ultrasonic/pepsin A	ND	55.06	ND	1.88	8.77	65.71	78.3
Heat-assisted/pepsin B	ND	58.01	ND	1.82	8.76	68.59	81.7
Heat-assisted/pepsin A	ND	56.53	ND	1.86	8.29	66.68	79.4
Heat-assisted/amylase A	ND	57.01	ND	1.91	8.54	67.46	80.3

Ultrasonic, 2 h; heat-assisted, 37°C for 24 h. Pepsin A and amylase A, water aqueous solution; pepsin B, 0.07 M HCl solution. ND, not detected; Sum, sum of all As species. Ext. rate: extracted total As/total As concentration with acid digestion (83.96 mg kg⁻¹).

Table 6 Water-soluble As species in hijiki extracted by ultrasonication with water

Sample ID	As(III)/ mg kg ⁻¹	As(V)/ mg kg ⁻¹	i-As/ mg kg ⁻¹	MMAA/ mg kg ⁻¹	DMAA/ mg kg ⁻¹	Other type/ mg kg ⁻¹	Sum/ mg kg ⁻¹	Total As ^a / mg kg ⁻¹	Ext. rate, %
1	ND	56.42	56.42	ND	1.83	6.71	64.96	83.96	77.4
2	ND	66.87	66.87	ND	1.09	6.13	74.10	86.77	85.4
3	4.73	111.62	116.34	ND	2.00	13.21	131.55	147.10	89.4
4	5.32	94.21	99.53	ND	3.03	14.51	117.07	137.37	85.2
5	0.90	16.89	17.79	0.17	0.97	17.89	36.81	49.05	75.1
6	ND	96.53	96.53	ND	0.80	9.08	106.41	124.91	85.2
7	ND	82.35	82.35	ND	1.77	14.29	98.40	114.13	86.2
8	ND	39.14	39.14	ND	1.31	15.53	55.98	72.55	77.2
9	ND	32.22	32.22	ND	1.10	13.91	47.24	65.07	72.6
10	2.14	42.15	44.29	ND	1.06	12.66	58.02	73.13	79.3
11	ND	52.60	52.60	ND	1.66	11.60	65.86	85.30	77.2
12	0.34	55.20	55.54	ND	1.07	20.03	76.65	96.15	79.7
13	1.22	66.73	67.95	ND	1.03	11.47	80.45	102.55	78.4
14	0.28	75.92	76.20	ND	0.58	16.01	92.78	113.15	82.0
15	0.52	16.55	17.06	ND	1.24	26.10	43.16	46.44	92.9

i-As: Sum of As(III) and As(V). ND, not detected; Sum, sum of all As species. a. Ext. rate: extracted total As/total As concentration with acid digestion (in Table 1).

degrades food proteins into peptides. α -Amylase is also a typical enzyme to catalyze the breakdown of starch into sugars and it is preset in human saliva.

Arsenic species were extracted by the ultrasonication with 10(w/w)% pepsin aqueous solution and by the heat-assisted extraction at 37°C with 10(w/w)% pepsin in 0.07 M HCl, 10(w/w)% pepsin aqueous solution and 10(w/w)% α -amylase aqueous solution. The results are shown in Table 5. The extraction amounts of As(V) were in good agreement with those obtained by the ultrasonic extraction with water. The total extraction rates were slightly higher than those by the ultrasonic extraction with water, because the concentrations of the other extracted types of As were increased by the enzyme digestion. These results indicate that the extraction of i-As from hijiki in human body may be stimulated by using the ultrasonic extraction with water.

Monitoring test

The ultrasonic extraction with water is very suitable as a monitoring method of i-As, because the mild extraction conditions do not impose any change in the naturally occurring As species, even if the total extraction rate is about 80%. Therefore, we define here the i-As extracted by ultrasonication with water as "water-soluble i-As". Fifteen hijiki samples were analyzed by the HPLC-ICP-MS following the ultrasonic extraction with water. The analytical results of water-soluble i-As are given in Table 6.

Arsenic extracted from the samples ranged from 73 to 93% of total As amounts. The average of the extraction ratio of 15 hijiki samples was 82% with a relative standard deviation (RSD)

of 7%. The extraction efficiency of the present method is sufficient for the monitoring of As in hijiki. The water-soluble i-As, that is the sums of As(III) and As(V), were 37 to 79% of the total As concentration in each sample. The concentrations of As(III), the most toxic As species, ranged from the LOD to 5.32 mg kg⁻¹, although half of them were less than the LOD. These amounts were less than 5% of water-soluble inorganic As and less than 4.5% of the extracted As.

For confirmation of the proposed technique, three of the samples (sample ID 3, 9 and 15; as examples of the higher, medium and lower total As concentration samples) were further analyzed with several other extraction (acid, alkaline and enzyme extraction) conditions in order to evaluate the results shown in Table 6. When the extractions were carried out with the ultrasonication and with the heat-assisted extraction at room temperature and 80°C using water and enzyme as solvents, all the determination results of extracted i-As were the same as those in Table 6. The extraction behaviors of all As species were the same as those mentioned previously in this paper regardless of samples.

The water extraction by ultrasonication technique was also applied to kombu, wakame and tengusa, which had been collected at the same location where hijiki sample ID 15 was collected. As they grew at the same location, the seawater and sediment conditions should have been similar for all of them. Therefore, differences in the amount and distribution of the As species among them should depend on the type of alga. The results are shown in Table 7. The concentrations of total As of hijiki, kombu and wakame were very similar and the total extracted amounts were also very similar among them. In this

- Chem.*, **2000**, 368, 116.
14. A. J. Signes-Pastor, C. Deacon, R. O. Jenkins, P. I. Harris, A. A. Carbonell-Barrachina, and A. A. Meharg, *J. Environ. Monit.*, **2009**, 11, 1930.
 15. M. Hamano-Nagaoka, K. Hanaoka, M. Usui, T. Nishimura, and T. Maitani, *Shokuhin Eiseigaku Zasshi*, **2008**, 49, 88.
 16. M. Rose, J. Lewis, N. Langford, M. Baxter, S. Origgi, M. Barber, H. MacBain, and K. Thomas, *Food Chem. Toxicol.*, **2007**, 45, 1263.
 17. C. Almela, J. M. Laparra, D. Vélez, R. Barberá, R. Farré, and R. Montoro, *J. Agric. Food Chem.*, **2005**, 53, 7344.
 18. J. M. Laparra, D. Vélez, R. Montoro, R. Barberá, and R. Farré, *J. Agric. Food Chem.*, **2003**, 51, 6080.
 19. P. A. Gallagher, J. A. Shoemaker, X. Wei, C. A. Brockhoff-Schwegel, and J. T. Creed, *Fresenius J. Anal. Chem.*, **2001**, 369, 71.
 20. A. Leufroy, L. Noël, V. Dufailly, D. Beauchemin, and T. Guérin, *Talanta*, **2011**, 83, 770.
 21. L. Hinojosa Reyes, J. L. Guzmán Mar, G. M. Mizanur Rahman, B. Seybert, T. Fahrenholz, and H. M. Skip Kingston, *Talanta*, **2009**, 78, 983.
 22. H. Castlehouse, C. Smith, A. Raab, C. Deacon, A. A. Meharg, and J. Feldmann, *Environ. Sci. Technol.*, **2003**, 37, 951.
 23. V. Devesa, A. Martínez, M. A. Súnier, D. Vélez, C. Almela, and R. Montoro, *J. Agric. Food Chem.*, **2001**, 49, 2272.
 24. R. Wahlen, *J. Chromatogr. Sci.*, **2004**, 42, 217.
 25. A. Moreda-Piñeiro, E. Peña-Vázquez, P. Hermelo-Herbello, P. Bermejo-Barrera, J. Moreda-Piñeiro, E. Alonso-Rodríguez, S. Muniategui-Lorenzo, P. López-Mahía, and D. Prada-Rodríguez, *Anal. Chem.*, **2008**, 80, 9272.
 26. V. Devesa, D. Vélez, and R. Montoro, *Food Chem. Toxicol.*, **2008**, 46, 1.
-