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The Identification of Some Water-Soluble Arsenic Species in the Marine Brown Algae *Fucus distichus*

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The extraction and clean-up procedures developed to isolate the water-soluble arsenic species present in the marine macroalga *Fucus distichus*, from British Columbia, Canada, are described. The arsenic species were extracted into methanol and then subjected to gel-permeation and ion-exchange chromatography. Fractions high in arsenic were identified by using graphite furnace atomic absorption spectroscopy (GF-AAS), and further investigated by using high-performance liquid chromatography coupled to inductively coupled plasma–mass spectrometry (HPLC–ICP MS). By using different HPLC columns and mobile-phase conditions, the four major arsenic-containing compounds present in the macroalga were positively identified as arsenosugars; one minor compound remained unidentified. © 1997 John Wiley & Sons, Ltd.

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INTRODUCTION

Arsenic is present in the natural environment and in living organisms in different chemical forms.[1] In the marine environment, high concentrations of arsenic occur in macroalgae,[2,3] which are able to accumulate inorganic arsenic or methylarsenicals and convert them to complex organoarsenic compounds.[2–4] Studies on macroalgae have shown that they all contain arsenoribosides (Fig. 1) to some extent, but none has been reported to contain arsenobetaine.[1–4] All marine animals that have been studied contain arsenobetaine;[5,6] tetramethylarsonium ion, trimethylarsine oxide, dimethylarsinic acid, arsenocholine and arsenoribosides have also been detected.[1–6]

In earlier work on marine macroalgae[7,8] methanol was used to release the majority of the arsenic present. This extract was then separated into different arsenic-containing fractions by using gel-permeation chromatography and ion-exchange chromatography with further purification by preparative layer chromatography and HPLC. Identification of the compounds as arsenosugars was facilitated by ^{1}H and ^{13}C NMR spectroscopy, and in one case by X-ray crystallography.[7,8] More recent studies have used similar methodology with identification by HPLC–ICPMS.[9]

The complete chromatographic isolation and identification of arsenosugars extracted from marine algae is difficult because of their similar chromatographic properties and labile nature, which can lead to interconversion during isolation.[4] Purification is also hindered by the presence of other sugar derivatives which do not contain arsenic. In the present work the problem of isolating pure compounds using column chromatography was overcome by analysing appropriate standards using HPLC–ICP–MS.

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However, for reliable identification the chromatographic behaviour of these compounds needs to be compared using a number of different columns and eluents. Some clean-up and isolation of the arsenoribosides is still necessary to increase the certainty of identification, but this can be less rigorous than when NMR is used.

To establish a more complete biogeochemical understanding of arsenic in the marine environment, the arsenic compounds in different species of marine macroalgae, from a variety of geographic regions, needs to be studied. The present work provides information on the marine brown alga *Fucus distichus* from the western coastal waters of Canada.

EXPERIMENTAL

Reagents

Methanol (HPLC grade, Fisher), tetrabutylammonium hydroxide (Aldrich), tetraethylammonium hydroxide (Eastman), malonic acid (BDH) and sodium borohydride (Aldrich) were used for HPLC and hydride generation AAS (HG AAS). Sephadex LH20, DEAE Sephadex A25 and CM Sephadex C25 (Pharmacia AB) were the column-chromatography packing materials. Sulphuric acid (98%), nitric acid (69%) and hydrogen peroxide (30%) (Fisher) were used for acid digestion.

Retention standards

Arsenobetaine was prepared from trimethylarsine.[10] Standard solutions of arsenobetaine and dimethylarsinic acid (Aldrich) were prepared in deionized water. Oyster tissue standard reference material NIST 1566a (Gaithersburg, US), a freeze-dried sample of a commercial kelp product (purchased in Vancouver, Canada) and a sample of arsenosugar 2 (donated by Dr K. A. Francesconi, Western Australian Marine Research Laboratory, Australia), were used as retention-time standards.

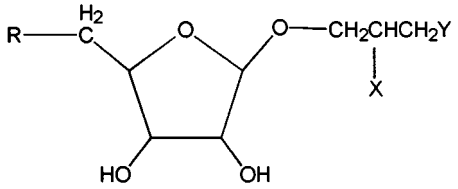
Samples

The macroalga *Fucus distichus* was collected from Hastings Arm, BC, Canada, during a cruise in May 1990. The sample was frozen immediately after collection and stored at -20 °C for seven days prior to extraction.

Extraction and clean-up of *F. distichus*

Where the use of rotary evaporation is mentioned, the conditions were: evaporation at 40 °C, under reduced pressure generated by aspiration.

As outlined in Fig. 2, the sample (6.77 kg wet weight) was homogenized in a blender with a small volume of methanol (50 ml). The resulting homogenate was placed in a glass column (15.3 cm i.d. $\times$ 100 cm) and 3 ml of methanol per gram of sample was continuously passed through, until the seaweed became a pale green colour. The methanol extract (approx 23 l) was filtered (Whatman No. 1) and then rotary-evaporated. The resulting oily syrup (90 g) was dissolved in water (1 l) and extracted three times with diethyl ether (3×300 ml). The dark green ether layer was discarded and the water layer was rotary-evaporated, to yield a dark brown syrup.



Arsenoriboside	R	X	Y
1	$(CH_3)_2As(O)$—	—OH	—SO_3H
2	$(CH_3)_2As(O)$—	—OH	—OH
3	$(CH_3)_2As(O)$—	—OH	—$OPO_3HCH_2CH(OH)CH_2OH$
4	$(CH_3)_2As(O)$—	—OH	—OSO_3H
5	$(CH_3)_2As(O)$—	—NH_2	—SO_3H
6	$(CH_3)_3As^+$—	—OH	—OSO_3H

Figure 1 The structures of some of the arsenoribosides found in the marine environment.

The syrup was dissolved in methanol (0.2 l) and this was poured into acetone (1.2 l) and allowed to stand for 1 h. The supernatant was decanted and discarded, and the residue was dissolved in deionized water (500 ml).

The removal of any remaining lipid material was attempted by extraction with water/methanol/chloroform (600 ml; 1:1:1 by vol). The pale green chloroform layer was discarded, while the aqueous layer was evaporated to a residue using a rotary evaporator. The residue was extracted twice with methanol (200 ml), and the residual material was discarded. The methanol solution was evaporated to dryness by rotary evaporation and the resulting material (31.1 g, 3.7 mg As) was dissolved in deionized water (100 ml). This solution was stored in plastic bottles at −20 °C for seven days.

Fractionation of the extract

The clean-up scheme (Fig. 3) is a modification of that described by Edmonds.[8] The *F. distichus* extract was fractionated by using a gel-permea-

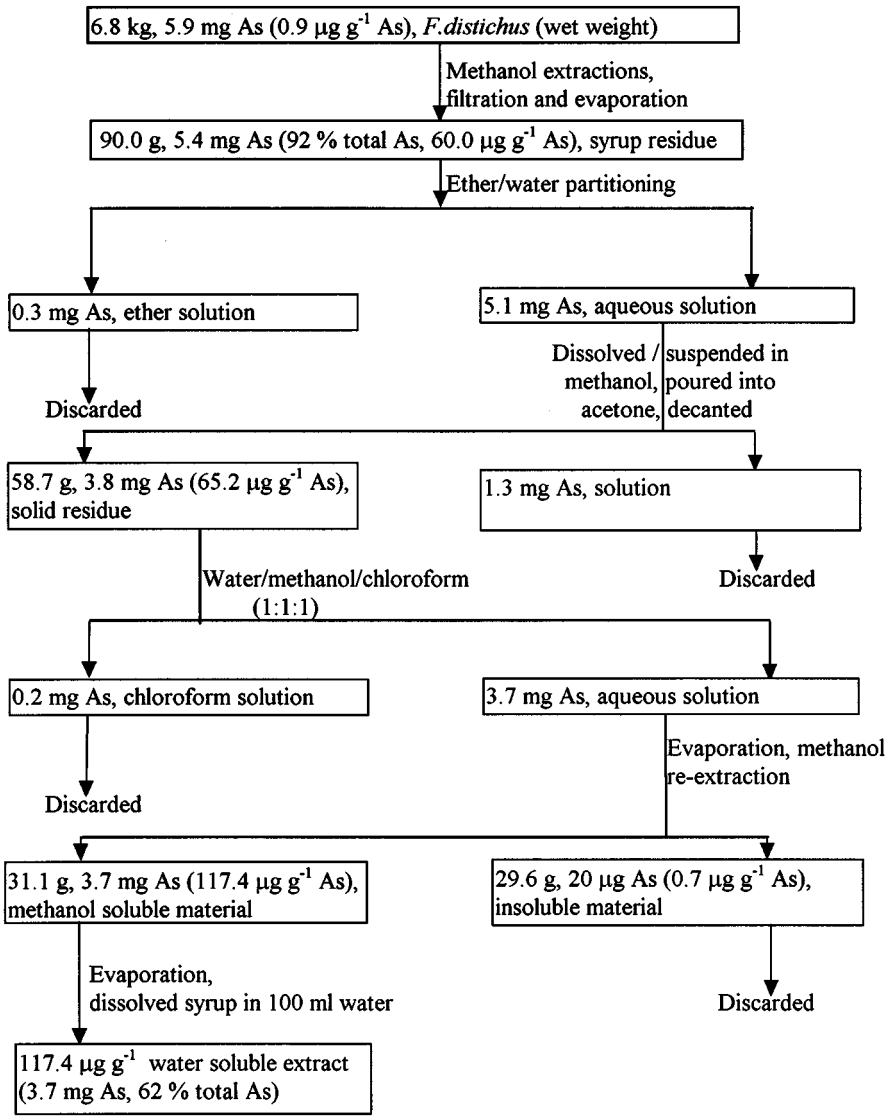


Figure 2 *Fucus distichus* extraction and initial clean-up scheme. Also shown is the mass of material isolated and the concentration of arsenic.

Table 1. HPLC columns and conditions used to analyse different arsenic-enriched bands. Detection by ICP-MS as outlined in the text.

Conditions	Analytical column	Mobile phase
I	μBondapak C_{18} (Waters), 33 cm × 3.9 mm i.d.	10 mM tetraethylammonium hydroxide 4.5 mM malonic acid at pH 6.8 Flow rate 0.8 ml min^{-1}
II	Bondclone C_{18} (Phenomenex), 33 cm × 3.9 mm i.d.	25 mM tetraethylammonium hydroxide 25 mM malonic acid at pH 6.8 Flow rate 1.0 ml min^{-1}
III	Inertsil ODS-2 (G.L. Sciences), 25 cm × 4.6 mm i.d.	10 mM tetraethylammonium hydroxide 4.5 mM malonic acid 0.1% methanol at pH 6.8 Flow rate 0.8 ml min^{-1}
IV	Inertsil ODS-2 (G.L. Sciences), 25 cm × 4.6 mm i.d.	10 mM tetrabutylammonium hydroxide 4.5 mM malonic acid at pH 6.8 Flow rate 1.0 ml min^{-1}

tion column (2.5 cm i.d. × 90 cm Sephadex LH20) with water as the eluent. Fractions of 8.5 ml were collected manually. Arsenic-containing fractions eluted between 159 and 743 ml and were combined and evaporated to a syrup by rotary evaporation. The syrup was dissolved in methanol and re-chromatographed on a Sephadex LH20 column (2.5 cm × 40 cm) using methanol as eluent. Arsenic-containing fractions eluted between 60 and 277 ml and were evaporated to a syrup (26.7 g, 3.1 mg As). The syrup was dissolved in 0.05 M Tris buffer and re-chromatographed using a weak anion exchange column (DEAE Sephadex A25, 2.5 cm × 40 cm). Arsenic-containing fractions eluted between 179 and 400 ml when the eluent was 0.05 Tris buffer, pH 8.0 (band **A**). After 1015 ml the eluent was changed to 0.5 M Tris buffer, pH 8.0, and less arsenic eluted between 1070 and 1476 ml (band **B**). The fractions corresponding to these two bands were separately combined and evaporated to syrups by rotary evaporation. (Syrups: **A**, 27.1 g; 3.0 mg As; **B**, 12.3 g, 0.2 mg As.)

Enrichment of the arsenic in bands A and B

Extraction of syrup **A** with methanol left some white solid (3.0 g, 7.7 μg As), which was discarded. The methanol solution was rotary-evaporated and the syrup (24.1 g) was dissolved in 50 ml of 0.05 M Tris buffer, pH 8.0, and chromatographed on DEAE Sephadex A25 (2.5 cm × 50 cm) by using 0.05 M Tris buffer, pH 8.0, as the eluent. The arsenic-containing fractions eluted between 156 and 330 ml and were divided into two: **Aa**, 156–330 ml and **Ab**, 331–770 ml. These were rotary-evaporated to

Table 2. Retention times for arsenobetaine (AB), dimethylarsinic acid (DMAA) and arsenosugars **1, 2, 3** and **4** in the retention standards, using the conditions in Table 1

Standard	Compound	Retention time(s) Cond. I	Cond. II	Cond. III	Cond. IV
Oyster SRM	AB	227	183	286	174
	DMAA	264	151	385	304
	2	290	249	461	174
	3	264	203	523	304
Kelp	**1**	—	—	493	420
	2	—	—	463	181
	3	—	—	493	310
	4	—	—	683	693
Isolate	**2**	287	249	—	—

syrups **Aa** (19.5 g, 2.9 mg As) and **Ab** (4.0 g, 0.2 mg As) which were separately dissolved in methanol/water (4:1, v/v) (35 ml). **Aa** was chromatographed on a Sephadex LH20 column [2.5 cm × 82 cm, isocratic elution using methanol/water (4:1, v/v)] and gave arsenic-containing fractions between 154 and 449 ml (**Aa**, 17.9 g, 2.7 mg As). **Aa** was next chromatographed on a weak cation exchanger, CM Sephadex C25 (25 cm × 42 cm) with 0.1 M ammonium acetate, pH 6.5, as the eluent. This afforded arsenic-rich fractions between 50 and 258 ml (**Aa**, 12.6 g, 2.2 mg As). **Ab** will not be considered further.

Band **B** was applied to a Sephadex G15 column (2.5 cm × 60 cm) using water as the eluent. Arsenic-containing fractions eluted between 54 ml and 342 ml, and were combined and reduced to a syrup as before (**B**, 8.0 g, 0.2 mg As).

Arsenic determination

The method developed for the determination of total arsenic in bivalves[11] was slightly modified for *F. distichus*. The freeze-dried, powdered sample (0.25 g) was placed in a 500-ml round-

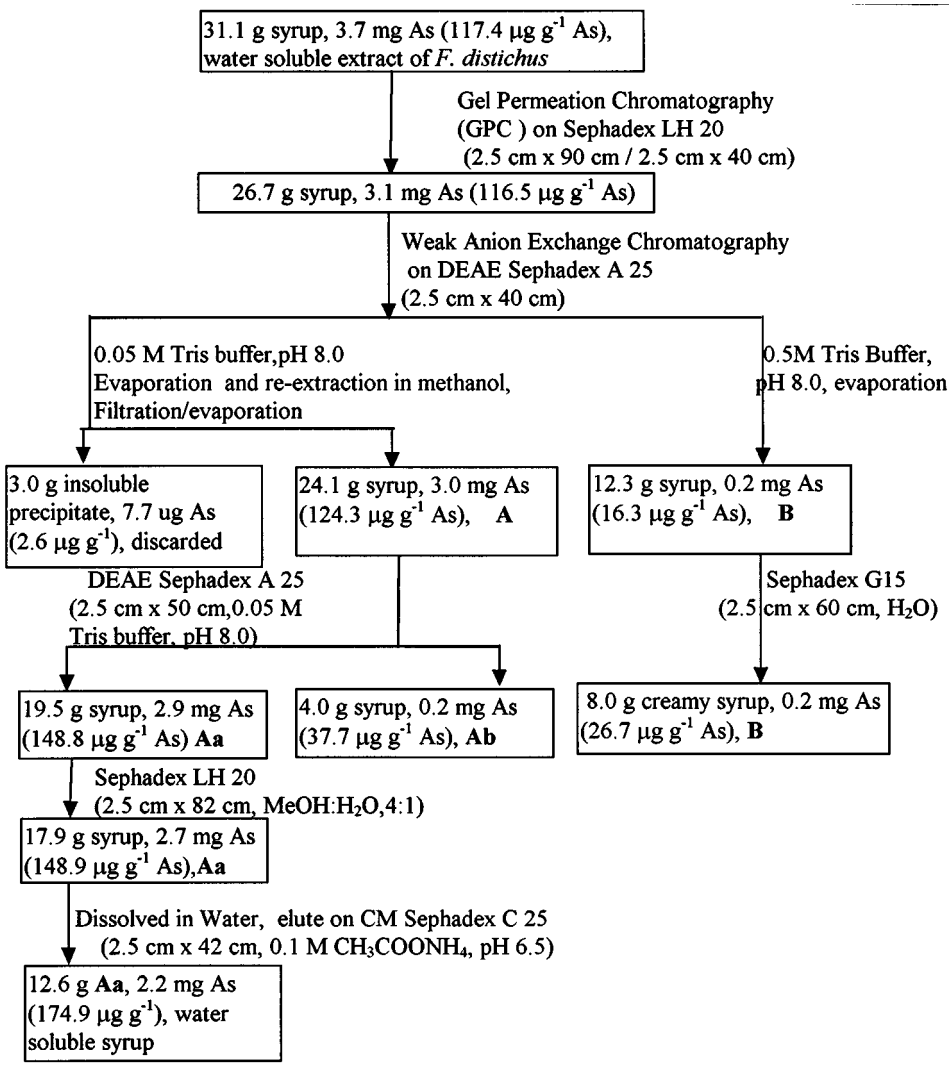


Figure 3 *Fucus distichus* chromatographic clean-up scheme. Also shown is the mass of material isolated and concentration of arsenic.

bottomed flask and 1 ml sulphuric acid (98%), 3 ml nitric acid (69%), and 3 ml hydrogen peroxide (30%) were added. The mixture was heated at 200 °C for 2 h, cooled, and made up to 100 ml with deionized water. The solution was analysed for arsenic by using HG AAS.[12] The arsenic present in the chromatographic fractions was directly determined by graphite furnace AAS using palladium as the matrix modifier.[13]

HPLC–ICP MS conditions

The HPLC system consisted of a Waters Associates model 510 reciprocating pump, a six-port Rheodyne injection valve, with a 20-μl sample loop and a C_{18} guard column. The conditions used are detailed in Table 1.

All ICP measurements were performed using a VG PlasmaQuad 2 Turbo Plus (VG Elemental, Fisons Instruments, Winsford, Cheshire, UK) equipped with a standard glass torch and a de Galan V-groove nebulizer. The plasma conditions were generated using a forward power of 1350 W and gas flow rates of: coolant 14.0 l min^{-1}; auxiliary 0.7 l min^{-1}; nebulizer 0.9 l min^{-1}. Nickel sampler and skimmer cones were used throughout. Measurements were made using single-ion monitoring mode at *m/z* 75 for arsenic.

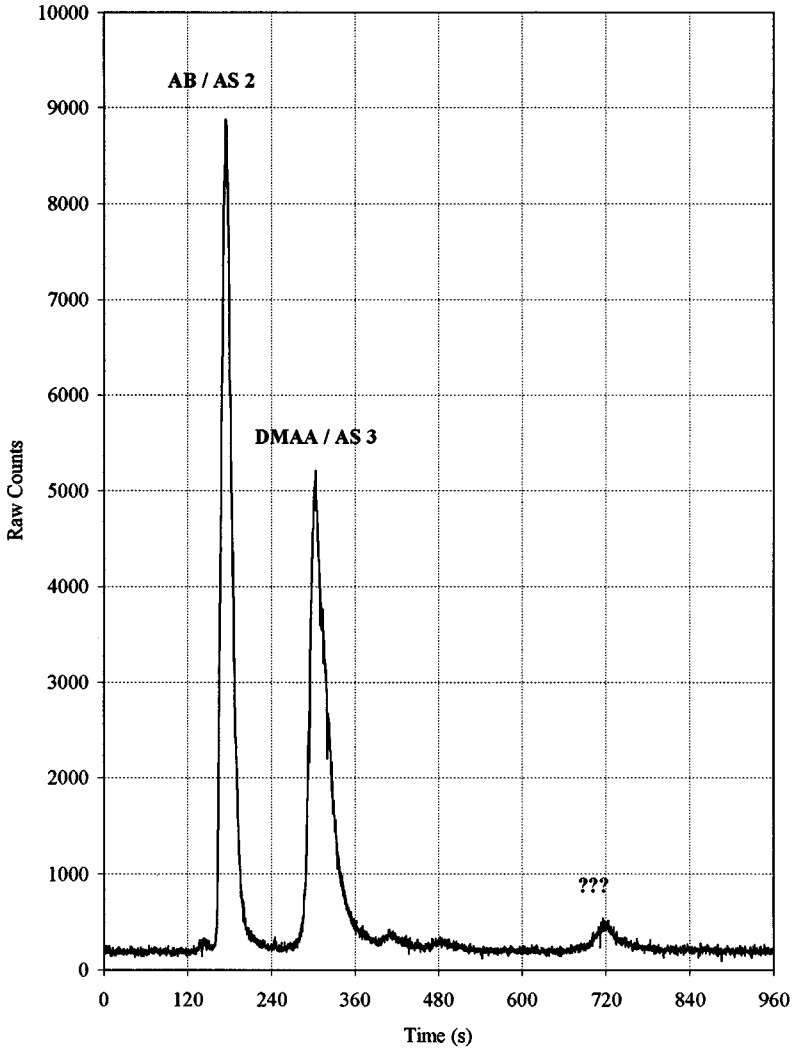


Figure 4 Analysis of the oyster tissue SRM using condition IV (see Table 1). As2, arsenosugar **2**; As3, arsenosugar **3**.

RESULTS AND DISCUSSION

Arsenic analysis

The total amount of arsenic present in the *F. distichus* sample was determined by using HG AAS to be 0.9 $\mu g\ g^{-1}$ (wet weight). Extraction of the seaweed with methanol yielded 92% of the total arsenic. The concentration of arsenic in the methanol residue was determined to be 60.0 $\mu g\ g^{-1}$ As.

HPLC–ICP MS analysis of the standards

The oyster SRM 1566a was analysed using all four of the conditions shown in Table I: I, II and III (results not shown) gave three, four and four

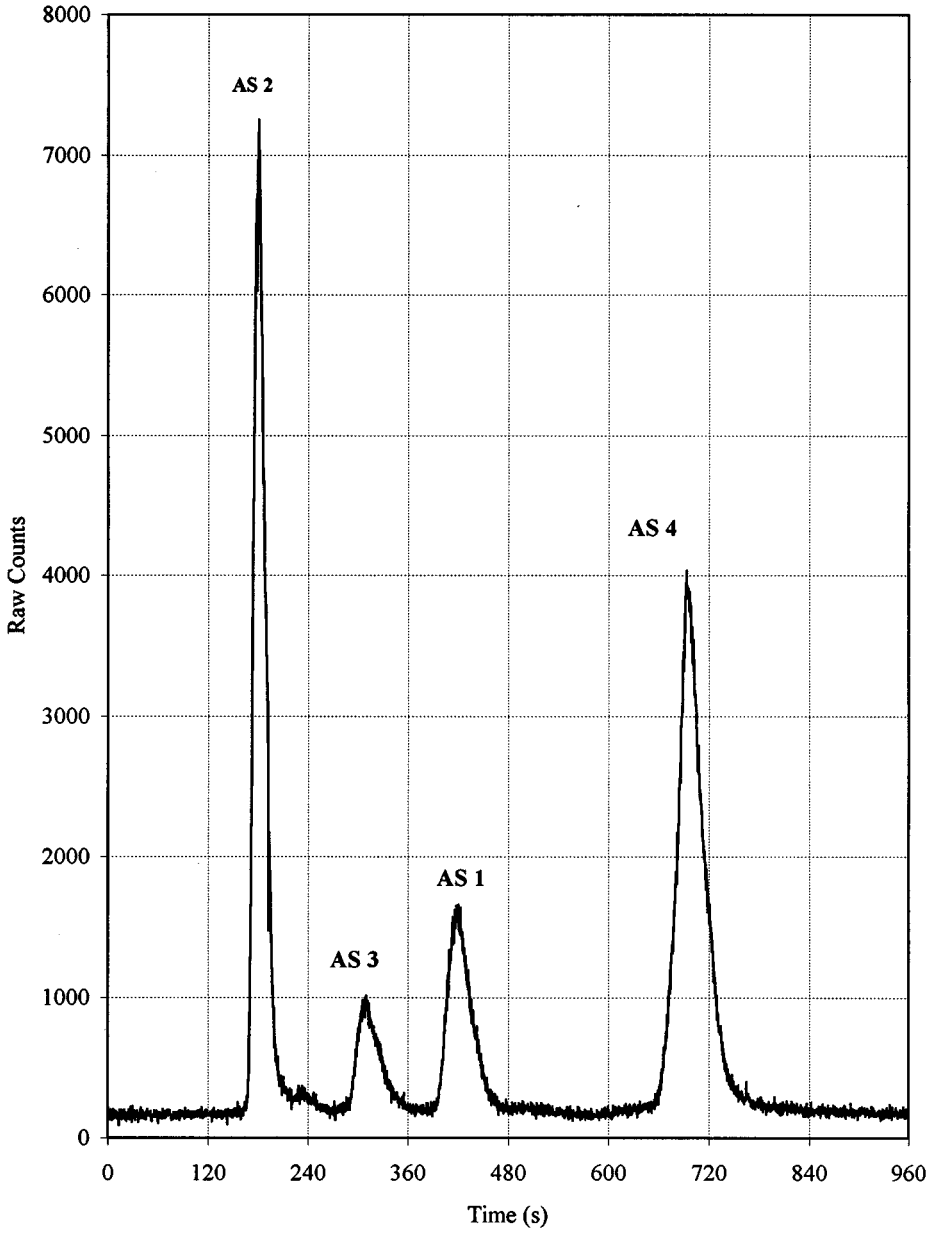


Figure 5 Analysis of the kelp sample using condition IV (see Table 1). **As1–As4**, arsenosugars **1**–**4**.

Table 3. Retention times for the arsenic-containing peaks found in the two main bands **Aa** and **B** using the conditions outlined in Table 1.[a]

Sample	Retention time (s) Cond. I	Cond. II	Cond. III	Cond. IV
Band **Aa**	PI = 257	P3 = 191	P6 = 466 (**2**)	P9 = 176 (**2**)
	P2 = 287 (**2**)	P4 = 223	P7 = 505	P10 = 318 (BLK)[b]
		P5 = 249 (**2**)	P8 = 693 (**4**)	P11 = 411 (**1**)
				P12 = 691 (**4**)
Band **B**	P13 = 261 (**3**)	P15 = 197 (**3**)	—	P17 = 305 (**3**)
	P14 = 311	P16 = 214	—	P18 = 475

[a] The arsenosugars with the same retention time (see Table 2) are given in parentheses

[b] BLK, peak present in reagent blank.

arsenic-containing peaks respectively, and the retention times are shown in Table 2. With condition III, four resolved peaks corresponding to arsenobetaine (AB), dimethylarsinic acid (DMAA) and arsenosugars **2** and **3** are evident, as reported previously.[6,9] The use of condition IV (Fig. 4) gave two major peaks due to co-elution of **2** plus AB and DMAA plus **3**, which was confirmed using standard solutions of AB and DMAA. Later work indicated that no AB or DMAA was present in *F. distichus*.

The four arsenic-containing compounds present in the kelp sample were tentatively identified on the basis of their relative retention times and magnitudes.[3] This designation was further confirmed for us by Edmonds and Shibata using HPLC–ICP MS (Asahipak GC-220 HQ gel-permeation column). In the present work, condition IV gives complete baseline resolution of arsenosugars **1, 2, 3** and **4** (Fig. 5).

The isolated sugar **2** gave a single peak using both conditions I and II (Table 2).

HPLC–ICP MS analysis of the arsenic enriched bands (Aa and B)

Analysis of band **Aa** using condition I gave two peaks; the retention time of peak P2 was 287 s, corresponding to sugar **2** in the oyster standard (290 s) and the isolate (287 s) (Table 3). When analysed using condition II, three arsenic-containing peaks (P3, P4 and P5) were apparent, with one peak (P5) again having the same retention time (249 s) as sugar **2** in the oyster (249 s) and isolate (249 s).

Further evidence for the presence of arsenosugar **2** in band **Aa** comes from the analysis of the kelp standard. With condition III, three arsenic-containing peaks are seen. The retention time of P6 (466 s) corresponds to sugar **2** in the kelp standard (463 s) and sugar **2** in the oyster standard (461 s). The last peak eluting from band **Aa** has a retention time (693 s) similar to arsenosugar **4** (683 s) in the kelp standard. Condition IV gives four peaks for band **Aa** (P9, P10, P11 and P12) (see Fig. 6) with retention times (s) 176, 318, 411 and 691 respectively. Analysis of the kelp standard under these conditions (Fig. 5) gave four peaks, **1** (420 s), **2** (181 s), **3** (310 s) and **4** (693 s), of which **1, 2** and **4** correspond to the peaks P9, P11 and P12 in band **Aa**. Peak P10 (318 s) appeared initially to correspond to **3**, but examination of a number of blank runs from different days showed the presence of a peak with a closer retention time (C. F. Harrington, unpublished results.)

The results from the analysis of **Aa** in conditions I, II, III and IV indicate that sugars **1** and **2** are the major arsenic-containing compounds, whilst **4** is the minor component (compare Figs 5 and 6).

Using a similar approach for the analysis of band **B** with conditions I, II and IV, it can be seen that peaks P13 (261 s), P15 (197 s) and P17 (305 s) have retention times corresponding to sugar **3** (264, 203 and 304 s respectively in the oyster and 310 s with condition IV for the kelp standards). Using condition III, arsenosugar **3** in the kelp standard co-eluted with arsenosugar **1**, due to the column degradation mentioned above.

The results for the analysis of bands **Aa** and **B** using the conditions outlined show that arsenosugars **1** and **2** comprise the major arsenic species in band **Aa**, and **4** is the minor species. In band **B** arsenosugar **3** was identified, while the other major arsenic compound remained unidentified. The arsenic content of band **Aa** was much larger

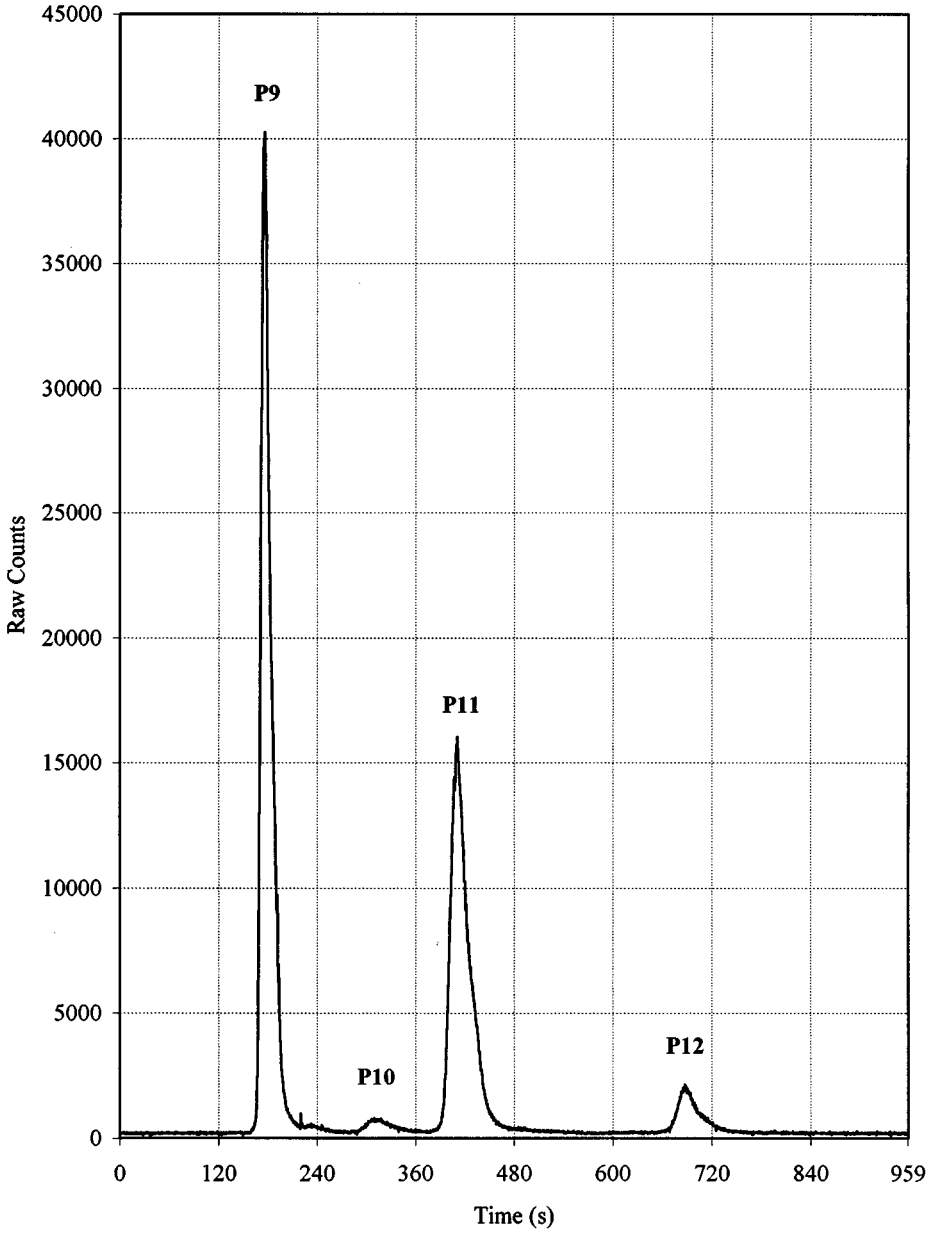


Figure 6 Analysis of the band **Aa** (1:25 dilution) using condition IV (see Table 1).

than band **B** (Fig. 3), indicating the relative magnitudes of the arsenosugars in *F. distichus*.

The confirmation of the presence of the arsenosugars **1, 2, 3** and **4** in *F. distichus* supports the general view that this class of compounds is ubiquitous in marine macroalgae.[1–4] The minor arsenic species present in band **B** was not characterized because of the unavailability of an appropriate standard, but it does seem likely that this compound is another arsenosugar, as it displayed similar chromatographic properties.

The occurrence of the various arsenic compounds in marine algae may possibly be related to algal taxonomy.[4] The major and minor arsenoribosides found in six different species of brown macroalgae from two different orders are shown in Table 4. *Ecklonia radiata* and *Laminaria japonica*, order Laminariales, contain **1** as their

Table 4. Summary of the arsenic species found in marine brown macroalgae

Order	Species	Arsenic species		Ref.
		Major	Minor	
Laminariales	*Ecklonia radiata*	**1**	**2, 3**	**7**
	Laminaria japonica	**1**	**2, 3**	14
Fucales	*Hizikia fusiforme*	**4**, arsenate	**1, 3, 5**	15
	Sargassum thunbergii	**4**	11	16
	Sargassum lacerifolium	**4**	1, 2, 3, 5, 7, 8, 12, DMAA, arsenate	17
	Fucus distichus	**1, 2, 3, 4,** unidentified sugar		This study

major arsenosugar. Three species of another brown alga from the order Fucales, namely *Hizikia fusiforme*, *Sargassum thunbergii* and *Sargassum lacerifolium*, contain **4** as the major arsenosugar and *H. fusiforme* contains arsenate as a major arsenic species (50% of total). *F. distichus*, order Fucales, contains the arsenosugars **1, 2** and **3** and very little inorganic arsenic is present. The minor arsenosugars present (Table 4) also vary with the species of brown alga. Thus it seems that a general taxonomic distinction cannot be made on the basis of the presence or absence of particular arsenic species. However, there is the possibility that some of the differences reported in this work are associated with geographical location.

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