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Working methods

Determination of arsenic species and arsenosugars in marine samples by HPLC-ICP-MS†

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Arsenic speciation analysis in marine samples was performed using high-pressure liquid chromatography (HPLC) with ICP-MS detection. The separation of eight arsenic species, viz. As^{III}, MMA, DMA, As^{V}, AB, TMAO, AC and $TeMAs^{+}$, was achieved on a Shiseido Capcell Pak C_{18} column using an isocratic eluent (pH 3.0), in which conditions As^{III} and MMA were co-eluted. The method was successfully applied to several marine samples, e.g. oyster, fish, shrimp and marine algae. For the extraction of arsenic from seafood products, low-power microwave digestion was employed, and for the extraction of arsenic from marine algae, sonicated extraction was employed. The separation of arsenosugars in the extracts from marine algae was achieved on an anion exchange column, Hamilton PRP X-100, using ammonium hydrogen carbonate solution (pH 8.4). The concentrations of arsenosugar-2, -3 and -4 in the extracts from the marine algae were analyzed to test the use of a *Fucus* sample as a reference standard material and were in the range 3.6–27.5 μg g^{-1}, which accounted for 6.0–34.9% of total arsenic. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: HPLC-ICP-MS; arsenic species; arsenosugars; marine algae; seafood; microwave extraction; sonication

INTRODUCTION

Speciation of arsenic has acquired significant attention over the past 20 years in both identification of arsenic species and environmental exposure assessment research. This is due to the species-dependent toxicity of arsenic. In marine organisms, more than 32 different inorganic and organic species have been reported.[1,2] Depending on the kind of organism, different kinds and concentrations of arsenic compounds have been observed. In fish and crustaceans, arsenobetaine (AB) is a dominant species along with traces of monomethyl arsenic acid (MMA), dimethylarsinic acid (DMA), tetramethylarsonium ion ($TeMAs^{+}$) and trimethylarsine oxide (TMAO).[3–6] In marine algae, arsenosurgars compounds (derivatives of dimethylarsonlribosides and trimethylarsonioribosides) are most abundant.[7–10] Bivalves[11] and marine sponge[12] contain AB and arsenosugars as major compounds.

The use of inductively coupled plasma–mass spectrometry (ICP-MS) as a chromatographic detector has produced much knowledge in specification, identification and characterization of trace element species in natural products such as arsenic, selenium, antimony, etc.[13–17] The considerable numbers and the wide concentration ranges of naturally existing arsenic species have been investigated by many researchers. Recently, electrospray mass spectrometry (ES-MS) has been used as a chromatographic detector[18–41] and for the characterization and identification of arsenosugars and unknown compounds. A number of researchers have employed separation of arsenic species by ion interaction reversed-phase chromatography, but separation of all of the arsenic species using one ion-pairing reagent is impossible. Anion-exchange HPLC seems to be the most often used technique for the separation of arsenic species, including arsinoyl ribosides, but most of the cationic species and As^{III} elute in or close to the void. Therefore, other mechanisms, such as cation-exchange or size-exclusion, have been employed.

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The simple arsenic species commonly found in marine animals, such as arsenobetaine and tetramethylarsonium ion, are commercially available. However, arsenosugars are difficult to obtain because of their chemical synthesis, or complex procedures of isolation from algae in a pure form. Arsenosugar-1 is a basic ion, and arsenosugars-2, -3 and -4 are anionic ions (Fig. 1). Recently, as a comparable reference material for the four arsenosugars, a sample of *Fucus* in which arsenosugars were identified was made by Professor Francesconi.[27] Using this reference sample, arsenosugars in ultrasonic extracts from marine algae were analyzed.

The present paper describes the use of Capcell Pak C_{18} ODS reversed-phase column to separate eight arsenic species, viz. As^{III}, MMA, DMA, As^{V}, AB, TMAO, arsenocoholine (AC) and $TeMAs^{+}$ in a single chromatographic run in combination with ICP-MS detection. The proposed method was applied to determine arsenic species in several marine samples, e.g. oyster, fish, shrimp and marine algae. The arsenosugars in water extracts from marine algae were determined using the anion exchange column, Hamilton RPR-X100, in combination with ICP-MS detection using a reference standard material, *Fucus*..

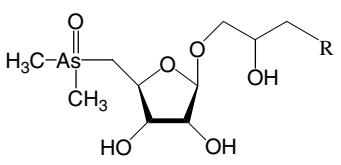
EXPERIMENTAL

Reagents and standards

For the preparation of reagents and standards, Milli-Q (Millipore, Milford, MA, USA) water of 18.3 MΩ cm was used. All reagents used were of analytical grades. The concentration of arsenic species is always given as the concentration of elemental arsenic. As^{III} (1000 mg l^{-1}) was made by dissolving 0.1733 g of $NaAsO_2$ in 100 ml deionized water and As^{V} (1000 mg l^{-1}) was made by dissolving 0.416 g of Na_2HAsO_4 in 100 ml of water. The stock solutions (1000 mg As l^{-1}) for MMA, DMA, AC, AB, TMAO and $TeMAs^{+}$ were prepared separately by dissolving corresponding amounts of salts, procured from Tri Chemical Laboratories Inc. (assay: >98% for all salts), Japan. All solutions were stored refrigerated and in the dark to prevent decomposition or oxidation. The final mixture prepared daily from a 20 mg l^{-1} standard solution each time.

Reference material

As the standard for major arsenosugars (Fig. 1), *Fucus*,[27] which was donated by Professor K. V. Francesconi, was used for evaluation of arsenosugars in marine algae.



1 R=OH
2 R=$OPO_3CH_2CH(OH)CH_2OH$
3 R=SO_3H
4 R=OSO_3H

Figure 1. Structures of the four arsenosugars.

Instrumentation

The chromatography system consisted of a Dionex pump (Dionex Corp, USA) and a Rheodyne Model 9125 six-port injection valve fitted with a 10 µl sample loop. A Capcell Pak C_{18} ODS analytical column (Shiseido, Japan) and Hamilton PRP-X100 anion exchange column (USA) were used. The former (250 × 4.6 mm i.d., 5 µm particles) has hydrophilic quaternary ammonium exchange sites on a divinyl benzene copolymer. The effluent from the column was connected to the concentric nebulizer in the cyclonic chamber of Elan 6000 ICP-MS (Perkin Elmer, USA). The ICP-MS instrument was first optimized off-line for arsenic with an aqueous As^{V} standard each time. The intensity of isotope at m/z 75 was measured and the isotope of $^{40}Ar^{37}Cl$ was used for the interference at all instances. PROLABO (MCS950) microwave and ultrasonic (Shibata, Japan) instruments were used for arsenic extraction and the former was also used for wet digestion.

Chromatographic conditions

To separate simple arsenic species, isocratic elution was employed on a Capcell Pak C_{18} ODS reversed phase column. The mobile phase was a 2 mM malonic acid, 0.3 mM 1-butanesulfonate sodium and 5 mM 1-hexanesulfonic sodium in 0.5% methanol (pH 3.0). To separate major arsenosugars, a 20 mM ammonium hydrogen carbonate buffer at pH 8.4 was used. The arsenic signals were monitored and their data were acquired using ELAN 6000 software. These raw data were later transferred into Total Chrom Work station version 6.2 (Perkin Elmer Inc, USA) to evaluate retention times and peak areas/peak heights. Peaks were identified according to retention time and confirmed by standard addition of arsenic compounds. The arsenic concentrations in real samples were quantified via peak areas on the basis of calibration curves of the known forms. The chromatographic gradient program and instrumental operating conditions of HPLC-ICP-MS are given in Table 1.

Samples

Oyster, fish and shrimp were purchased from local market. These samples were directly freeze-dried continuously for 3 days to a constant weight and finely powdered then stored in desiccators. Marine algae [*Sargassum piluliferum* (*Hondawara*), *Sargassum piluliferum* (*Mamedawara*), *Undariapinnatifida* (*Wakame*), kelp, *Hizika fusiformis* (commercial), *Pelvetia wrightii* (*Ezoishige*) and *Myelophycus simplex* (*Iwahige*)] were collected from Hiroshima Bay. The samples were cleaned thoroughly with tap water to remove salt and dirt and further rinsed with milli-Q water. The algae were freeze-dried to a constant weight and then ground in a mixer. The resulting powder was used for extraction of arsenic.

Total arsenic concentration

The total arsenic concentrations in natural samples were determined after microwave acid digestion using a closed vessel system following this procedure: Aliquots of ~100 mg powdered samples were weighed into the pre-cleaned, Teflon

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Table 1. Instrumental operation conditions for HPLC-ICP-MS

ICP-MS parameters:	
Rf power	1200 W
Lens voltage	13 eV
Ar plasma gas flow rate	15 L min^{-1}
Ar nebulizer gas flow rate	0.85 L min^{-1}
Sample introduction	Concentric nebulizer
Dwell time	500 ms
Mass (m/z)	^{75}As, ^{77}Se
HPLC Parameters:	
Flow rate	1.0 mL min^{-1}
Injection volume	10 μL
As eight species;	
Analytical column	Capcell Pak C18 (250 × 4.6 mm i.d., 5 μm)
Mobile Phase	5 mM 1-butanesulfonic acid, 0.3 mM sodium hexane sufonic acid and 2 mM malonic acid in 0.5% methanol
Arsenosugsrs;	
Analytical column	Hamilton PRP X-100 (250 × 4.1 mm i.d., 10 μm)
Mobile Phase	20 mM NH_4HCO_3 (pH 8.4)

vessels. After addition of 5.0 ml of suprapure nitric acid and 2.0 ml of H_2O_2 (30%), the vessels were closed and kept at room temperature for 15 min. The vessels were then mounted in the rotor, which was placed in the microwave oven (PROLABO MCS950), and eight-step digestion program was started (time in min, power in W): 2, 250; 0.5, 0; 5, 300; 0.5, 0; 5, 450; 0.5, 0; 5, 600; 10, 0 (ventilation).[42] After cooling, the digests were transferred into 50 ml calibrated flasks and diluted with MQ water. Total arsenic contents in these sample solutions were determined by direct ICP-MS after a further 10 dilutions. The analytical results are shown in Table 4.

Extraction of arsenic compounds

Natural marine samples

An aliquot of ~100 mg dried powder of various samples such oyster, fish muscle, shrimp muscle and shrimp shell were separately weighed into Teflon vessels in triplicates and 10 ml of distilled water were added into those vessels. These samples were digested using low-power microwave (50 W, 10 min) in a closed system using a PROLABO microwave instrument. After cooling, these suspensions were centrifuged at 2500 rpm for 15 min. The supernatant extracts were filtered through 0.45 μm Millipore filter and refrigerated at 4 °C until analysis. In the case of marine algae, ~100 mg of dried samples were weighed in triplicate into 50 ml glass vials. An aliquot of 10 ml of water was added and the sample was extracted using ultrasonication (15 min) instead of microwaves. Further procedures were followed as described above. These extracts were further diluted 10 times with milli-Q water prior to the analysis. The speciation analysis of all sample extracts was carried out within 3 days of sample preparation.

RESULTS AND DISCUSSION

Chromatographic separation

The separations of eight arsenic species, which are available commercially, were investigated. The pH of elution was changed by addition of amount of tetraethylammonium hydroxide and ammonium water. The template separation of eight species containing 5 ng As ml^{-1} at pH 3 is shown in Fig. 2. In this separation, MMA and As^{III} could not be separated, and the second peak contained both MMA and As^{III}.

Detection limits and precision

The analytical characteristics of the method, i.e. detection limit, precision and linearity, were evaluated for each arsenic species. The detection limits (DLs) were calculated based on 3σ of baseline noise at the peaks retention time ($n = 5$). The DLs obtained were from 0.03 μg l^{-1} (AC) to 0.23 μg l^{-1} ($TeMAs^+$). The repeatability of HPLC-ICP-MS was examined by five consecutive runs at 10 μg l^{-1} and RSD values were 2.4–8.0%. The retention time of each arsenic compound was also shown to be reproducible. An eight-point calibration in the range 1–200 μg As l^{-1} was obtained for all eight species. Peak areas were used for the calibration data. The linearity was calculated by least-squares fit using Excel software and the square correlation coefficient (r^2) was 0.990 or better for all eight species.

Arsenic extraction

The extraction procedure was evaluated by determining the ratio between extractable arsenic (sum of As from single

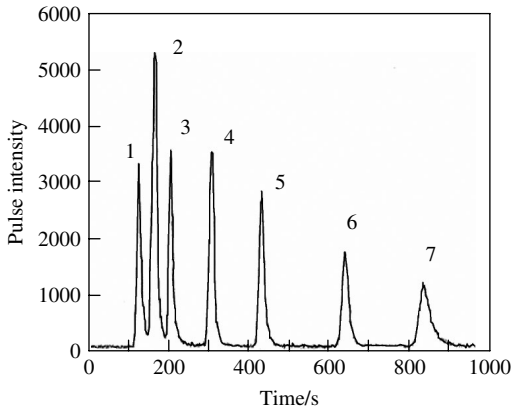

Figure 2. HPLC-ICP-MS chromatograms of mixtures of eight standards; 5 μg l^{-1} As; Shiseido Capcell Pak C_{18} column (250 × 4.6 mm i.d., 5 μm); mobile phase, 5 mM 1-butanesulfonic acid, 0.3 mM 1-hexanesulfonic acid and 2 mM malonic acid in 0.5% methanol (pH 3); sample loop, 10 μl; **1**, As^V; **2**, MMA + As^{III}; **3**, DMA; **4**, AB; **5**, TMAO; **6**, AC and **7**, $TeMAS^+$.

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Table 2. Arsenic speciation analysis in the extracts from marine animals by HPLC-ICPMS

Compound	As^V	As^{III} + MMA	DMA	AB (μg As dry g^{-1})	TMAO	AC	TeMAs+	Sum of arsenic species (μg As dry g^{-})	Total As* (μg As dry g^{-1})	Extraction efficiency (%)
Fish										
Red drum	0.05 ± 0.01	ND	0.05 ± 0.01	0.6 ± 0.1	ND	ND	ND	0.7 ± 0.1	2.2 ± 0.1	30.2
Schrimp-A	0.10 ± 0.02	0.1 ± 0.02	0.09 ± 0.01	5.0 ± 0.6	0.06 ± 0.01	0.08 ± 0.01	ND	5.4 ± 0.6	6.6 ± 3.0	81.3
Fish										
Chub mackerel	0.06 ± 0.01	0.1 ± 0.01	0.46 ± 0.03	3.8 ± 0.2	0.05 ± 0.01	0.04 ± 0.01	0.01 ± 0.01	4.5 ± 0.2	7.6 ± 0.9	59.4
Scallop	0.50 ± 0.03	0.4 ± 0.03	0.70 ± 0.05	5.1 ± 0.8	0.23 ± 0.01	ND	ND	6.9 ± 0.8	12.4 ± 0.7	56.0
Shrimp-B	0.44 ± 0.05	ND	ND	32.7 ± 6.4	ND	1.05 ± 0.19	ND	34.2 ± 6.4	35.1 ± 1.7	97.4
Fish	0.07 ± 0.03	ND	0.13 ± 0.01	10.6 ± 1.9	ND	ND	ND	10.8 ± 1.9	10.8 ± 1.1	99.5
(Black sea bream)										
Oyster	2.10 ± 0.20	ND	0.60 ± 0.20	5.4 ± 0.2	ND	ND	ND	8.1 ± 0.2	7.6 ± 0.2	106.9
DORM-2	0.65 ± 0.49	0.20 ± 0.28	0.41 ± 0.05	13.2 ± 0.2	0.10 ± 0.00	ND	0.43 ± 0.40	15.0 ± 0.5	17.5 ± 1.1	85.7
(Dogfish)				[16.4 ± 1.1]			[0.248 ± 0.054]		[18.0 ± 1.1]	
TORT-2	0.50 ± 0.10	0.50 ± 0.10	1.10 ± 0.30	12.2 ± 0.3	ND	0.80 ± 0.04	ND	15.1 ± 0.3	20.0 ± 0.5	75.5
(Lobster)									[21.6 ± 1.8]	

Column: Capcell Pak C18; The values are average of triplicates; The values in the brackets are certified values.
* Acid digestion

Table 3. Arsenic speciation analysis in the extracts from marine algae by HPLC-ICPMS

Compound	As^V	As^{III} + MMA	DMA	Unknown (μg As dry g^{-1})	TMAO	AC	TeMAs+	Sum of arsenic species (μg As dry g^{-1})	Total As* (μg As dry g^{-1})	Extraction efficiency (%)
Sargassum fulvellum (Hondawara)	112.4 ± 2.3	1.7 ± 0.2	1.05 ± 0.40	9.15 ± 0.43	ND	ND	6.3 ± 1.9	130.6 ± 2.3	206.9 ± 11.2	63.1
Sargassum piluliferum (Mamedawara)	47.4 ± 3.3	1.4 ± 0.2	2.7 ± 0.8	5.79 ± 0.82	ND	ND	4.2 ± 0.2	61.5 ± 3.3	288.0 ± 16.8	21.4
Undaria pinnatifida (Wakame)	1.7 ± 0.1	ND	0.56 ± 0.03	17.83 ± 2.21	ND	ND	3.9 ± 1.2	23.9 ± 2.2	26.2 ± 7.3	91.2
Kelp	0.78 ± 0.06	35.1 ± 1.2	2.9 ± 0.4	ND	ND	ND	2.3 ± 0.4	41.0 ± 1.2	47.6 ± 1.2	86.2
Myelophycus simplex (Iwahige)	4.2 ± 5.1	0.8 ± 0.5	27.6 ± 4.6	3.26 ± 4.48	14.6 ± 2.7	ND	ND	49.4 ± 5.1	52.2 ± 4.9	94.6
Hizikia fusiformis (Hiziki)	16.0 ± 1.6	0.50 ± 0.10	ND	ND	ND	ND	ND	16.5 ± 1.6	60.4 ± 0.4	27.3
Pelvetia wrightii (Ezoisige)	0.6 ± 0.4	0.13 ± 0.05	0.3 ± 0.2	0.21 ± 0.06	ND	0.12 ± 0.03	ND	1.4 ± 0.4	14.5 ± 0.5	9.3

Column: Capcell Pak C18; The values are average of triplicates; The values in the brackets are certified values.
* Acid digestion

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species) and total arsenic after acid digestion. Extraction efficiencies were calculated for all sample types, which ranged from 51 to 105% (Tables 2 and 3). The various sample extracts were analyzed for arsenic species following the proposed HPLC-ICP-MS procedure.

Seafood samples (microwave extraction)

The chromatograms of extracts from oyster, shrimp, fish and scallop are shown in Fig. 3. Although AB is dominant here, reasonable concentrations of As^V and DMA were detected in the range 0.60–2.10 μg As g^{-1} (Table 2). The fish extract was mainly detected for AB with a concentration of 10.6 μg As g^{-1} while minor concentrations of As^V and DMA were also presented. Similarly the extract from shrimp muscle has mainly shown the presence of AB at higher concentrations, i.e. 32.7 μg As g^{-1}.

Marine algae (sonication)

Aqueous extracts of *S. fulvellum* (*Hodawara*), *S. piluliferum* (*Mamedawara*), *Undariapinnatifida* (*Wakame*), kelp, commercial *H. fusiformis* (*Hiziki*), *M. simplex* (*Iwahige*) and *P. wrightii (Ezoishige)* were analyzed following the proposed HPLC-ICP-MS. The chromatograms of *S. piluliferum (Mamedawara), U. pinnatifida (wakame), P. wrightii (Ezoishige),* and *M. simplex*

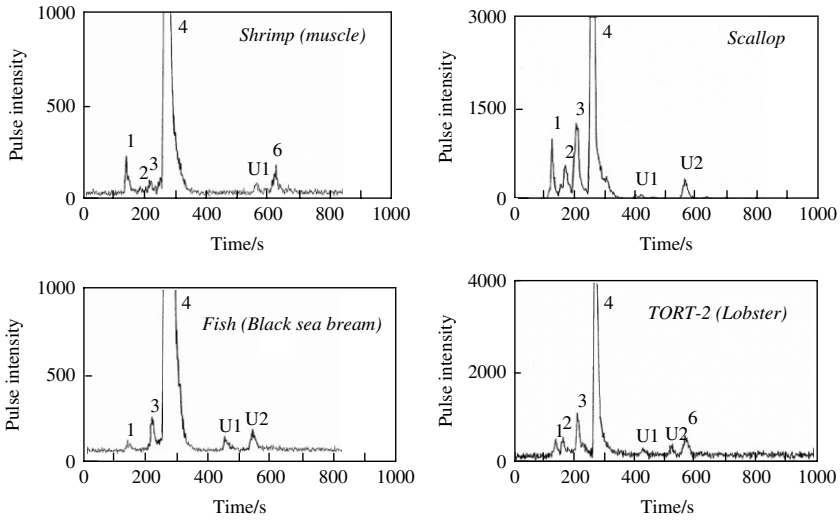

Figure 3. HPLC-ICP-MS chromatogram of arsenic species in the extract from shrimp, scallop, fish and TORT-2; column, Shiseido Capcell Pak C_{18} (250 × 4.6 mm i.d., 5 μm); mobile phase, 5 mM 1-butanesulfonic acid, 0.3 mM 1-hexanesulfonic acid and 2 mM malonic acid in 0.5% methanol (pH 3); sample loop, 10 μl; 1-7, equivalent to the numbers in Figure 2; U1, U2, unknown peaks.

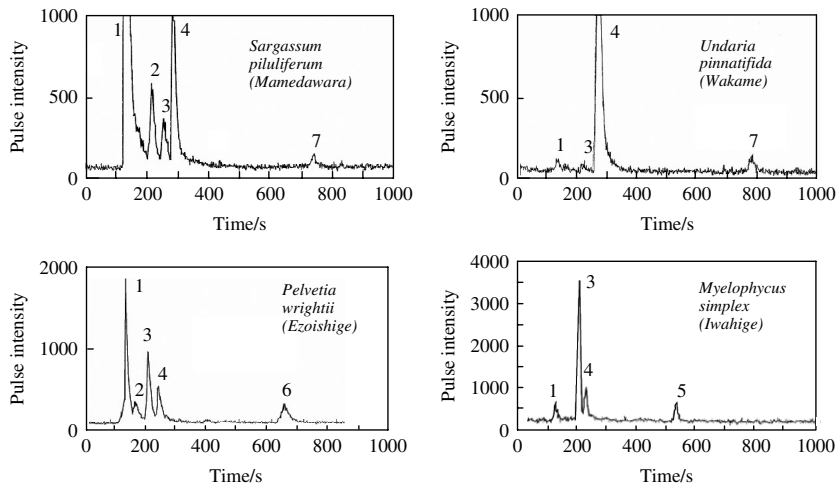

Figure 4. HPLC-ICP-MS chromatogram of arsenic species in the extract from *S. piluliferum (Mamedawara)*, *U. pinnatifida (Wakame)*, *P. wrightii (Ezoishige)*, and *M. simplex (Iwahige)*; column, Shiseido Capcell Pak C_{18} column (250 × 4.6 mm i.d., 5 μm); mobile phase, 5 mM 1-butanesulfonic acid, 0.3 mM 1-hexanesulfonic acid and 2 mM malonic acid; 1-7, equivalent to the numbers in Figure 2.

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(Iwahige) extracts are shown in Fig. 4. Arsenate (As^V) was dominant with concentrations of 112.4 and 47.4 μg As g^{-1}. Considerable concentrations of As^{III}, MMA, DMA and $TeMAs^+$ in the range 1.05–6.31 μg As g^{-1} were detected (Table 3). Lower recoveries for marine algae (51–72%) are due to the difficulty of complete extraction in marine algae matrix.

pH dependence on separation of arsenosugars and arsenic species

The separation of arsenic species and arsenosugars was investigated by changing the pH of the ammonium hydrogen carbonate solution using an anion exchange resin, Hamilton PRP X-100 column. The retention times of As^V depended

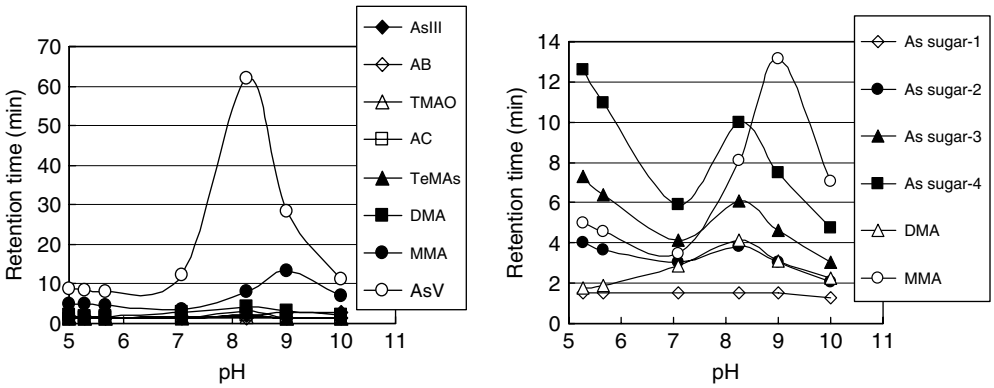

Figure 5. Relationship of eight arsenic species and arsenosugars between retention times and pH of mobile phase; column, Hamilton PRP X-100 anion-exchange column (250 × 4.1 mm i.d., 10 μm); mobile phase, 20 mM NH_4HCO_3 at 1.0 ml min^{-1}; sample loop, 10 μL.

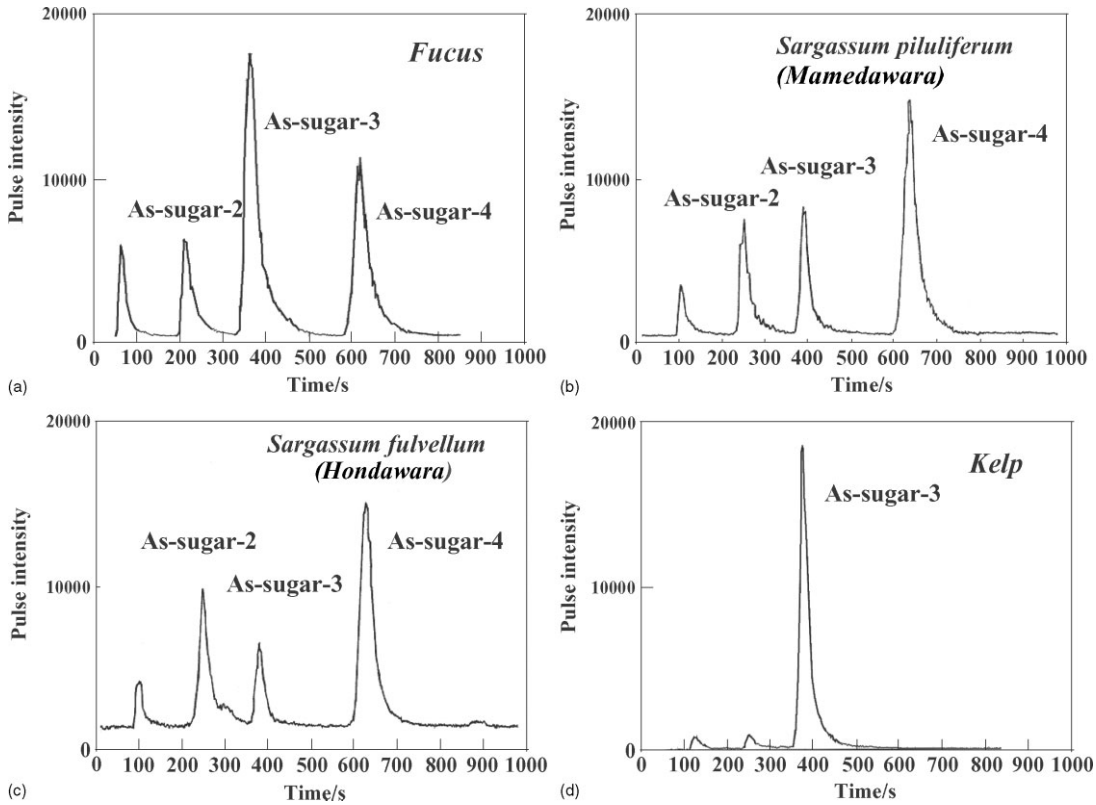

Figure 6. HPLC-ICP-MS chromatograms of arsenosugars in the extracts from marine algae; column, Hamilton PRP X-100 anion column (250 × 4.1 mm id., 10 μm); mobile phase, 20 mM NH_4HCO_3 (pH 8.4); sample loop, 10 μL. (a) *Fucus*, (b) *S. piluliferum (Mamedawara)*, (c) *S. fulvellum* (*Hondawara*) and (d) Kelp; G **1**, arsenosugar-1; **2**, arsenosugar-2; **3**, arsenosugar-3; and **4**, arsenosugar-4.

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Table 4. Determination of arsenosugars in the marine algae extracts by HPLC-ICPMS

Marine algae	As-Sugar 2	As-Sugar 3 (μg As dry g^{-1})	As-Sugar 4	Found As-Sugars (μg g^{-1})	As-Sugars (%)
Sargassum fulvellum	9.66 ± 0.67	2.71 ± 0.38	11.9 ± 2.84	27.5 ± 2.84	13.3
Sargassum piluliferum	5.62 ± 0.39	2.14 ± 0.30	9.44 ± 2.26	18.8 ± 2.26	6.5
Undaria pinnatifida	6.40 ± 0.04	ND	ND	17.8 ± 5.09	24.4
Kelp	2.23 ± 0.52	14.4 ± 0.87	ND	31.5 ± 2.73	34.9
Hizika fusiformis	1.09 ± 0.23	0.17 ± 0.07	1.17 ± 0.43	3.61 ± 0.43	6.0
Myelophycus simplex	2.60 ± 0.21	5.59 ± 0.86	ND	9.72 ± 0.86	18.6

largely on pH of the mobile phase. As^V was eluted at 60 min by mobile phase at pH 8.4, whereas below pH 6 and over pH 9.5, As^V was eluted within 20 min, as shown in Fig. 5(a). Also, MMA depended on the pH of mobile phase. Since arsenosugars and arsenic species have similar chemical natures, the separation of arsenosugars from another arsenic species was complicated. Arsenosugar-3 and -4 could be separated with eight other arsenic species. DMA overlapping arsenosugar-2 and arsenosugar-1 could not be identified from other arsenic species.

Arsenosugars

The chromatograms of *Fucus, S. piluliferum, S. fulvellum*, and kelp extracts are shown in Fig. 6. The concentrations of arsenosugars-2, -3 and -4 were evaluated by comparison with concentrations of arsenosugars-2, -3 and -4 in the *Fucus*. The analytical results are shown in Table 4. Arsenosugar-2 was contained in the extracts from *S. fulvellum, S. piluliferum* and *Undariapinnatifida*, which were in the range 1.09–9.66 μg As g^{-1}. Arsenosugar-3 was contained in the extracts from kelp and *M. simplex*, at 14.4 μg As g^{-1} for kelp and 5.59 μg As g^{-1} for *M. simplex*. Arsenosugar-4 had high concentration in the extracts from *S. fulvellum* and *S. piluliferum* and their concentrations were 11.9 μg As g^{-1} for *S. fulvellum* and 9.44 μg As g^{-1} for *S. piluliferum*. Summations of arsenosugar-2, -3 and -4 contents accounted for 6.0–34.9% of total arsenic of these marine algae. These arsenosugars may play an important role in metabolism of arsenic in marine organisms.

CONCLUSIONS

The proposed HPLC-ICP-MS offers sufficient detection limits and reproducibility for the investigation of species of As^{III}, As^V, MMA, DMA, AB, TMAO, AC, $TeMAs^+$ and arsenosugars in the marine samples. The method was successfully applied to various marine samples, e.g. oyster, fish, shrimp and marine algae. The summations of arsenosugar-2, -3, and -4 in the extracts from marine algae accounted for 6.0–34.9% of total arsenic. Further, a confirmation of the compound can be achieved by coupling the chromatography to an ES-MS/MS system. The coupling of this system to ES-MS/MS is essential for characterization of the unknown species and in future the role of arsenogugars will be clarified.

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