ARSENIC SPECIATION IN ALGAE

by

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Abstract

Arsenic speciation in a variety of commercial algal products and a brown alga, *Fucus gardneri*, collected in Vancouver, B.C., was carried out by using high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) and hydride generation atomic absorption spectrometry (HGAAS).

Water-soluble organoarsenic compounds present in commercially available food products made from red algae, brown algae and blue-green algae were analyzed by using HPLC-ICP-MS. By the application of two HPLC columns and two mobile phase conditions, arsenosugars (arsenoribofuranosides) in a variety of algae were identified by comparing the retention times with the organoarsenic compounds previously identified in an oyster tissue standard reference material, NIST 1566a. A commercial brown algal product, kelp powder, was found to contain four different arsenosugars. This product may have potential as a "standard reference material" for identification purposes. A terrestrial blue-green alga, *Nostoc commune var flagelliforme*, was also analyzed and found to contain an arsenosugar, a compound which was previously known only in marine organisms.

The total arsenic content as well as the amounts of water-soluble arsenic compounds in all commercial products were determined by using a continuous flow HGAAS system. Commercial marine algae were found to contain high amounts of total arsenic, from 7.6 $\mu g\ g^{-1}$ to 49.3 $\mu g\ g^{-1}$. The terrestrial product was found to contain only 2.7 $\mu g\ g^{-1}$ of total arsenic.

Seasonal changes in the arsenic speciation of *Fucus gardneri* were also investigated. Fresh *Fucus* was collected in different seasons from Brockton Point, Stanley Park, Vancouver, B.C. Qualitative analysis was performed by using HPLC-ICP-MS. The relative amounts of some arsenosugars were found to be different in growing tips as compared to the remainder of the plant.

Quantitative analysis was performed by using a continuous flow HGAAS system. The total arsenic content, as well as the extraction efficiency, which relates to the amount of water-soluble arsenic compounds in the *Fucus,* varied between the growing tips and the rest of the plant and between different seasons.

The results add to our knowledge of arsenic cycling in terrestrial and marine environments.

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LIST OF ABBREVIATIONS

AAS	-	atomic absorption spectrometry/atomic absorption spectrometer
ADP	-	adenosine diphosphate
AES	-	atomic emission spectrometry
AsB	-	arsenobetaine
As(V)	-	arsenate/arsenic acid
ATP	-	adenosine triphosphate
DC	-	direct current
DMAA	-	dimethylarsinic acid
HG	-	hydride generation
HPLC	-	high performance liquid chromatography
ICP	-	inductively coupled plasma
MeOH	-	methanol
MMAA	-	monomethylarsonic acid
MS	-	mass spectrometry/mass spectrometer
m/z	-	mass to charge ratio
ODS	-	octadecylsilica
ppb	-	parts per billion (10^{-9}g g^{-1} or 10^{-9} g mL^{-1})
ppm	-	parts per million (10^{-6}g g^{-1} or 10^{-6} g mL^{-1})
PTFE	-	polytetrafluoroethane (Teflon)
RF	-	radiofrequency
SAM	-	S-adenosylmethionine
scfh	-	standard cubic foot per hour (ft^3/h)
TBAH	-	tetrabutylammonium hydroxide
TEAH	-	tetraethylammonium hydroxide
TRA	-	time resolved analysis

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1. Introduction

1.1 General Introduction

Chemical speciation refers to the identification and quantification of different molecular forms of an element in environmental compartments. These forms include compounds that have different oxidation states of the element and/or different groups bound to the central atom. Because the necessity exists to predict species-dependent properties such as the toxicity and the fate of pollutants in environmental studies, knowledge of the total concentration of a particular element or some elements will not be adequate.[1]

Arsenic is an example of a pollutant that can exist as an extremely toxic form, arsine (AsH_3), moderately toxic forms, inorganic arsenicals, and relatively non-toxic forms, arsenobetaine, arsenocholine and arsenosugars (Sections 1.3, 1.4 and Table 1.1). In this project, the speciation of a number of arsenic compounds in the environment was studied in order to have a better understanding of the arsenic cycle in the environment and its impact on the environment.[2]

1.2 Arsenic in the Environment

Arsenic is a well-known toxic metalloid which exists as a free element as well as in many complex forms with other elements in the environment.[2] Arsenic is found in many types of mineral deposits, particularly those containing sulfides and sulfosalts.[2, 3, 4] It accompanies many metals including Cu, Ag, Au, Pt and Fe. Hence, arsenic is a good

indicator in geochemical prospecting surveys for elements of commercial importance.[4] The most commonly found ores are arsenopyrite (FeAsS), enargite (Cu_3AsS_4), orpiment (As_2S_3) and realgar (As_4S_4). Weathering of arsenic containing rocks liberates arsenic in the form of inorganic compounds.[2]

The average concentration of arsenic in the earth's crust is 2-5 mg kg^{-1} ($\mu g\ g^{-1}$).[2, 3, 5, 6] Sedimentary rocks including coal have been found to contain 0.1 to 2 900 mg kg^{-1} of arsenic.[2] This concentration varies widely in the environment.[3]

The concentration of arsenic in freshwater also varies widely, and depends on the exposure of the water to minerals. The average concentration is approximately 1.7 $\mu g\ L^{-1}$ (ng mL^{-1}) with a range of 1.0 to 10 $\mu g\ L^{-1}$ in most freshwater environments.[2] In contrast, the concentration of arsenic in seawater is more homogeneous ranging from 1.5 to 5.0 $\mu g\ L^{-1}$.[2, 5]

Terrestrial plants and animals contain relatively low concentrations of arsenic (0.06 to 0.5 mg L^{-1}).[7] In contrast, marine plants and animals contain higher levels of arsenic (0.78 to more than 100 mg L^{-1}).[7, 8]

There are four possible oxidation states for arsenic: -3, 0, +3 and +5 although only +3 and +5 have environmental importance.[2, 4] The toxicity of different arsenic compounds is related to the oxidation state which will be discussed in more detail later.[2, 8]

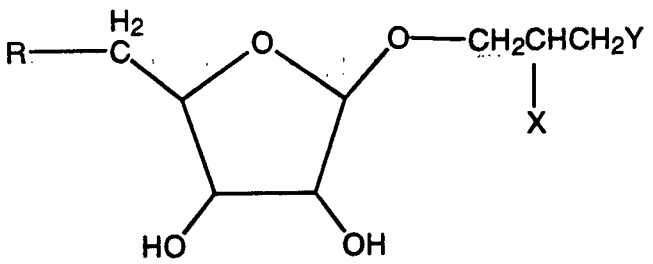
Little information is available on the chemical forms of arsenic in terrestrial plants and animals, except for the amount of inorganic and simple methylated species.[2,9] In the marine environment more complex organometallic forms are found in plants and animals. It has been suggested that the conversion from inorganic forms to methylated forms is a

process of detoxification.[2, 10] Table 1.1 shows some arsenic compounds found in environmental and biological systems. The actual species present will depend on pH.

Table 1.1 Some Arsenic Compounds in Environmental and Biological Systems

No.	Name	Abbreviation	Chemical Formula
I	Arsenous acid	As(III)	H_3AsO_3
II	Arsenic acid	As(V)	H_3AsO_4
III	Monomethylarsonic acid	MMAA	$CH_3AsO(OH)_2$
IV	Dimethylarsinic acid	DMAA	$(CH_3)_2AsO(OH)$
V a	Trimethylarsine	TMA	$(CH_3)_3As$
V b	Trimethylarsine oxide	TMAO	$(CH_3)_3AsO$
VI	Tetramethylarsonium ion	Me_4As^+	$(CH_3)_4As^+$
VII	Arsenocholine	AsC	$(CH_3)_3As^+CH_2CH_2OH$
VIII	Arsenobetaine	AsB	$(CH_3)_3As^+CH_2COO^-$
IX	Dimethylarsinylethanol	DMAE	$(CH_3)_2As(O)CH_2CH_2OH$

Arsenosugars



	R	X	Y
X	$(CH_3)_2As(O)$-	-OH	-OH
XI	$(CH_3)_2As(O)$-	-OH	$-OPO_3HCH_2CH(OH)CH_2OH$
XII	$(CH_3)_2As(O)$-	-OH	$-SO_3H$
XIII	$(CH_3)_2As(O)$-	-OH	$-OSO_3H$
XIV	$(CH_3)_2As(O)$-	$-NH_2$	$-SO_3H$
XV	$(CH_3)_3As^+$-	-OH	$-OSO_3H$

1.3 History of Biological Arsenic Chemistry

Arsenic compounds are notorious for their toxicity. Arsenious oxide (As_2O_3) was used as a weapon to get rid of enemies in the Middle Ages.[11] In 1815, several accidental arsenic poisonings occurred in Germany. These and others were eventually ascribed to a gas generated from the wallpapers which were coated with arsenic-containing pigments, such as 'Schweinfürt green' (copper arsenite plus copper acetate).[8, 12] The volatile gas with a garlic-like odor was identified by Challenger in 1932 as trimethylarsine (TMA).[2, 8, 12] Since then, a number of fungi species, including *Scopulariopsis brevicaulis*, were found to be able to volatilize arsenic. The proposed mechanism by Challenger for the formation of trimethylarsine is as shown in Figure 1.1.[8, 12]

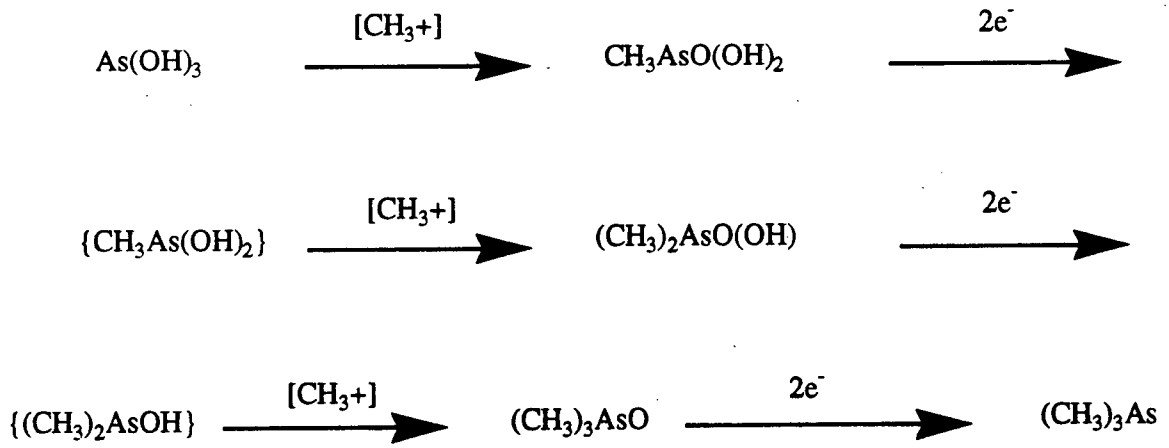


Figure 1.1 Proposed Mechanism of Methylation of Arsenous Acid by Challenger [8, 12]

In later experiments by Challenger *et al.*[13], it was found that $^{14}CH_3$-labelled methionine transferred its label to arsenite to a large extent.[8, 13] The "active methionine" was subsequently identified as S-adenosylmethionine (SAM) with the structure shown in Figure 1.2. More recently, Cullen *et al.*[8] demonstrated that the CD_3 group in L-methionine-methyl-d_3 was transferred to arsenite, arsenate, methylarsonate and dimethylarsinate by cultures of *S. brevicaulis*.[8, 14] Similar results were obtained for *C. humicola*. These results strongly indicate that SAM is the source of CH_3^+ in Challenger's proposed mechanism.[8]

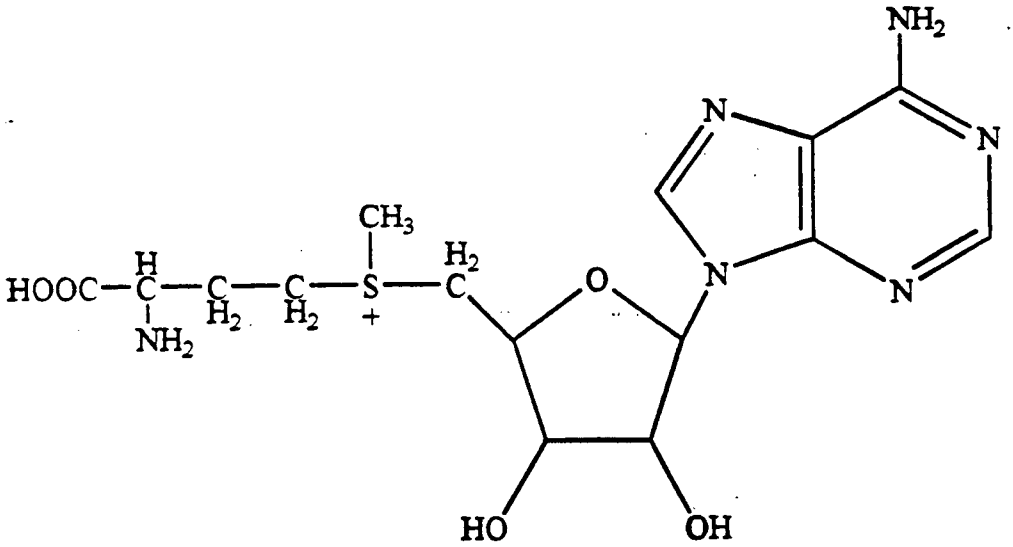


Figure 1.2 Structure of S-adenosylmethionine (SAM)

1.4 Toxicity and Utilization of Arsenic Compounds

1.4.1 Toxicity

The toxicity of arsenic compounds is related to the oxidation state. The relative toxicity is in the trend of R_3As (R=H, Me, Cl, etc.) > As_2O_3 (As(III)) > $(RAsO)_n$ > As_2O_5 (As(V)) > $R_nAsO(OH)_{3-n}$ (n=1,2) > R_4As^+ > As(0).[8, 15, 16] The extreme toxicity of arsine (AsH_3) is due to its ability to cause rapid hemolysis.[2, 17]

Trivalent arsenic compounds have a high affinity for thiol groups (S-H) and can interact with the active sites in a number of enzymes, thus inhibiting the enzyme activity.[2, 11, 18, 19] Lewisite ($ClCH=CH-AsCl_2$) is an example of toxic trivalent compound, which was developed in the Second World War as a war gas, that likely exerts its negative effects in this manner.[11, 18]

Arsenate inhibits ATP synthesis by formation of an unstable arsenate ester of ADP instead of the phosphate ester, ATP. The energy of the ester bond cannot be recovered metabolically.[2, 11, 18]

The organometallic compounds, such as arsenobetaine, arsenocholine, and arsenosugars have not been observed to show toxic effects.[11, 18]

1.4.2 Utilization of Arsenic Compounds

The poisonous character of arsenic allows for its use in herbicides, fungicides, and insecticides.[2] In ancient Chinese medicine, realgar ($(AsS)_n$) was used to cure malaria and parasitism, or as an external antidote for snake bites;[20, 21] orpiment (As_2S_3) and

arsenopyrite (FeAsS) were used for detoxification and treating asthma respectively.[21] *p*-Arsanilic acid was applied to the treatment of sleeping sickness.[18]

Arsenic is also used industrially in the manufacture of semiconductors, colored glass and as a wood preservative.[6, 18, 22]

1.5 Previous Studies of Arsenic in Freshwater and Marine Environments

1.5.1 Terrestrial and Freshwater Environment

Levels of arsenic in the terrestrial environment are generally less than that encountered in the marine environment. Very little is known about the biochemical behavior of arsenicals in terrestrial plants.[8] Inorganic arsenicals as well as some simple methylated species have been found in terrestrial plants.[8, 23] Arsenobetaine has been recently found in mushrooms.[24] In humans who do not eat seafood, arsenite, arsenate, methylarsonate and dimethylarsinate are present in urine.[8] Seafood intake results in the presence of arsenobetaine, a major arsenic compound found in marine animals, which is rapidly excreted unchanged in urine.[9] Previous studies have shown that freshwater algae can tolerate up to 10 000 ppm of arsenic in a contaminated site.[8]

Very few papers on the chemical structure of organoarsenic compounds in freshwater organisms have been published.[25] Dimethylarsenic compounds have been found in freshwater algae and trimethylarsenic compounds have been found in freshwater animals.[23, 25] It has been suggested that arsenosugars may be present in a freshwater alga, *Rizoclonium* sp. However, the chemical forms have not been confirmed yet.[23] The

presence of arsenosugars in freshwater algae remained uncertain until discoveries during the course of the present study.

1.5.2 Marine environment

The distribution of arsenicals in the marine environment has been studied for more than half a century.[7, 9] However, the chemical forms of arsenic in marine organisms remained unknown until 1977 when arsenobetaine was isolated and identified from the Western rock lobster (*Panulirus cygnus*) by Edmonds *et al.*[8, 9, 26] Since then, arsenobetaine has been found as a major arsenic compound in most marine animals investigated.[2, 8, 9] Arsenocholine has been found in some shrimps. The tetramethylarsonium ion (Me_4As^+) has been found in a clam (*Meretrix lusoria*) and trimethylarsine oxide (TMAO) has been found as a minor component in a number of fish species.[8] Varying amounts of arsenobetaine and the tetramethylarsonium ion have been found in some clams in B.C. by Cullen and Dodd.[59] Arsenosugars (X-XV, Table 1.1) have been found to be present in marine algae.[9] A summary of arsenic compounds found in marine algae is seen in Table 1.2

Table 1.2 Previous Discoveries of Arsenic Compounds in Marine Algae[6,27]

Brown Algae		
Wakame	*Undaria pinnatifida*	X, XI, XII
Makonbu	*Laminaria japonica*	X, XI, XII
Hijiki	*Hizikia fusiforme*	X, XII, XIII, XIV
Umitoranoo	*Sargassum thunbergii*	X, XI, XIII, XV
Green Algae		
Miru	*Codium fragile*	X, XI
Red Algae		
Nori	*Porphyra tenera*	X, XI, DMAA

Arsenobetaine and arsenosugars are present in the California mussel (*Mytillus californianus*) and oyster (*Crassostrea gigas*).[27] It is believed that the presence of arsenosugars in the giant clam is due to the symbiotic green algae living in the clam tissue.[8] Arsenosugars have been found in most marine bivalves so far investigated.[9]

Organoarsenicals in higher trophic levels are believed to result from the accumulation of compounds that have been synthesized from arsenate at lower levels.[8] Figure 1.3 shows a proposed mechanism of the formation of arsenosugars from DMAA and SAM.[8, 28, 29] It has been suggested that arsenobetaine and arsenocholine are formed from arsenosugars, based on the observation that an anaerobic incubation of *Ecklonia radiata* with seawater and beach sand results in the production of dimethylarsinylethanol (DMAE, Table 1.1). The structure of this compound suggests that it might be a precursor for arsenobetaine.[8] Figure 1.4 shows the proposed mechanism.[28] However, DMAE has not been identified in the natural environment. It is not obvious where any of these transformations would occur in a natural ecosystem. [8, 30]

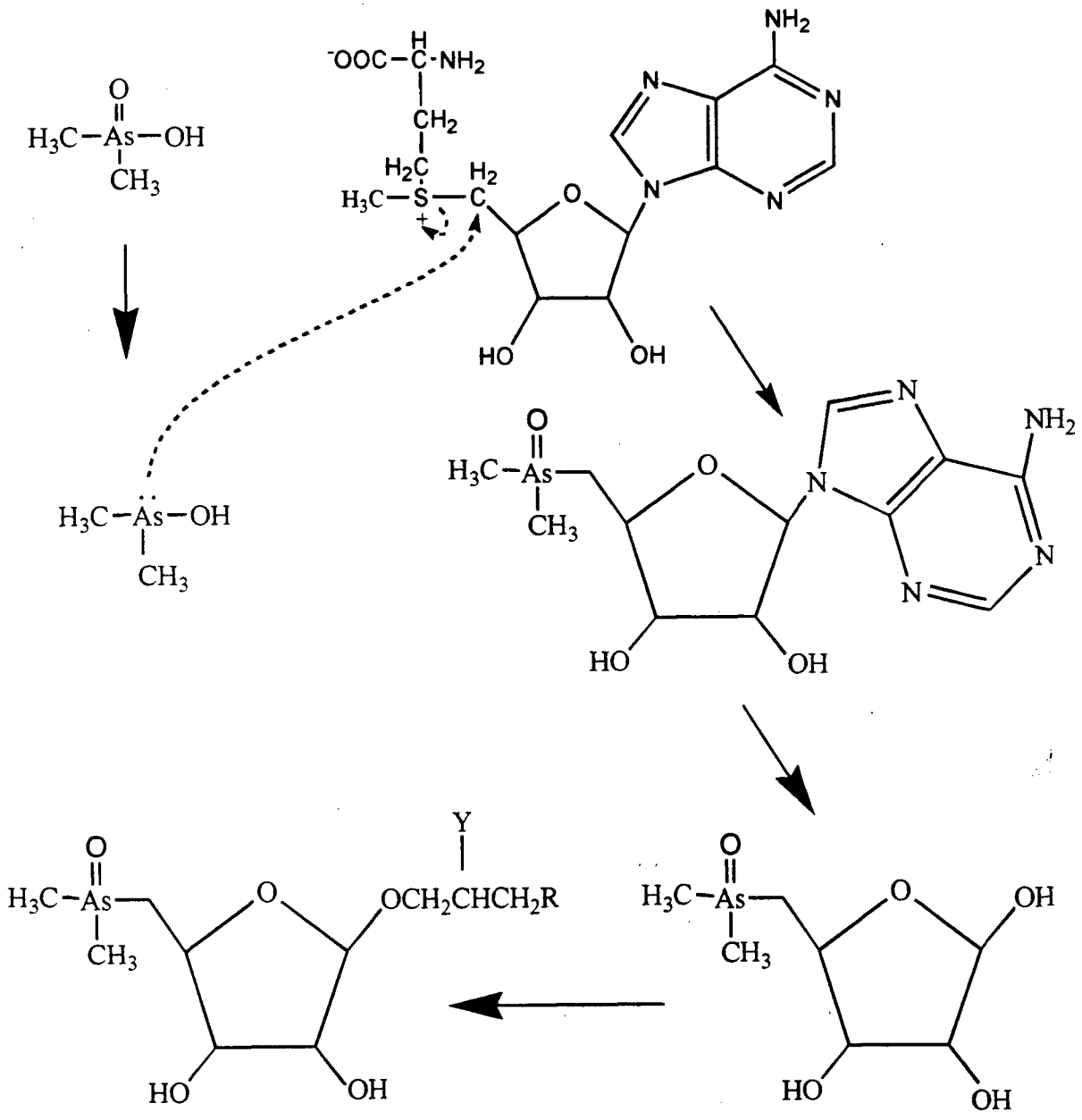


Figure 1.3 Proposed Mechanism, DMAA to Arsenosugars[28]

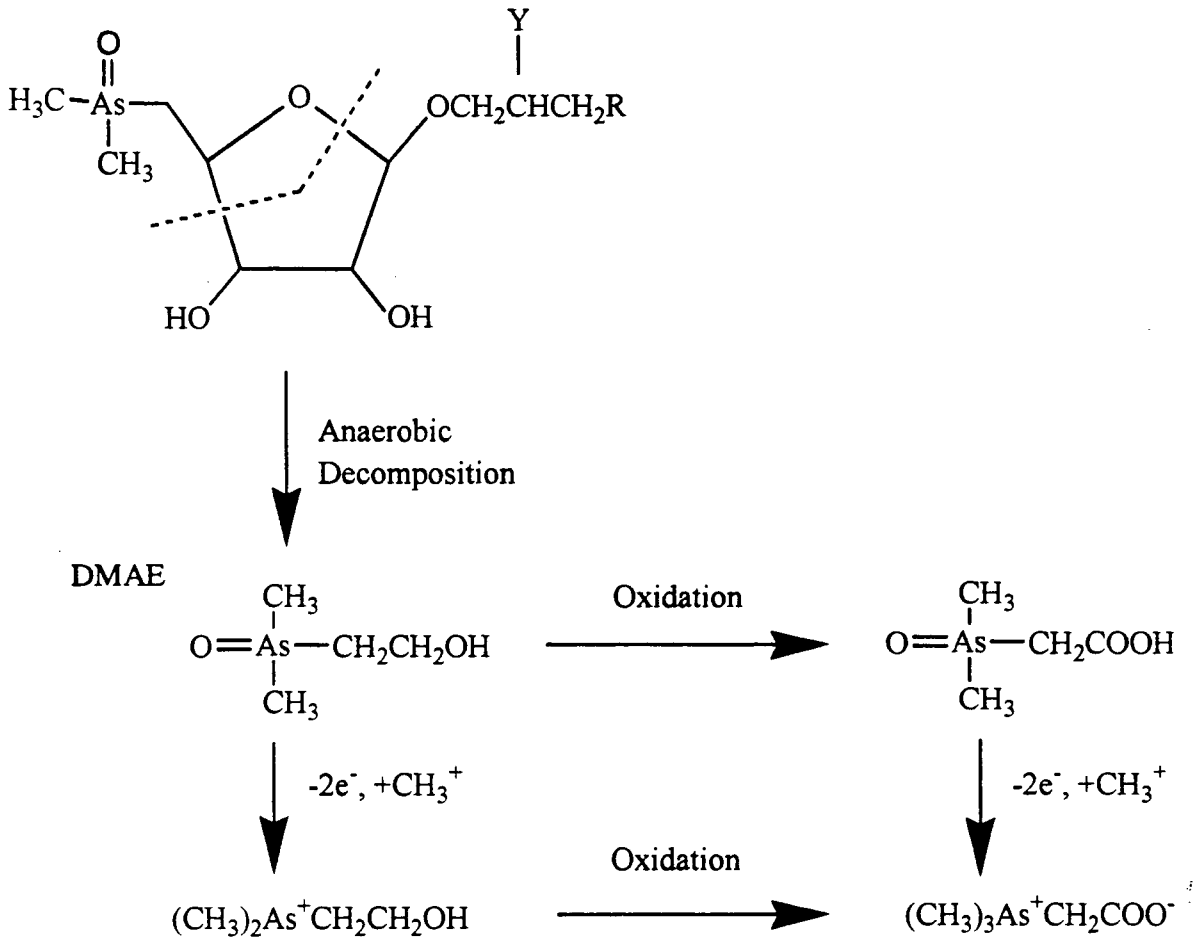


Figure 1.4 Proposed Mechanism, Arsenosugars to AsB and AsC[28]

1.6 Introduction to Algae

The term algae (sing. alga) means different things to different people. Colloquially they have been given names such as 'pond scums', 'frog spittle', 'water mosses' and 'seaweeds'. Algae share many characteristics with other plants, while their unique features are more subtle.[31] They can be found in freshwater and marine environments, as

well as in damp soil and dry tree trunks. The major feature of algae is that their bodies are not obviously divided into root, stem and leaf as is the case for the higher plants.[32]

The classification system of living organisms most widely used is the five kingdom system, consisting of Monera (bacteria and viruses), Protista, Fungi, Plantae and Animalia. Chlorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae) are included in the Protista kingdom. On the other hand, the blue-green algae (cyanobacteria) belong to another kingdom, Monera. Each species has its characteristic photosynthetic pigments in the body.[33]

The brown algae are almost exclusively marine, for example, Arame (*Eisenia bicyclis*), Kombu (*Laminaria groenlandica*), Bull kelp (*Nereocystis leutkeana*) and *Fucus gardneri*.[31, 32, 33] Freshwater brown algal species are quite rare.[34] All brown algae are multicellular, and most are macroscopic. *Laminaria* are commonly called kelps. They can grow to a length of 45 meters or more.[34] *Nereocystis* are also macroalgae which can be 25-30 meters in length.[31] The young specimens of this species have a *Laminaria*-like appearance, but by the progressive splitting of the original blade and a modification of the stipe, the distinct form of *Nerocystis* is assumed. Comparatively, the size of *Eisenia* is smaller than the two species mentioned previously.[31]

Fucus is characterized by the branching filamentous thallus.[34] Mature *Fucus* possesses receptacles at the ends of the branches, which are the fertile areas. Receptacles may be enlarged and swollen when they are mature. Scattered over the surface of these receptacles are minute openings, called conceptacles, in which are produced the eggs and the sperm. In some species a single plant may produce eggs and sperm and is thus a

bisexual individual; in other species, eggs and sperm may be restricted to separate plants. The fertilization of zygotes may happen in the sea water or within the plant body, depending on the species.[7] The swollen receptacles may appear yellowish.[35]

The red algae are the seaweeds that can be seen growing along shores and cast up on beaches. Almost all red algae are multicellular.[36] Dulse (*Palmaria palmata*) and Nori (*Porphyra tenera*) belong to this division. Both species are marine algae.[31, 37]

Blue-green algae are a remarkable group of simple photosynthetic micro-organisms. They are prokaryotic, characterized by the lack of nuclear membrane. This characteristic separates them from other algae. Their oxygen-evolving photosynthesis, however, is similar to that of eukaryotic algae. Some blue-green algae possess symbiotic associations with other organisms. A great number of blue-green algae have the capability of nitrogen fixation.[38]

Nostoc belongs to this division. *Nostoc* species live in freshwater, and only rarely in the marine environment. The species, *Nostoc commune var flagelliforme* (*Nostoc flagelliforme*), lives in the terrestrial environment in areas with sandy soil.[39, 40, 41]

1.7 Commercial Utilization of Algae

The earliest utilization of algae was probably as food for humans and animals.[32] There are historical references to the use of algae as human food in China as early as 600-800 BC.[42]

Marine algae (seaweeds) are eaten for their food value, flavors, colors and textures and are typically combined with other types of food.[42] For example, Arame is eaten in

soups or taken with soya-sauce in Japanese food.[43] Kelps, which are represented by *Laminaria spp.*, are also used as a food product. Nori is an ingredient for the 'sushi' in Japanese food. Analyses of certain edible seaweeds show that many contain significant amounts of protein, vitamins and minerals essential for human nutrition.[42] Some ingredients in seaweeds are extracted for use in pharmaceuticals. Kelps are rich in iodine and they were once the major source of iodine in the drug industry.[32, 42] Seaweeds are also used as fertilizers, as food products for other animals, and for the production of agar-agar which is used extensively for culturing bacteria and fungi in the laboratory.[32, 42] One of the terrestrial algae, *Nostoc*, is used as food product in China.[32, 39, 40]

The uses of algae have led to its large scale commercial cultivation in Japan, the Philippines and China.[42]

1.8 Analytical Methods

1.8.1 Hydride Generation Atomic Absorption Spectrometry (HGAAS)

AAS is a common detection method used in trace analysis, because of its elemental specificity. Hydride generation techniques coupled with atomic spectrometry have been widely used in trace analysis.[44] The system consists of a hydride generation unit in which the sample, often acidified, and the reducing reagent react and hydrides are generated.[44, 45] The reducing reagent can be Zn-HCl or the more commonly used $NaBH_4$.[44, 46] Typically, a flow of inert gas, N_2 or Ar, transports the metallic hydrides into a quartz tube, where the flame (air/H_2 or air/acetylene) atomizes the hydrides.[44, 45] The use of hydride generation

has been found to enhance the sensitivity by 10-1000 fold over the more commonly used liquid sample nebulization procedures.[44] The limitation of this method with respect to arsenic analysis is that it can be applied only to hydride-forming species, such as arsenite, arsenate, MMAA and DMAA. For some environmentally important species such as arsenobetaine, arsenocholine and arsenosugars, which do not form hydrides by reaction with borohydride, modification of the method must be made. Chromatography systems can also be coupled to this system for qualitative analysis of one or more arsenic species.[47]

1.8.2 Chromatographic Methods - High Performance Liquid Chromatography (HPLC)

Liquid chromatography is a widely used technique for separating non-volatile compounds. In arsenic speciation, the commonly used chromatography methods include ion-pair chromatography, ion chromatography and gel permeation chromatography.[9] Anion and cation chromatographic methods have been used to separate inorganic and simple methylated compounds. The separation is based on the different interactions between the ionic species and the stationary phase bonded with oppositely charged ions to the ionic analytes.

Ion-pair chromatography is a common technique applied to arsenic speciation. In this technique, the separation of ionic solutes on a lipophilic stationary phase (typically a C_{18} column) can be achieved by the addition of an ion-pairing reagent to the eluent. The ion-pairing reagent contains a lipophilic component (usually a quaternary amine for anion ion-pairing or an alkylsulfonate for cation ion-pairing) having an opposite charge to that of the solute ions. One theory of the mechanism of ion-pair chromatography is based on the

hydrophobic interaction between the stationary phase and the target ionic compound-hydrophobic pairing ion complex. A more recent theory involves the concept of 'ion interaction' implying an interaction resulting from Coulombic and other forces that impose on an analyte an affinity for the mobile phase or the stationary phase. In this model, the ion-pairing reagent is adsorbed evenly over the stationary phase to create a charged primary layer, to which is attracted a secondary layer of opposite charged counter-ions (sample ions). Transfer of solute through this 'electrical double layer' to the stationary phase is a function of electrostatic effects for retention in reverse phase chromatography. The actual mechanisms are dependent on some variables, such as the length of the alkyl chain in the ion-pairing reagent and pH.[48, 49]

1.8.3 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

The application of plasma as an ionization source for the analysis of biological and biomedical materials has grown with the development of inductively coupled plasma (ICP), direct current plasma (DCP) and microwave induced plasma (MIP). Nowadays, ICP sources continue to be widely used in trace metal analysis.[50] The advantages of ICP include good excitation and ionization of metals and metalloids, an ability to cope with matrix effects of the samples, low detection limits, and excellent accuracy.[9, 50]

The ICP is formed in a quartz torch made from three concentric tubes supplied with a flow of argon at one end and open at the other end. A water cooled induction coil (0.5 mm-1 mm diameter) is wrapped around the torch. Power is applied to the torch via the coil by using a radiofrequency (RF) generator that operates at 27 or 41 MHz.

Ionization of the flowing argon is initiated by a spark from a Tesla coil. The Ar^+ ions, and their associated electrons, then interact with the fluctuating magnetic field produced by the induction coil. The Ar^+ ions accelerated by the RF field transfer energy to the entire gas by collisions between atoms. The inductive heating of the flowing gas maintains the plasma 'burning' at temperatures of 6 000-10 000 K. Samples are introduced to the plasma via the nebulizer. Only 1% of the sample actually reaches the ICP and 99% of the sample drains out as waste. The ICP heats up the sample, and desolvation, dissociation, atomization and ionization take place.[51, 52, 53, 54]

ICP is often coupled with atomic emission spectrometry (AES) or mass spectrometry (MS). ICP-AES has a wider linear dynamic range than typical AAS, and has less severe chemical interferences. However, spectroscopic interferences still exist.[9] By comparision, ICP-MS does not have as many spectroscopic interferences as ICP-AES. Instead of monitoring particular wavelengths, specific mass to charge ratios (m/z) are monitored, enabling isotopic determination. Other advantages include simple spectra, even for complex matrices, superior detection limits and wide linear dynamic ranges. However, compared with ICP-AES, ICP-MS is less tolerant of the presence of large amount of salts in the sample.[9, 53] Mass interferences remain problematic for some particular elements.[50]

ICP operates at atmospheric pressure and a temperature of 7 000 K, whereas MS operates at lower than 10^{-5} torr and 300 K. Coupling between ICP and MS is achieved by using a sampling cone and a skimmer cone.[52] Sample ions created in the plasma are extracted through orifices, with diameters of about 0.5 mm to 1 mm in shallow water

cooled cones.[51, 52] A fraction of the ions is sampled through the differentially pumped region at about 1 torr, and enters the ion optics.[54] The ion lenses focus the ions through a final aperture into the quadrupole. A mass scan is accomplished by varying the amplitude of RF and DC voltages while keeping RF/DC ratio constant. The signals from the detector are collected and processed.[51, 53]

1.9 Objectives of the Thesis

The objectives of the thesis are twofold: arsenic speciation of commercial algal food products available in retail markets (Chapter 3), and determining the seasonal changes in arsenic speciation in the tips, which contain the reproductive organs, and the remainder of *Fucus sp.* (Chapter 4).

The techniques used involved some modifications of two previously developed methods, HGAAS for quantitative analysis and HPLC-ICP-MS for qualitative analysis. The detailed procedures are described in Chapter 2.

This project is a study of organometallic chemistry in the environment. The results add to our understanding of the biogeochemistry of arsenic, an important element in the environment. The results also add to our knowledge of arsenic species present in commercially available algal products.

2. Experimental

2.1 Instrument

2.1.1 High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometry (HPLC-ICP-MS)

The HPLC system consisted of a Waters Model 510 delivery pump, a Reodyne Model 7010 injector valve with a 20 μL sample loop, and an appropriate column. The columns used included two reverse phase C_{18} columns (GL Sciences Inertsil ODS, 250 mm x 4.6 mm; and Phenomenex Inertsil 5 ODS-2, 250 mm x 4.6 mm). A guard column packed with the same material (Supelco) was used preceding the analytical column. The HPLC system was connected to the ICP nebulizer via a PTFE tube (2.5 cm) and appropriate fittings.

A VG Plasma Quad 2 Turbo Plus inductively coupled plasma-mass spectrometer (VG Elemental, Fisons Instrument) equipped with a SX 300 quadrupole mass analyzer, a standard ICP torch, and a de Galan V-groove nebulizer was used as the detector. The mass analyzer was operated in time resolved analysis (TRA) mode. Signals at m/z 75 corresponding to As^+ were monitored and data were transferred to the VG data system. The TRA mode operation allowed for the possibility of simultaneous monitoring of more than one m/z at a time. All signals were collected and the data were transferred to the computer. The data were then exported to a Microsoft Excel 5.0 program for further processing. Figure 2.1 shows the schematic diagram of the HPLC-ICP-MS system and Table 2.1 shows the commonly used operating parameters.

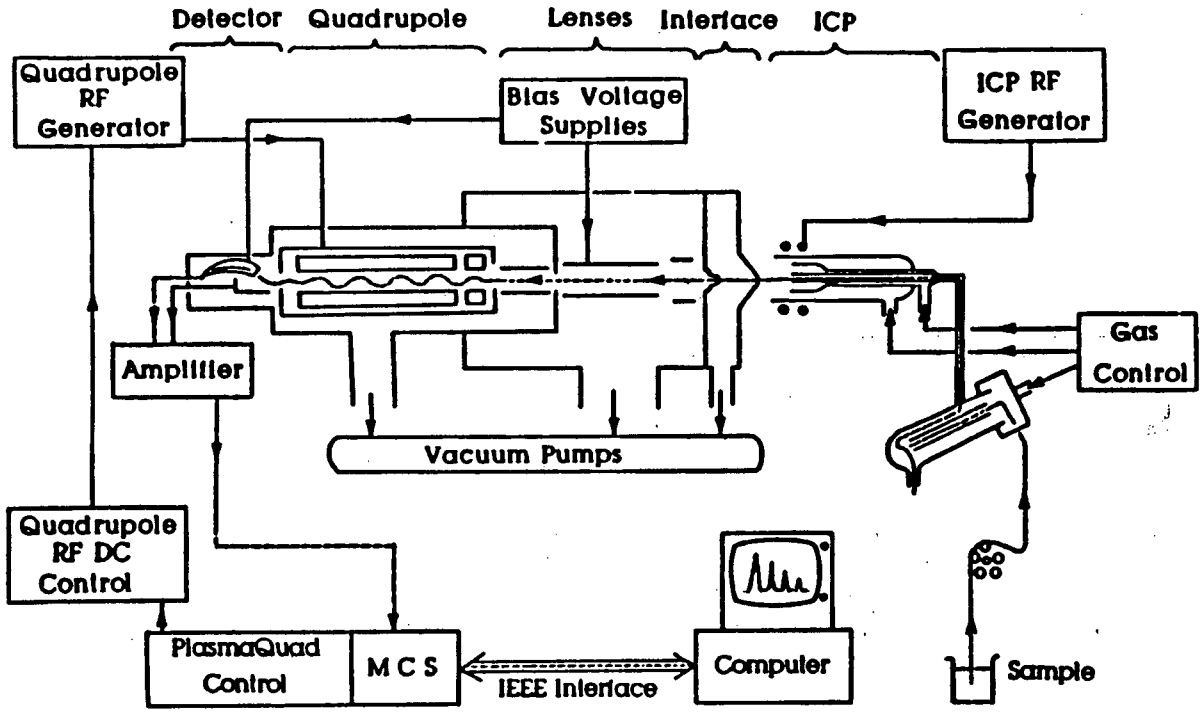


Figure 2.1 The Schematic Diagram of ICP-MS (VG Elemental, Fisons Instrument)[55]

Table 2.1 Operating Paramaters of ICP-MS

Forward r.f. power	1350 W
Reflected power	<10 W
Outer (cooling) gas flow rate	13.8 L/min
Intermediate (auxiliary) gas flow rate	0.65 L/min
Nebulizer gas flow rate	1.002 L/min
Analysis mode	TRA, 1 sec time slice
Quadrupole pressure	9×10^{-7} mbar
Expansion pressure	2.4 mbar

2.1.2 Hydride Generation Atomic Absorption Spectrometry (HGAAS)

Atomic absorption measurements were performed with a Jarrell Ash 810 atomic absorption spectrometer equipped with a hydrogen-air flame atomizer. A spectral band width of 1 nm and a wavelength of 193.7 nm were used. Background correction was not available. A Hewlett Packard 3390A integrator was connected to the AA spectrometer to record signals. An open-ended T-shaped quartz absorption tube (optical path: 9.5 cm x 0.8 cm i.d.) was mounted in the flame of the burner. Light from the arsenic hollow cathode lamp (Varian) was aligned to pass through the wide end of the flame-heated quartz tube. Hydrides from the reaction were introduced through the side arm of the quartz tube (9 cm x 0.4 cm i.d.).

A peristaltic pump (Cole-Parmer) was used to introduce the sample, acid and $NaBH_4$ solutions into the system. Table 2.2 shows the operating parameters of the system.

Table 2.2 Operating Parameters of HGAAS

Gas	Conditions
Nitrogen (Carrier Gas)	90 mL/min
Nitrogen (Drying Gas)	90 mL/min
Hydrogen (Flame)	20 scfh (recommended by the operation manual of the AAS)
Air (Flame)	8 scfh (recommended by the operation manual of the AAS)
Sample, Acid, $NaBH_4$	4 mL/min

A combined hydride-generating/gas-liquid separating apparatus as shown in Figure 2.2 was used in the system.[44] This glass apparatus consisted of an inner Buchner funnel and an outer glass cylinder with a side arm for waste. Carrier gas (N_2, Table 2.2) was connected to continuously flow through the fritted disc of the inner Buchner funnel and hydrides formed were carried into the quartz tube in the flame. The excess liquid from the reaction overflowed from the inner funnel to the outside of the cylinder.

A commercial Nafion tube (Perma Pure Inc.) was used to dry the hydrides before they reached the quartz tube: nitrogen (Table 2.2) was used as the carrier gas. Figure 2.3 shows a schematic diagram of the system.

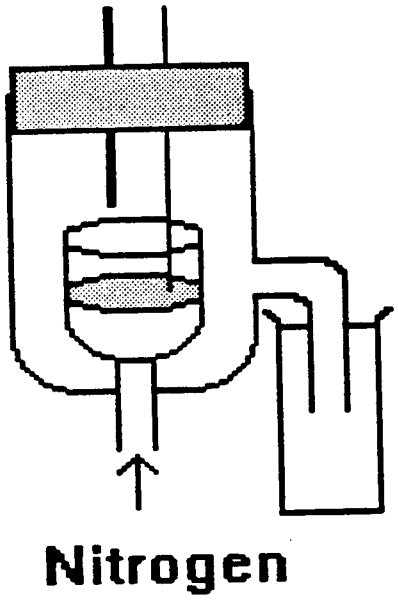


Figure 2.2 Gas-Liquid Separator of HGAAS[44]

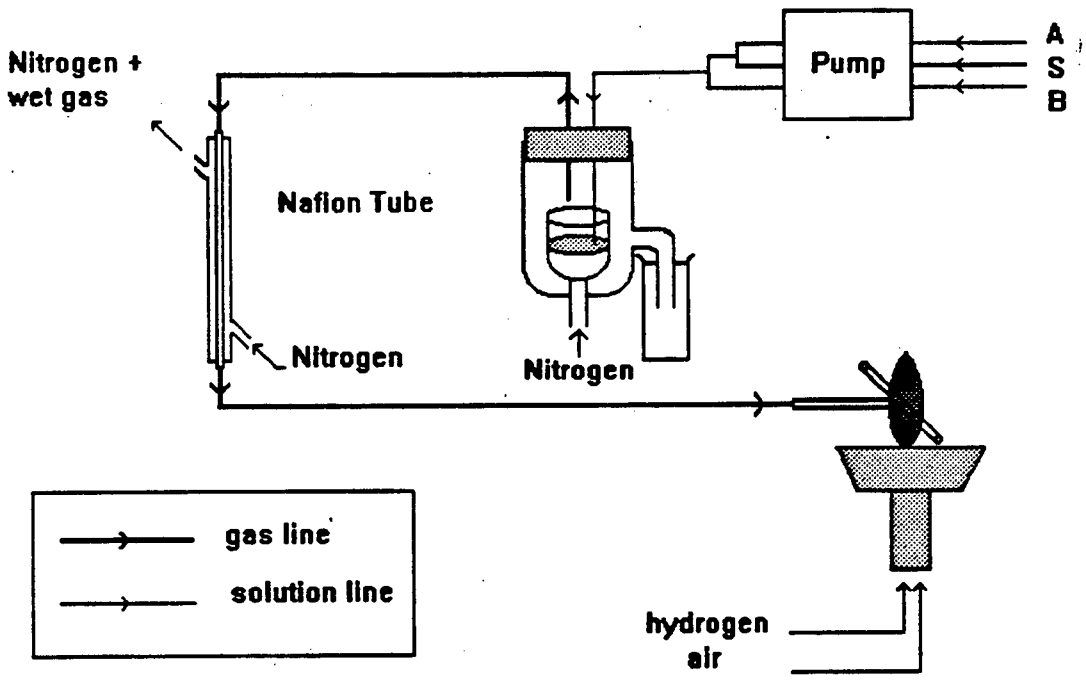


Figure 2.3 The Schematic Diagram of HGAAS (A: acid, B: $NaBH_4$, S: sample)

2.2 Reagents and Chemicals

All chemicals used were of analytical grade unless otherwise stated. The chemicals used included methanol (HPLC grade, Fisher), tetraethylammonium hydroxide (20 wt%, Aldrich), tetrabutylammonium hydroxide (Aldrich), malonic acid (BDH), sodium borohydride (Aldrich), hydrochloric acid (36.5%, Fisher), phenol (Fisher), diethyl ether (ACS reagent grade, Fisher), sulfuric acid (98%, Fisher), nitric acid (69%, sub-boiling distilled, Seastar Chemicals) and hydrogen peroxide (30%, Fisher).

Arsenobetaine (AsB) was prepared from trimethylarsine.[26] Standard solutions of arsenobetaine and dimethylarsinic acid (DMAA) (Aldrich) were prepared in deionized water.

The water used to make up the eluent for HPLC, sample extraction and preparation was deionized using a deionizer with resistivity better than 1 MΩ. The eluent for HPLC was adjusted to pH 6.8 by using 3 M HNO_3 solution and filtered through a millipore 0.45 μm filter. All samples analyzed by using HPLC were prefiltered through a 0.45 μm filter.

The glassware and plasticware were cleaned by soaking in 2% Extran solution overnight, rinsing with water and deionized water, and soaking in 0.1 M HNO_3 solution overnight. They were then rinsed with deionized water and air-dried.

2.3 Samples

2.3.1 Sample Collection

Fucus samples were collected at Brockton Point, Stanley Park, Vancouver B.C., at mid tide level in July 1995, February 1996 and May 1996. Commercial algal products were purchased at food stores in Vancouver, B.C., Richmond, B.C., Guangzhou, China as well as Hawaii. They consisted of products from Japan, China, N.B., Canada and B.C., Canada. Oyster tissue standard reference material (SRM NIST 1566a) was purchased from the National Institute of Standards and Technology, U.S. Department of Commerce.

2.3.2 Sample Treatment prior to Storage

Fresh *Fucus gardneri* collected in July 1995 was separated into two different batches, the swollen tips (reproductive organs) and the remainder.

Fresh *F. gardneri* collected in February 1996 was firstly sorted into two subsamples, the whole young *Fucus* without observable conceptacles (minute openings) on the tips (Section 1.6), and the whole mature *Fucus* with observable conceptacles on the tips. Each subsample was further separated into two batches, the tips, and the remainder.

F. gardneri collected in May 1996 was separated into three batches. Batch 1 contained the whole young *Fucus* without swollen tips, batch 2 contained the swollen tips of mature *Fucus*, and batch 3 contained the remainder of mature *Fucus*.

Fucus samples were stored at 4°C prior to separation into the tips and the remainder. After they were separated into different batches, they were kept frozen and then freeze-dried prior to analysis.

2.3.3 Storage

The freeze-dried samples were kept frozen until further analysis. Commercial products were kept in their packages in a cool area in the laboratory.

2.4 Sample Preparation

2.4.1 Extraction

2.4.1.1 MeOH/H_2O

The commercial products Nori (*Porphyra tenera*), Dulse (*Palmaria palmata*), Arame (*Eisenia bicyclis*), *Laminaria sp.* from China, Bull Kelp (*Nereocystis leutkeana*), Kombu (*Laminaria groenlandica*), dried *Nostoc* (*Nostoc commune var flagelliforme*), freeze-dried *Fucus gardneri*, as well as oyster tissue SRM (0.5-1 g dry weight) or fresh *F. gardneri* (5-10 g wet weight), each in duplicate, were extracted by using a procedure similar to that described by Shibata and Morita.[56] Each sample was weighed into a 15 mL or 50 mL centrifuge tube. To each tube was added 10 mL/g dry sample or 2 mL/g wet sample of a methanol/water mixture (1:1, v/v). The tube was sonicated for 10 min and centrifuged for 10 min. After centrifugation, the extract was removed by using a Pasteur

pipette and placed in a round bottom flask. The extraction procedure was repeated an additional four times for each sample. The combined extract was evaporated to dryness and then dissolved in 10 mL of deionized water prior to further analysis.

2.4.1.2 Phenol-diethyl ether Extraction

In order to probe the possible presence of arsenobetaine in *Fucus* and the effect on the arsenic compounds by this particular combination of solvent, a phenol-diethyl ether extraction was performed. A sample of the remainder of *Fucus* collected in July (10 g wet weight) was extracted by the procedure described in section 2.4.1.1. From the resulting 10 mL extract, 7 mL were taken for phenol extraction as described by Cullen and Nelson.[57] The 7 mL extract after MeOH/H_2O extraction was further extracted with phenol (2 x 50 mL). The combined phenol extracts were diluted with diethyl ether (400 mL) and the water soluble compounds were back-extracted into deionized water (2 x 50 mL). The aqueous extracts were combined and evaporated to dryness and redissolved in deionized water (5 mL).

2.4.1.3 Chromatographic clean-up of wet *Fucus*

One sample from the extraction of *Fucus* tips collected in July (10 mL extract) was evaporated to dryness by a rotary evaporator. It was then dissolved in a minimum amount of deionized water (1 mL). The sample was then applied to a gel permeation column made of sephadex LH-20 in a 30 cc plastic syringe (3 cm x 10 cm). The column was eluted with methanol. The eluent was collected until a total of 100 mL of methanol had

been applied to the column. The combined eluent was evaporated to dryness and dissolved in 10 mL of deinoized water prior to HPLC analysis. A second extract from another sample of the tips of *Fucus* was not applied to the Sephadex column as a comparison.

2.4.2 Acid Digestion of Samples

Dry samples (0.25 g-0.5 g), wet samples (2.5 g), or samples after extraction (0.5 g original for dry samples, 5 g original for wet samples) were placed in a 250 mL or 500 mL round bottom flask. A glass condenser was fitted to the round bottom flask, as shown in Figure 2.4. This set-up was similar to those described by Bajo *et al.*[58]

An acid mixture of H_2SO_4:HNO_3:H_2O_2 (1:3:3, v/v/v) with a total volume of 7 mL was added to each sample.[59] After refluxing for 2 hours[60], the reaction mixture was cooled and made up to 25 mL or 50 mL until further analysis.

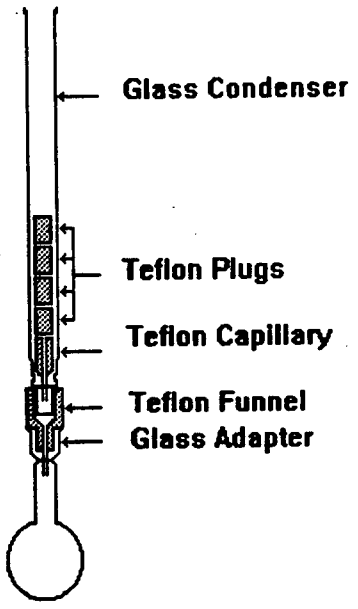


Figure 2.4 The set-up of the Total Digestion Apparatus

2.5 Analytical Procedures

2.5.1 HPLC-ICP-MS

The HPLC-ICP-MS system consisted of two parts, the HPLC used for separation of arsenic compounds and the ICP-MS used for element-specific detection. The ODS and ODS-2 columns were equilibrated with the eluent for one hour prior to analysis. Table 2.3 shows the experimental conditions of HPLC.

Table 2.3 Summary of Experimental Conditions (HPLC)

Conditions		Column	Mobile Phase	Flow Rate (mL/min)
(1)	(a)	Inertsil ODS (GL Sciences, Japan)	10 mM tetraethylammonium hydroxide (TEAH), 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	0.8
	(b)	Inertsil ODS (GL Sciences, Japan)	10 mM tetrabutylammonium hydroxide (TBAH), 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	1.0
(2)	(a)	Inertsil 5 ODS-2 (Phenomenex)	10 mM tetraethylammonium hydroxide (TEAH), 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	0.8
	(b)	Inertsil 5 ODS-2 (Phenomenex)	10 mM tetrabutylammonium hydroxide (TBAH), 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	1.0

Filtered samples (20 μL) were injected into the HPLC column. The solution which passed through HPLC entered the nebulizer via the PTFE tubing and connection. The m/z=75 signal peak corresponding to As^+ generated from each arsenic compound at a given time was recorded as retention times of the chromatogram. Arsenic compounds in the samples were identified by matching the retention times of the peaks in the chromatograms with those of standards.

2.5.2 HGAAS

The samples after total digestion were diluted and then analyzed by using HGAAS. The blank solution was made up with appropriate amounts of H_2SO_4, H_2O_2 and HNO_3 in order to resemble the acidic condition of the samples after total digestion and dilution. Standard arsenate (As(V)) solutions were also made up in a similar way. After acid digestion of the samples, the only arsenic species should be the oxidized form, As(V).[58] Therefore, the use of As(V) solution as calibration was appropriate to the experiment.

To determine the total arsenic in the marine samples before extraction, 3 M HCl and 3% $NaBH_4$ (in 0.1 M NaOH) were used as the reagents at the optimized concentrations obtained by Le. [44] The samples, after a further tenfold dilution contained approximately 0.3 M acid. For the determination of samples that contain lower arsenic concentrations as found in *Nostoc* and all samples after extraction, the tenfold dilution step was omitted and a lower acid concentration (1.5 M HCl instead of 3 M HCl) was used. Figure 2.5 shows the hydride generating reaction.

$$BH_4^- + AsO_4^{3-} + 4H^+ \rightarrow AsH_3 + H_2O + H_3BO_3$$

$$BH_4^- + 3H_2O + H^+ \rightarrow H_3BO_3 + 4H_2$$

Figure 2.5 Formation of Hydride

The samples were aspirated (Figure 2.3) for 30 seconds resulting in the uptake of approximately 2 mL of sample in each analysis. Triplicate analyses were performed. The hydrides formed in the gas-liquid separator were purged by the carrier gas (N_2) to pass through the Nafion tube. The Nafion tube (Figure 2.3) consists of an inner microporous PTFE tube and an outer tube fitted with two side arms. The outlet of the gas/liquid separator was connected to the inner PTFE tube. Drying gas (N_2) was connected to the side arms to enable a flow of nitrogen to purge in the opposite direction outside the inner PTFE tube. The water from the reaction mixture diffused into the flow of drying gas through the micropores in the PTFE tube and the drying gas carried the water to the exhaust. It should be noted that the Nafion tube and the tubings preceding the quartz tube needed to be cleaned after 15 to 20 runs because of clogging from the accumulated water. The assembly of the Nafion tube was easy so that frequent cleaning up of the Nafion tube did not affect the overall efficiency of the system. Dry hydrides were carried by the carrier gas to the quartz tube in the flame. Detailed experimental parameters are listed in Table 2.2. The hydrides were then atomized by the flame and the signals were recorded continously. Both peak height and peak area of signals were measured. The standard deviations from these two sets of data were compared to decide which would be used for quantification.

3. Arsenic Speciation in Commercial Algal products

3.1 Introduction

Marine animals (seafood) and marine algae (seaweeds) are popular food for humans. Marine algae have been used for food products by humans for centuries. In times of famine associated with failure or destruction of terrestrial food crops, seaweeds have often been eaten by coastal inhabitants to ward off starvation.[42] Nowadays, seaweeds have become a popular food since they contain large amounts of protein, vitamins and carbohydrates which are relevant to health.[61] In studies of arsenic chemistry, marine animals and marine algae are common subjects for speciation analysis since they generally contain high amounts of arsenic. Arsenobetaine has been found in marine animals, such as lobster and fish, in previous studies. Arsenosugars have been found in a variety of marine algae. It has been suggested that the arsenobetaine found in the consumers, such as marine animals, is formed from arsenosugars present in the primary producers, marine algae.[62]

The relative amounts of different arsenosugars vary from one algal species to another. Arsenosugar XII has been found to be the major arsenic compound in brown algae, *Laminariales*.[20] Arsenosugars X and XI have been found to be major arsenic compounds in Nori (*Porphyra tenera*).[63] Inorganic arsenicals and simple methylated arsenicals are believed to be the precursors to arsenosugars in the environment.[8, 28]

Very few chemical species of arsenic have been found in freshwater organisms.[25] The arsenic species found in terrestrial plants have previously been limited to simple methylated arsenic species. Recently, mushrooms have been found to contain

arsenobetaine[24], a form which had previously been found only in marine animals. However, most studies tended to suggest that the arsenic cycle in freshwater systems might be different from that in marine systems.

All the algae investigated in the first part of this study are commercial food products available in retail markets from a wide range of locations in the world, ranging from B.C., Canada, N.B., Canada, Japan and China. They constitute of a variety of marine algae in addition to *Nostoc* sp., a terrestrial blue-green alga. It was anticipated that detailed study of these samples could give a better understanding of the arsenic cycle in the environment as well as the relationships between different algal species, different arsenic compounds, and different geographical locations.

3.2 Results and Discussions

3.2.1 Qualitative Analysis by HPLC-ICP-MS

3.2.1.1 Analysis of a Standard Reference Material

One major problem of arsenic speciation in environmental samples is the absence of pure organoarsenic compounds as standards. Many of the organoarsenic compounds important in environmental and biological systems (Table 1.1) are not commercially available. This problem was partially solved by the use of oyster tissue NIST 1566a as the standard reference material for the analysis. Figure 3.1 shows a chromatogram of NIST 1566a oyster tissue obtained in the present study with the use of TEAH as ion-pairing reagent and the ODS column (condition 1a, Table 2.3). The major arsenic compounds

AsB, DMAA, arsenosugars X and XI present in this standard reference material were previously identified by Shibata *et al.*[56] A sample from the same batch that was used locally was sent to Dr. Y. Shibata for analysis and he confirmed the assignments as shown in Figure 3.1.[64] Thus in the absence of pure AsB, DMAA, arsenosugars X and XI standards, this standard reference material can be adopted for identification purposes and Figure 3.1 was used as one of the standard chromatograms for the present study.

Previous studies of arsenic speciation in marine algae showed that arsenosugars X-XV (Table 1.1) were present in a number of algal species. It would be very useful to find another commercially available product, which contains more arsenosugar species, that can be used as a standard reference material. Figure 3.2 shows the chromatogram of a commercially available food product, kelp powder, under condition 1a (Table 2.3). It was found that it contains four major peaks; arsenosugars X and XI accounting for two of them. By comparing the relative retention times of the peaks with the chromatograms by Shibata *et al.*,[9] arsenosugars XII and XIII were also identified in this sample. *A* sample from the same batch was sent to Dr. Y. Shibata's group and the identification of arsenosugars X, XI, XII and XIII were confirmed by Dr. J. Edmonds and Dr. Y. Shibata who ran samples of the kelp powder extract against their standards by using HPLC-ICP-MS (Asahipak GC-220 HQ gel permeation column).[65]

Figure 3.3 and Figure 3.4 show the chromatograms of the oyster tissue SRM and kelp powder using TEAH as ion-pairing reagent and the ODS-2 column (condition 2a, Table 2.3). It was found that the elution orders of the peaks are similar to Figure 3.1 and Figure 3.2 respectively; however, condition 1a (Table 2.3) gave a better separation of the

peaks. Arsenosugars XII and XI co-elute under condition 2a. The differences in the separation can be related to the difference in the structure of the two columns. The ODS column, having a slightly larger carbon load and surface area,[66] is superior in ion-pair chromatographic separation of organoarsenic compounds.

Figure 3.5 and Figure 3.6 show the chromatograms of the oyster tissue SRM and kelp powder using TBAH as the ion-pairing reagent and the ODS-2 column (condition 2b, Table 2.3). Arsenic compounds were identified by matching the retention times with those of standard AsB and DMAA solutions and comparing the relative peak areas and peak heights of the peaks obtained under the two conditions, 2b and 1a. When using condition 2b, AsB co-elutes with arsenosugar X and DMAA co-elutes with arsenosugar XI. Arsenosugars XII and XIII are well separated. This system can be used as a complement for condition 2a. This HPLC condition is particularly useful when arsenosugars XII and XI are not separated on a particular column when TEAH is used as the ion-pairing reagent.

The separation obtained by using TBAH as ion-pairing reagent and the ODS column (condition 1b, Table 2.3) is very similar to that obtained using condition 2b. The results seen in Figures 3.1 to 3.6 and the discussion above reveal that the choice of column is very critical when TEAH or TBAH is used as the ion-pairing reagent. TBAH, being more hydrophobic than TEAH, may increase the capacity factor of the chromatography.[49] The application of a more hydrophobic ion-pairing agent will delay the elution of some compounds. As a result, arsenosugars are spread out more widely in the chromatogram as can be seen in Figure 3.6. When TBAH is used as the ion-pairing reagent, the peak shapes

of the late-eluting compounds, such as arsenosugars XII and XIII, are relatively broad. A slightly faster flow rate (1.0 mL/min) improves peak shapes when TBAH is used as eluent.

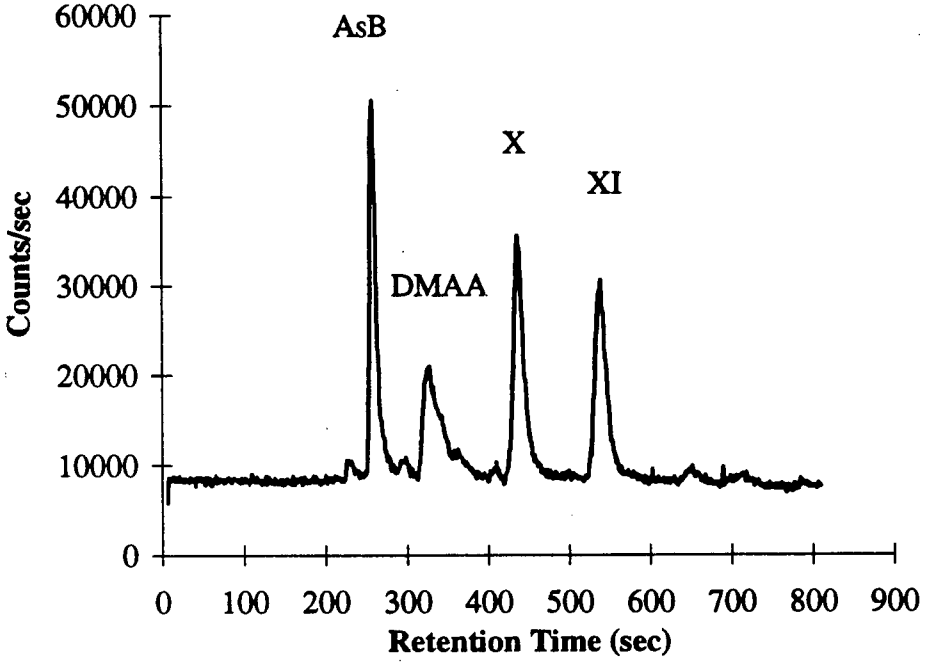


Figure 3.1 A Chromatogram of Oyster Tissue SRM, condition 1a

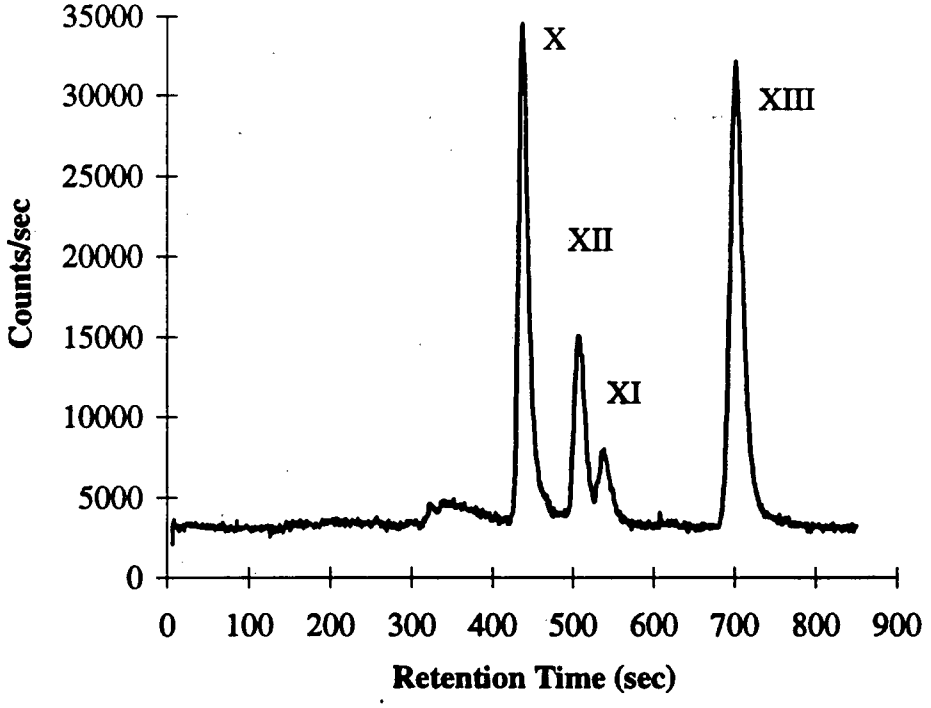


Figure 3.2 A Chromatogram of Kelp Powder, condition 1a

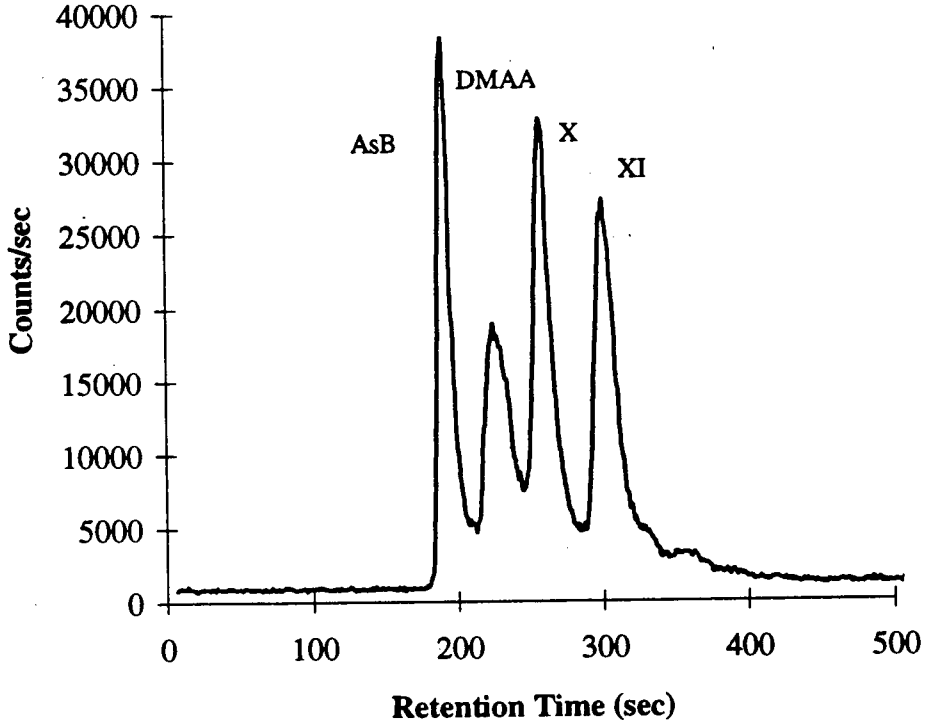


Figure 3.3 A Chromatogram of Oyster Tissue SRM, condition 2a

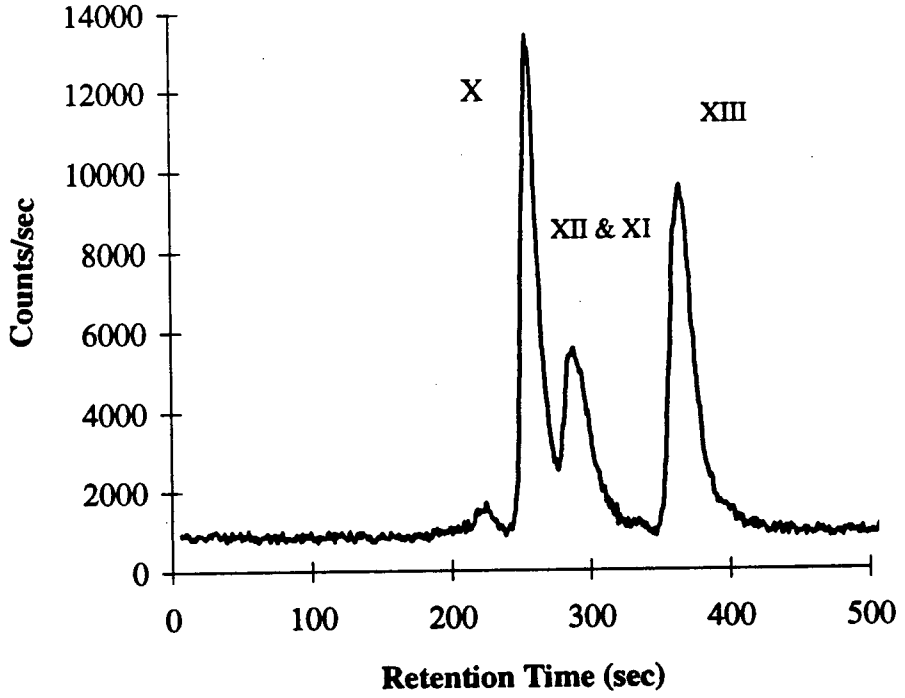


Figure 3.4 A Chromatogram of Kelp Powder, condition 2a

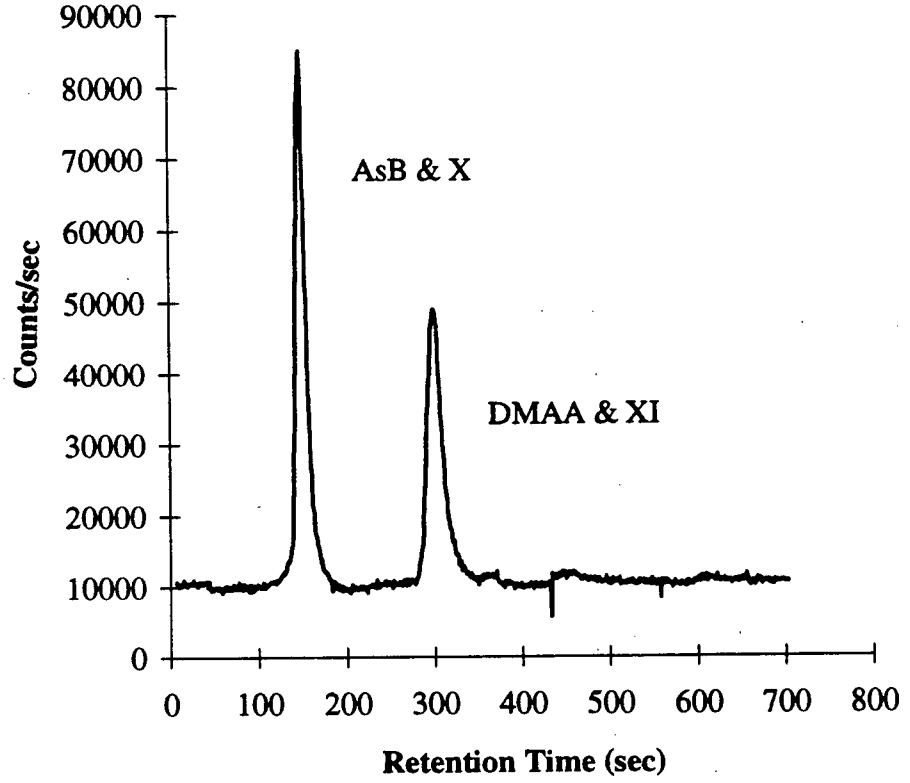


Figure 3.5 A Chromatogram of Oyster Tissue SRM, condition 2b

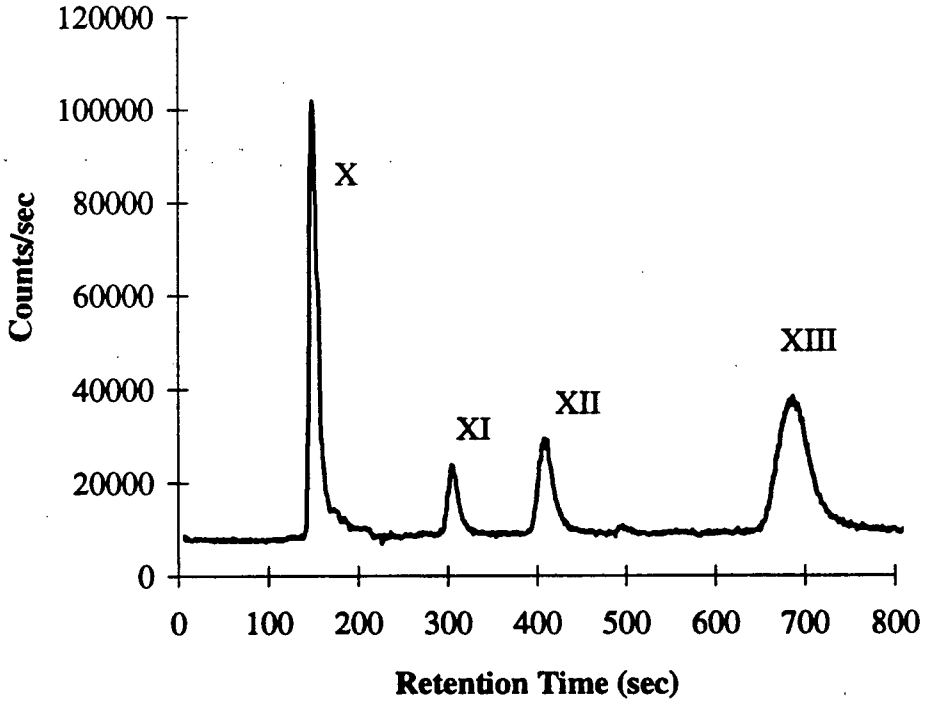


Figure 3.6 A Chromatogram of Kelp Powder, condition 2b

3.2.1.2 Analysis of Marine Red Algae

The marine red algal products, Dulse from N.B., Canada and Nori from Japan, Taiwan and China, were extracted and filtered as previously described in Sections 2.4.1.1 and 2.2. No further separation methods were applied prior to HPLC-ICP-MS analysis. The samples were analyzed by using HPLC-ICP-MS as described in Section 2.5.1.

The predominant arsenicals found in the extracts of these samples are arsenosugars X and XI. The relative amounts vary widely. Other arsenosugars such as XII and XIII which were found in the kelp powder, a brown algal species, were not found in these red algal samples. A previous study of Nori by Shibata *et al.*[63] showed that arsenosugars X and XI were present in Nori from Japan although arsenosugar XI was the major species.[63] The wide variation of relative amounts of arsenosugars X and XI in the samples could result from the decomposition of arsenosugar XI to X during storage (even at - 20°C) and extraction.[63] This hypothesis is strengthened by the observation during the present investigation of the disappearance of arsenosugar XI from an extract of the oyster tissue SRM after a 10-month storage at a temperature below 4°C and by the decomposition of arsenosugar XI in the Nori extract to arsenosugar X after a 30-month storage. These samples were stored in the same environment as used during the present study. Previous studies also indicated that arsenosugars could be decomposed to DMAA at extreme pH values.[67] These phenomena make precise quantification of individual arsenosugars suspect because the final extracts may not represent the original proportions of the arsenic compounds in the algae. With this caveat in mind, Table 3.1 shows the relative peak areas of the arsenic compounds in the chromatograms obtained from the extracts of red algae.

Table 3.1 Relative Amounts of Arsenicals in Marine Red Algae

Algae	DMAA	X	XI
Dulse	5.8%	87.8%	6.4%
Nori (Japan)	-	82.0%	18.0%
Nori (China)	-	58.1%	41.9%
Nori (Taiwan)	2.1%	69.8%	28.1%

Until now, there is no information about how the seasonal changes and the food processing procedures affect the relative amount of the arsenosugars in commercial algal products.[27, 63]

3.2.1.3 Analysis of Marine Brown Algae

Four different brown algal species, Bull Kelp (*Nereocystis leukeana*) and Kombu (*Laminaria groenlandica*) from B.C., Canada, Arame (*Eisenia bicyclis*) from Japan and *Laminaria* sp. from China, were analyzed after extraction and filtration (Sections 2.4.1.1 and 2.2).

Figure 3.7 and Figure 3.8 show the chromatograms of Kombu and Bull kelp under condition 1a, and Table 3.2 shows the relative peak areas of the arsenic compounds in the chromatograms from the extracts of all four brown algal species. Arsenosugar XII was found to be the major arsenical in the extracts of all four algal species. The relative amounts of other arsenic compounds, DMAA, arsenosugars X and XI, vary depending on the algal species. The DMAA content in Arame is relatively high when compared to other

brown algae. As previously mentioned in Section 3.2.1.2, the decomposition of arsenosugar XI to arsenosugar X or DMAA is one possible explanation for the variations of relative amounts of those three arsenic compounds.

The *Laminaria* sp. from China and Arame from Japan are from locations remote from B.C., Canada. Both algal species contain lower amounts of arsenosugar XI than do the algal species from B.C. Differences in the relative amounts of each arsenic compound can be seen in four algal species. However, there is not enough evidence to show how geographic variations, food processing, and storage affect arsenic speciation.[27, 63]

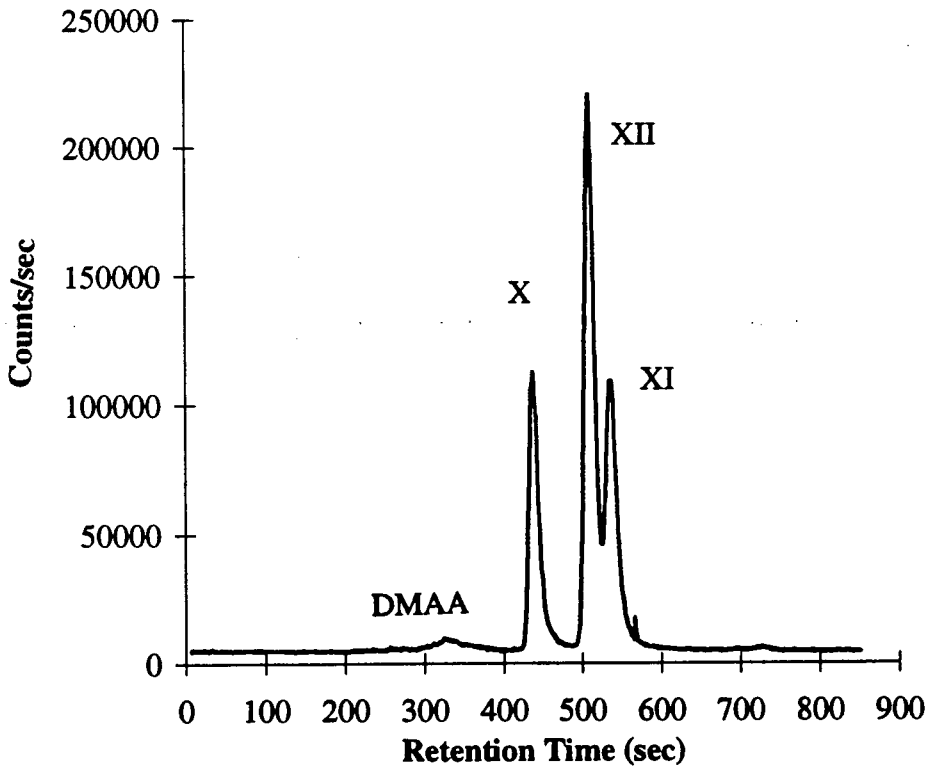


Figure 3.7 A Chromatogram of Kombu, condition 1a

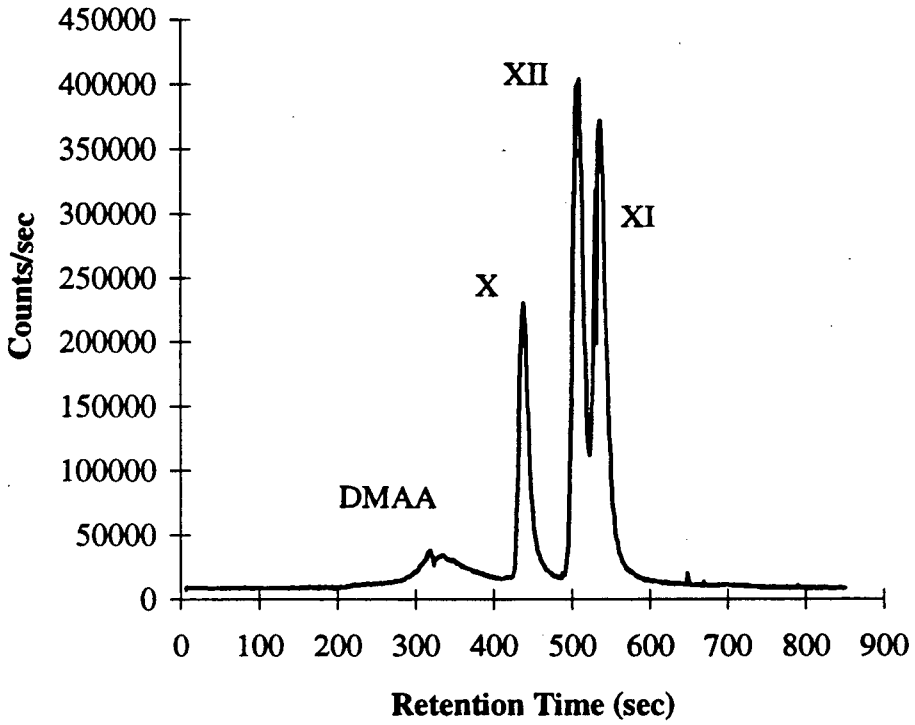


Figure 3.8 A Chromatogram of Bull Kelp, condition 1a

Table 3.2 Relative Amounts of Arsenicals in Marine Brown Algae

Algae	DMAA	X	XII	XI
Bull Kelp	0.6%	19.7%	53.8%	25.9%
Kombu	1.7%	23.5%	60.2%	14.6%
Laminaria sp.	1.5%	16.5%	77.5%	4.5%
Arame	11.8%	17.8%	67.5%	2.9%

3.2.1.4 Analysis of a Terrestrial Blue-Green Alga

There are few studies of the arsenic speciation in edible terrestrial algae when compared with the volume of work done on marine algae. Previous work by Maeda[23, 25] showed that dimethylarsenic compounds were present in aquatic plants. In one study of arsenic metabolism in a freshwater food chain, *Nostoc* sp., a blue-green alga, was found to accumulate inorganic arsenic species present in the medium as dimethylarsenic compounds.[25] Apart from this work the chemical structures of arsenic in the freshwater plants are essentially unknown.

The present study involves arsenic speciation in *Nostoc commune var flagelliforme* purchased from three different locations (Hawaii, Richmond, B.C. and Guangzhou, China). Figure 3.9 and Figure 3.10 show the chromatograms of the extract of one of the samples (from a market in Hawaii) under conditions 2a and 2b (Table 2.3). It can been seen that it contains arsenosugar X, an arsenic compound which was previously found only in marine environments, as the major compound (93.6%); DMAA is also present as a minor compound (6.4%). Samples from other markets in Canada and China were also found to contain arsenosugar X as the major arsenical.

The discovery of arsenosugar X in a terrestrial organism is important for our understanding of the arsenic cycle in freshwater environments. The presence of the arsenosugar in the terrestrial environment shows that the conversion from arsenosugars to arsenobetaine and arsenocholine might be possible outside the marine environment. A recent study by Byrne *et al.*[24] showed that arsenobetaine was present in terrestrial mushrooms. Arsenosugars were not found in those mushroom samples. The actual

mechanism of the formation of arsenobetaine in mushrooms remains uncertain. However, the discovery of arsenosugar X in the terrestrial environment in the present study at least makes the overall route of the conversion from arsenosugars to arsenobetaine possible.

The intensity of the peaks in the chromatograms shown in Figures 3.9 and 3.10 is lower than that found for marine algal extracts under the same dilution. This could indicate either a lower total arsenic content in the sample or a lower extraction efficiency. More details will be described later in Section 3.2.2.2.

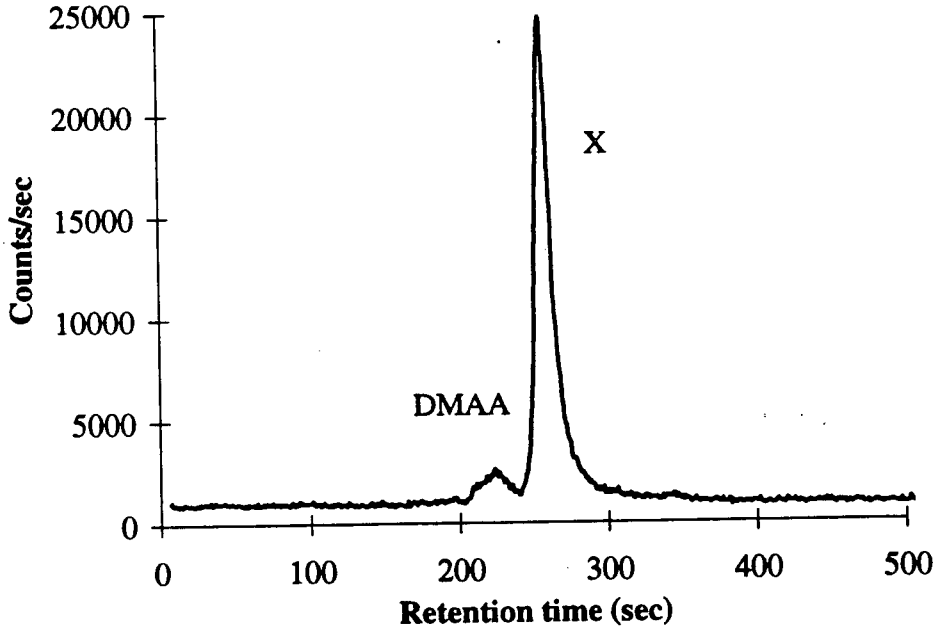


Figure 3.9 A Chromatogram of *Nostoc* sp., condition 2a

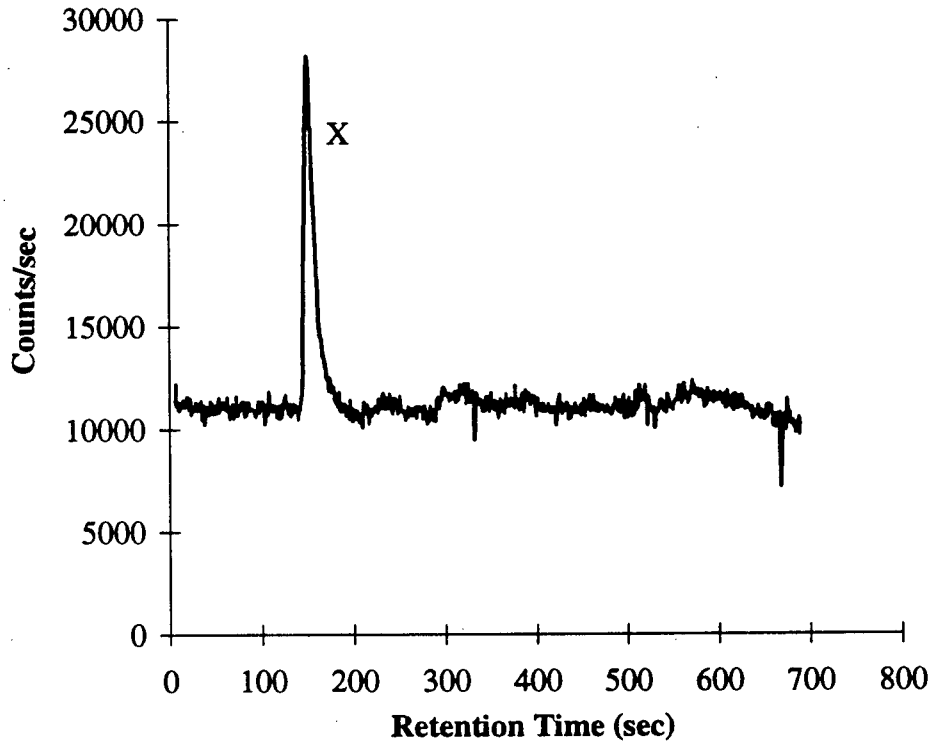


Figure 3.10 A Chromatogram of *Nostoc* sp., condition 2b

3.2.2 Quantitative Analysis by HGAAS

3.2.2.1 Calibration

Hydride generation atomic absorption spectrometry was used for quantitative analysis of the samples. From a rough quantitative analysis of the samples by using HPLC-ICP-MS, it was found that the total arsenic concentration has a wide range from several $\mu g\ g^{-1}$ to $10^2\ \mu g\ g^{-1}$. In a previous study with Nori, Shibata *et al.* showed that

64% to 76% of the total arsenic content was extracted by $MeOH/H_2O$.[56] Because the arsenic content remaining in the residue after extraction could be relatively low, all samples were kept in a small volume and were diluted if necessary.

Arsenate (As(V)) was chosen to be the arsenic species used for the calibration. It was assumed that the only arsenic species in the samples after two hour-acid refluxing was As(V).[58]

The HGAAS system is a modified version of the system developed by Le.[44] The same gas/liquid separator-hydride generator was used. A commercial Nafion tube (Perma Pure Inc.) was adapted to dry the hydrides preceding introduction into the flame. An H_2/air flame was used instead of air/acetylene as recommended by the user manual of the AAS. The analysis of arsenic generally shows a better detection limit with hydrogen as a fuel because of its lower temperature flame and less background absorption in the far ultraviolet.

Because a rather large number of samples were processed in the present study, a methodology that can analyze many samples conveniently and accurately on a routine basis was required. This means that a wide linear dynamic range and short analysis time are necessary. The sensitivity of this system is dependent on the concentration of HCl used, the acid concentration of the samples, the concentration of $NaBH_4$ as well as the actual arsenic species being analyzed. Care should be taken to consider that diluting the samples by using deionized water will also dilute the acidic content of the samples and may eventually affect the amount of hydrides formed.

In the first part of the experiment, 3 M HCl and 3% $NaBH_4$ were used as previously optimized by Le[44] in a similar continuous flow hydride generating system. All marine samples were further diluted and the standards were also made up using the same acid-digestion mixture in order to resemble the acid matrix in the samples.

Figure 3.11 shows a typical calibration curve for As(V) using 3 M HCl and 3% $NaBH_4$ in the continuous flow HGAAS system (Section 2.5.2). The correlation coefficient r^2 is equal to 0.9956 and the linear range is from 0 ppb to 50 ppb for As(V). The detection limit, defined as the concentration which will give a signal equivalent to blank + $3SD_b$ (Appendix I) is 2 ppb. Peak heights of signals were used instead of peak areas for quantification since the calibration curve from peak heights of signals has a smaller standard deviation and better correlation.

The second part of the experiment involved the use of 1.5 M HCl and 3% $NaBH_4$. The lower concentration of HCl was used because the samples already had a relatively high H^+ concentration. Those conditions are suitable for the analysis of those samples with lower arsenic concentration, such as *Nostoc* sp., and the sample residues after extraction, which may have very low concentrations of arsenic. The calibration curve is very similar to Figure 3.11. The detection limit is also found to be 2 ppb.

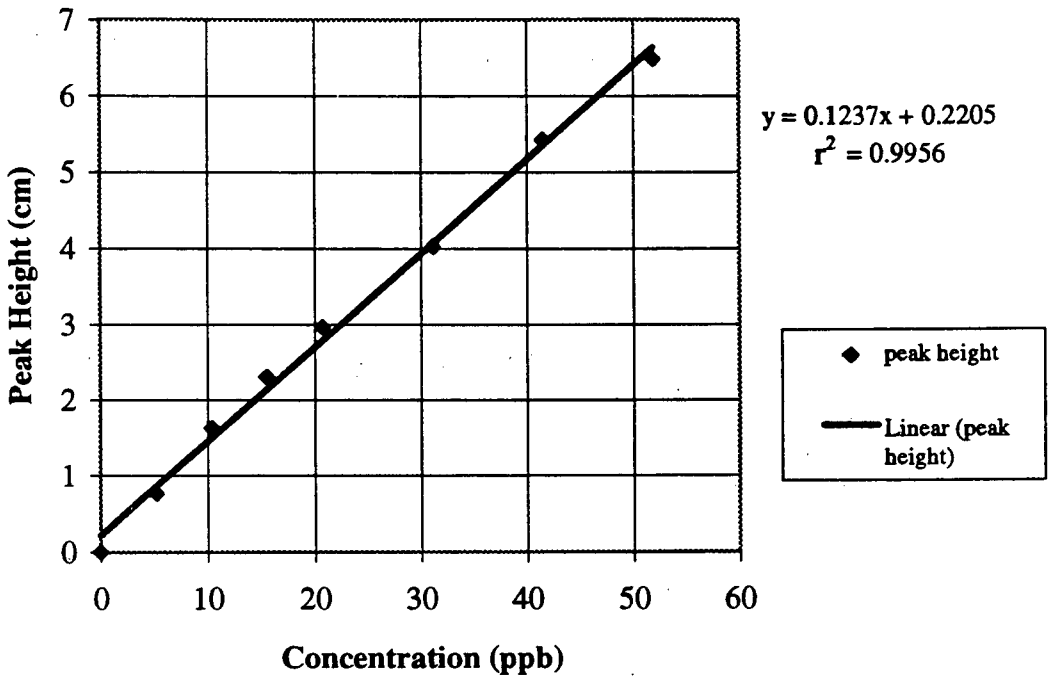


Figure 3.11 A Typical Calibration Curve for As(V) using HGAAS

3.2.2.2 Arsenic Concentrations in the Samples

All commercial products, each in duplicate, were digested as described in Section 2.4.2. Table 3.3 shows the arsenic concentration of samples before and after extraction, as well as the extraction efficiency ((([As] $_{\text{before extraction}}$ - [As] $_{\text{after extraction}}$)/[As] $_{\text{before extraction}}$) x 100%). The certified arsenic concentration in oyster tissue SRM NIST 1566a is 14.0 ± 1.2 ppm and the experimental result is 11.0 ± 0.2 ppm* . The arsenic concentrations in marine brown algae range from 15 ± 1 ppm to 49 ± 3 ppm. Arsenic concentrations in marine red algae range from 7.6 ± 0.6 ppm to 21 ± 1 ppm. These results show that marine brown algae generally contain higher total arsenic content than marine red algae. The

* ± standard deviation of triplicate analyses

arsenic concentration in *Nostoc* sp. is only 2.70 ± 0.08 ppm. The results agree with the observation that terrestrial organisms usually contain lower amounts of arsenic than marine organisms.[23]

Table 3.3 Arsenic Concentrations in the Samples before and after Extraction

Samples	$[As]_{before\ extraction}$ (ppm)*	$[As]_{after\ extraction}$ (ppm)*	Extraction Efficiency
Oyster Tissue SRM**	11.0 ± 0.2	1.29 ± 0.05	88.3%
Brown Algae			
Kelp Powder	27.7 ± 0.8	4.0 ± 0.3	85.4%
Kombu	18.4 ± 0.4	5.1 ± 0.8	72.3%
Bull Kelp	39 ± 2	1.0 ± 0.1	97.5%
Arame	15 ± 1	1.6 ± 0.1	89.4%
Laminaria sp. from China	49 ± 3	4.5 ± 0.2	90.9%
Red Algae			
Dulse	7.6 ± 0.5	0.65 ± 0.09	91.4%
Nori from China, P.R.C.	16 ± 1	0.46 ± 0.03	97.1%
Nori from Japan	7.6 ± 0.6	0.20 ± 0.09	97.4%
Nori from Taiwan, R.C.	21 ± 1	0.4 ± 0.1	98.1%
Blue-Green Algae			
Nostoc sp.	2.70 ± 0.08	1.74 ± 0.07	34.2%

* ± standard deviation of triplicate analyses

** The certified arsenic concentration in oyster tissue SRM is 14.0 ± 1.2 ppm.

The extraction efficiency of marine algae ranges from 72.3% to 98.1%. This indicates that most of the arsenic compounds in marine algae are water-soluble. However, the extraction efficiency of *Nostoc* sp. is only 34.2%. This may be because of the differences in matrices between marine and terrestrial organisms, or that most of the arsenic compounds exist as water-insoluble forms in terrestrial environments.

4. Seasonal Changes in Arsenic Speciation in the Tips and the Remainder of *Fucus* Sp.

4.1 Introduction

Marine brown algae have been found to contain arsenosugars. [9, 63] In a previous study by Ojo[60], methods were developed to isolate some arsenic compounds from extracts of *Fucus* sp., a brown algal species which is abundant in the coastal shores of British Columbia.

The present study involves an investigation of the seasonal changes in arsenic speciation in the tips and the remainder of *Fucus gardneri*. *Fucus gardneri* (Appendix II) is commonly found in British Columbia. This species grows on rocks in the middle and lower intertidal zones. The plant is olive green to yellowish green in color. The swollen tips of the branches of *Fucus* sp., termed receptacles, are the reproductive areas of branches. Scattered over the surface of these receptacles are minute openings, called conceptacles, in which are produced the sperm and eggs.[31, 67] Receptacles are generally yellowish in colour.[35] This species is the major *Fucus* sp. growing in the sampling site, Brockton Point, Stanley Park. Samples collected in July, February and May represent the algae collected in summer, winter and spring. The same sampling site was chosen for all collections in order to minimize the differences in arsenic speciation due to geographical variations. Algal samples were separated into different subsamples as previously described in Section 2.3.2.

4.2 Results and Discussions

4.2.1 Sample Collection

Fucus samples collected in July 1995 had obvious receptacles. The samples referred to as 'the tips', contain the receptacles with conceptacles, and the samples referred to as 'the remainder', contain the remaining parts of the plant.

Fucus samples collected in February 1996 were first separated into two subsamples, the young *Fucus* sp. and the mature *Fucus* sp. The young *Fucus* samples do not show observable conceptacles, and mature *Fucus* has observable conceptacles. The mature *Fucus* samples and the young *Fucus* samples were further separated into the tips and the remainder. Mature *Fucus* is usually brown in colour, and young *Fucus* is greenish brown. The method of selecting mature 'tips' was the same as for the July collection. It is more difficult to decide which part of young *Fucus* is the reproductive organ since conceptacles are not well developed. In this case, the swollen ends of the branches were picked as the tips. Occasionally, only flat non-swollen branches can be seen. For these samples, the parts from the end of the branches to 1.5 cm towards the main rib were regarded as the tips.

Fucus samples collected in May 1996 were first separated into young and mature samples based on whether there were conceptacles on the receptacles. The mature *Fucus* collected appeared to be greenish yellow, similar to that collected in July 1995. The young *Fucus* was not subsampled because of the small size of the alga. The tips and the

remainder of mature *Fucus* were separated in the same way as in the July collection. Figure 4.1 shows the scheme of the sample collection of *Fucus*.

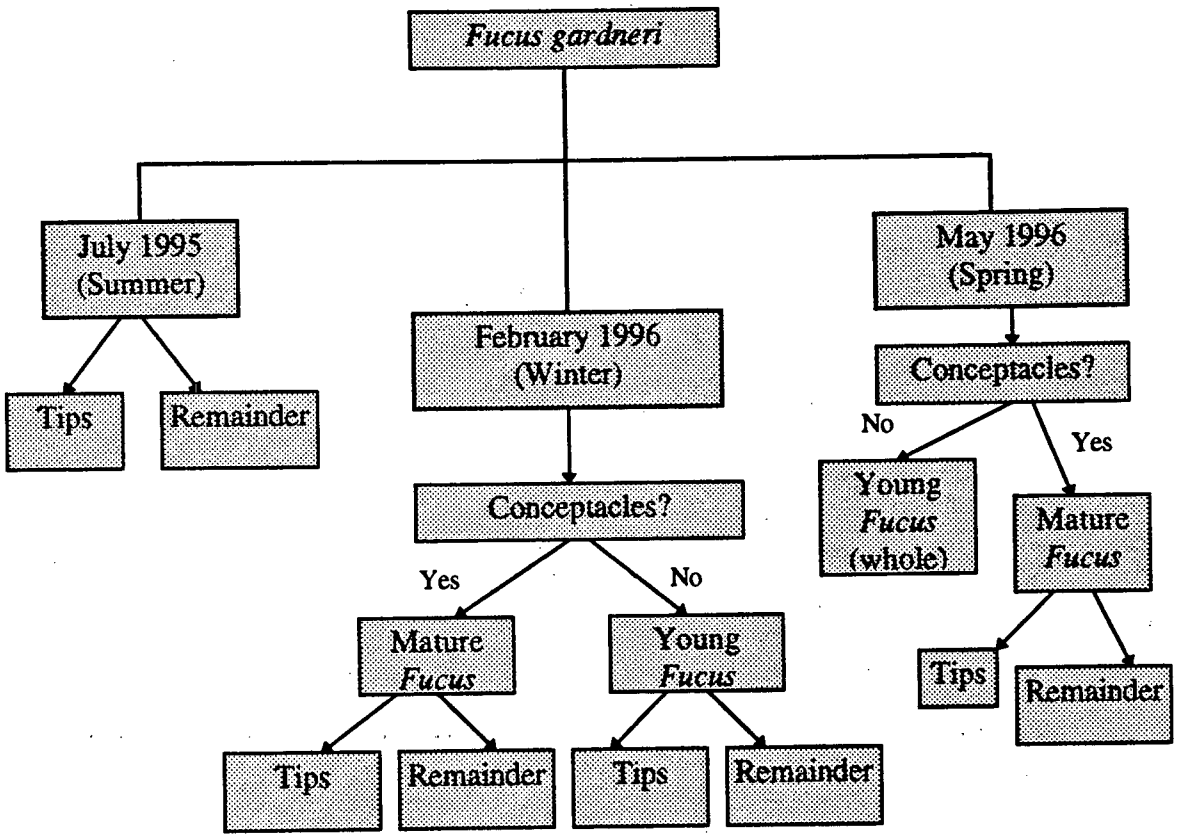


Figure 4.1 The Sample Collection Scheme of *Fucus*

4.2.2 Sample Treatment prior to Storage and Sample Preparation

4.2.2.1 Freeze-Drying Procedure

Freeze-drying is a common method to preserve samples in environmental studies. It provides a convenient mode of preservation because it results in the elimination of water and the denaturation of protein.[69] One aim of the present experiment was to find out if the freeze-drying procedure would affect arsenic speciation. Wet *Fucus* and freeze-dried *Fucus* were extracted as prevously described in Section 2.4.1.1. Figure 4.2 shows the chromatogram obtained by using HPLC-ICP-MS (condition 1a, Section 2.5.1), from an extract of the tips of *Fucus* collected in July 1995 and Figure 4.3 shows the chromatogram from the extract of the freeze-dried *Fucus* from the same batch under same HPLC-ICP-MS conditions. Arsenosugars X, XII and XIII were identified by matching the retention times of the peaks with those obtained from the kelp powder and oyster tissue standard reference material as previously described in Section 3.2.1.1. It can be seen that the chromatograms are very similar, indicating that the freeze-drying procedure does not significantly alter arsenic speciation.

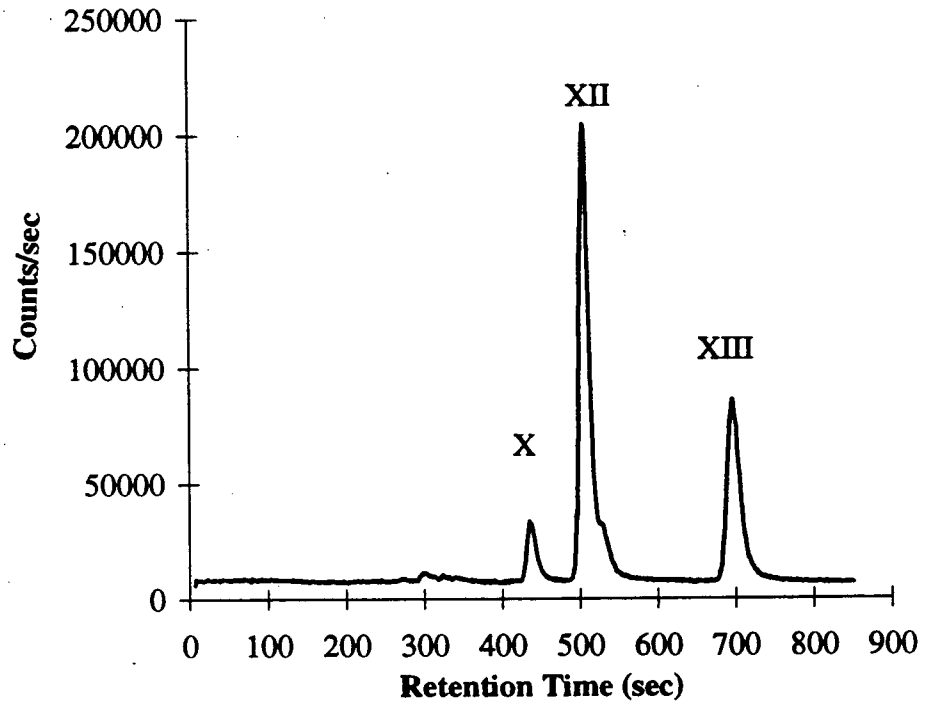


Figure 4.2 A Chromatogram of Wet Tips of *Fucus*, condition 1a

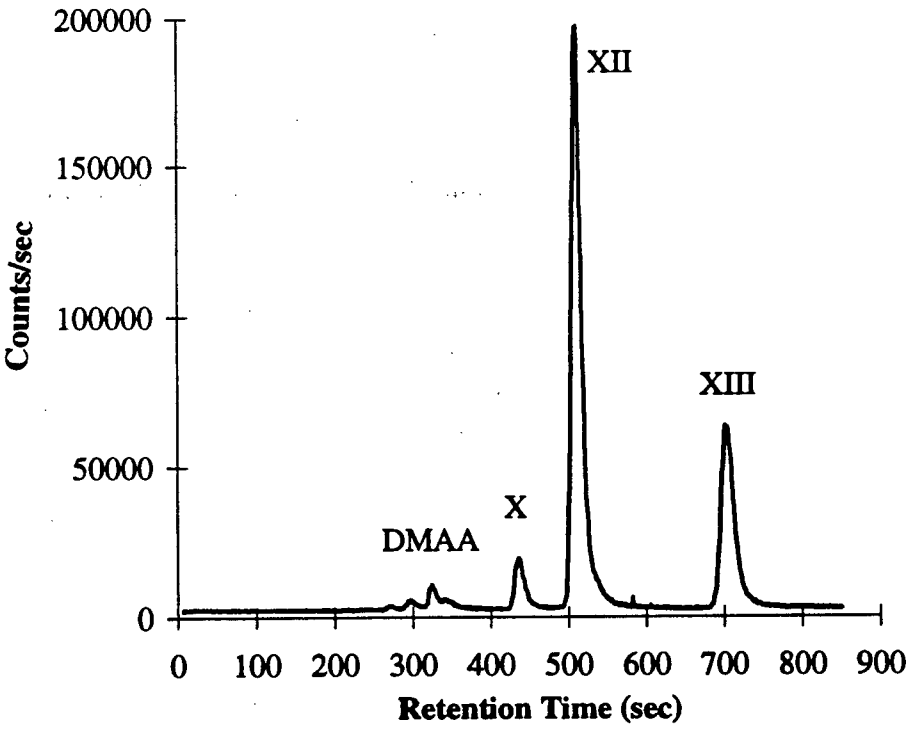


Figure 4.3 A Chromatogram of Freeze-Dried Tips of *Fucus*, condition 1a

4.2.2.2 Sephadex LH-20 cleanup

The wet tips of *Fucus* from the July collection were extracted as previously described in Section 2.4.1.1. The extract was applied to a gel permeation column made of Sephadex LH-20 as described in Section 2.4.1.3. The chromatogram of the eluted sample obtained by using HPLC-ICP-MS (condition 1a, Section 2.5.1) is shown in Figure 4.4. Figure 4.2 and Figure 4.4 represent extracts of different samples from the same batch with and without Sephadex clean-up. It can be seen that they are very similar, indicating that the composition of the arsenosugars was not changed after applying the sample to a Sephadex LH-20 column.

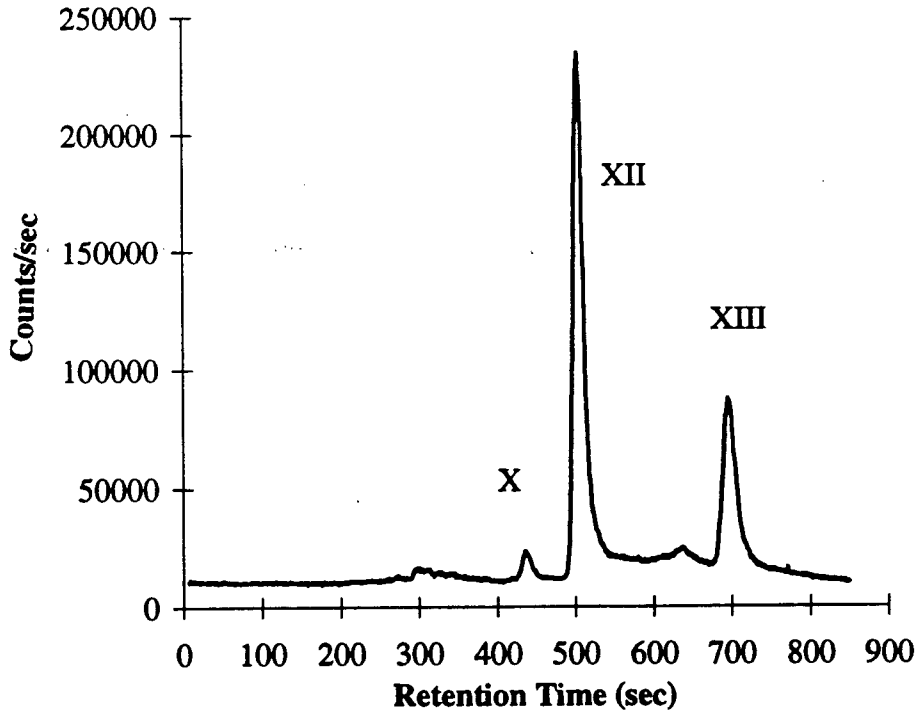


Figure 4.4 A Chromatogram of Wet Tips of *Fucus* after Sephadex LH-20 treatment, condition 1a

The separation that is achieved by gel permeation chromatography is based on differences in molecular sizes of molecules. It is a mild method for purifying proteins, polysaccharides, nucleic acids and enzymes from small salts and other small molecules.[70] It is also used as a preparatory technique to concentrate arsenic-containing fractions for further purification. [59, 60, 71]

Based on the theory, the compounds with higher molecule weights have shorter retention times on the column. Small biomolecules and salts tend to be retained longer in the column. [70] In the present study, it was found that some greenish yellow substance which could be the pigment in the algae was retained in the gel permeation column. A common plant pigment, chlorophyll, has a strong affinity for Sephadex LH-20 gel due to the HO-H bonding between the pigment and the gel. For large scale arsenosugar isolation, Sephadex LH-20 may be a useful step to clean up the extract since some large biomolecules, which elute in the void volumn, and the pigment, which is retained by the column can be eliminated from the extract. This should enhance the purity of the extract for further isolation and analysis. In the present study, Sephadex clean-up was not used for this small scale analysis in order to avoid changing the concentration as well as the speciation.

4.2.2.3 Phenol-Ether Extraction

Arsenobetaine has been found in marine animals.[2, 8, 9] The existence of arsenobetaine in marine plants is an open question. The preliminary chromatograms from

extracts of the remainder of *Fucus* sp. by using HPLC-ICP-MS show that trace amounts of arsenobetaine might be extracted by MeOH/H_2O. In order to verify the results, a phenol-ether extraction as previously described in Section 2.4.1.2 was performed. This is a commonly used extraction method for arsenobetaine and has been applied by a number of workers. [70, 71] Another objective of this experiment was to monitor the effects of this particular combination of solvents on the arsenosugars in the extract.

Figure 4.5 shows the chromatogram from the extract of the wet remainder of *Fucus* collected in July by using HPLC-ICP-MS (condition 1a, Section 2.5.1). Figure 4.6 shows the chromatogram from the same extract after phenol-ether treatment by using the same HPLC-ICP-MS conditions. It can be seen that those two chromatograms are similar, except that a rise in the baseline is observed in Figure 4.6. It is possible that a trace amount of the phenol-ether solvent mixture remained in the sample and this difference in solvent composition between the sample and the mobile phase results in the different conditions of the plasma.

There is no dramatic increase in the amount of arsenobetaine being extracted into the analytical sample after phenol-ether treatment. A very small peak which matches the retention time of arsenobetaine is still present in both figures. Therefore, the existence of arsenobetaine in brown algae, ***Fucus gardneri***, is still questionable. The composition of arsenosugars in the extract is not changed by phenol-ether treatment.

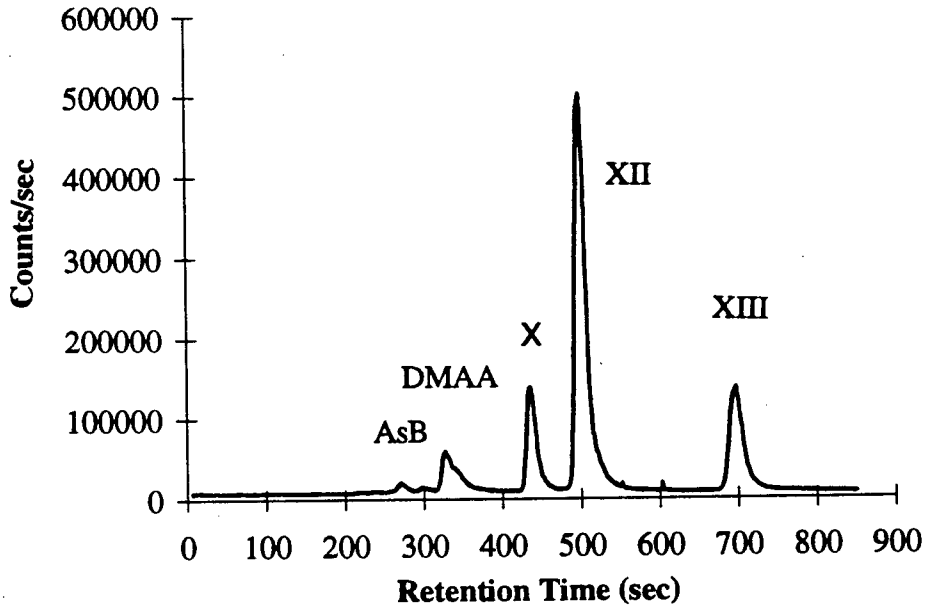


Figure 4.5 A Chromatogram of the Wet remainder of *Fucus*, before Phenol-Ether Extraction, condition 1a

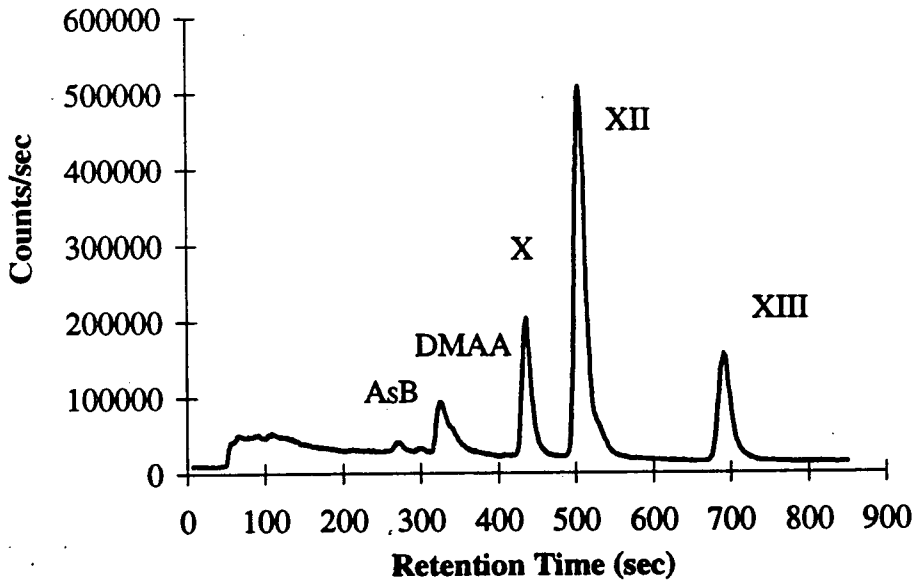


Figure 4.6 A Chromatogram of the Wet remainder of *Fucus*, after Phenol-Ether Extraction, condition 1a

4.2.3 Seasonal Changes in Arsenic Speciation in the Tips and the Remainder of *Fucus* sp.

4.2.3.1 Water Content

Table 4.1 shows the water content of the *Fucus* samples collected in summer, winter and spring between 1995 and 1996 in Brockton Point, Stanley Park. It is found that the water contents* of *Fucus gardneri* remain relatively constant from season to season. The tips of *Fucus*, which are sometimes swollen, contain a higher amount of water (88% to 93%) than does the remainder (80% to 82%).

Table 4.1 Water Content of *Fucus gardneri*

Samples	Water Content (%)*
July Collection (Summer)	
Reproductive Organs	93
Other Parts	80
February Collection (Winter)	
Young Reproductive Organs	89
Young Other Parts	82
Mature Reproductive Organs	89
Mature Other Parts	81
May Collection (Spring)	
Young Whole Plant	82
Mature Reproductive Organs	88
Mature Other Parts	80

* Water content $= \left({}^{\text{(weight before freeze-drying - weight after freeze-drying)}} / {}_{\text{weight before freeze-drying}} \right) \times 100\%$

4.2.3.2 Qualitative Analysis by HPLC-ICP-MS

Because the freeze-drying procedure was found to have no effect on arsenic speciation of *Fucus* sp., freeze-dried samples were used for the rest of the present study. They were easy to handle, and also gave consistent results for quantitative analysis.

Figure 4.3 and Figure 4.7 show chromatograms of the extracts from the tips and the remainder of *Fucus* from the July collection. The arsenic compounds in the extract were identified by matching the retention times of the peaks with those in the standard reference material as previously described in Section 3.2.1.1. It can be seen that the extract from the tips (Figure 4.3) contains a smaller amount of arsenosugar X than the extract from the remainder of *Fucus* (Figure 4.7). There is no detectable amount of arsenobetaine in either sample. The tips contain a slightly lower amount of DMAA than does the remainder of *Fucus*.

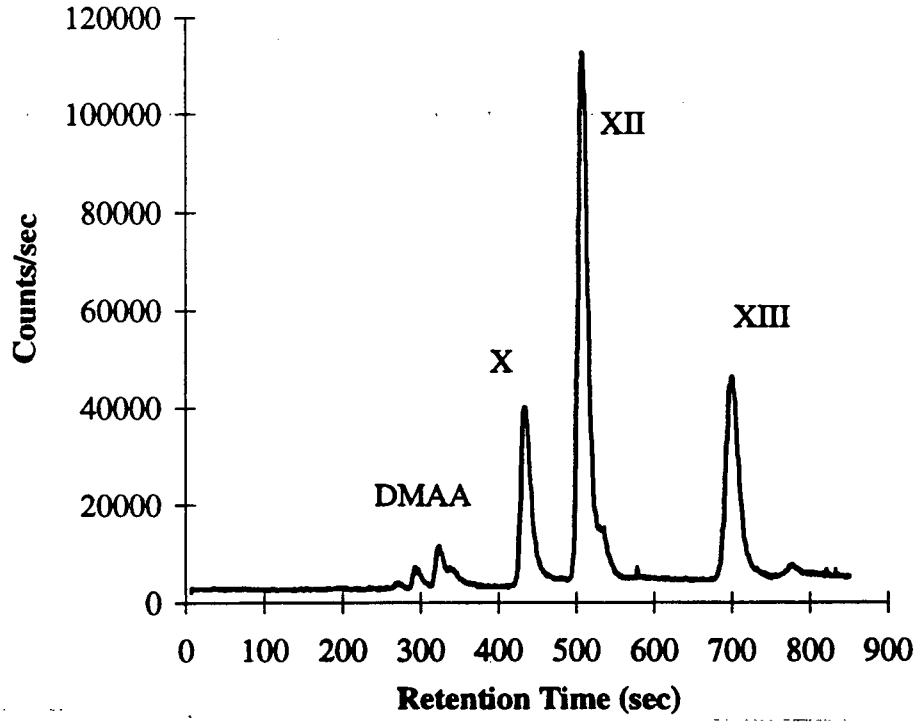


Figure 4.7 A Chromatogram of the Freeze-Dried Remainder of *Fucus*, condition 1a

Figure 4.8 and Figure 4.9 show the same two samples analyzed under conditions 2b (Section 2.5.1). With the use of TBAH as the ion-pairing reagent, arsenobetaine, if present, will co-elute with arsenosugar X and DMAA will co-elute with arsenosugar XI (Table 1.1). However, no detectable amounts of arsenobetaine and arsenosugar XI are observed in Figures 4.3 and 4.7. The first and second major peaks in Figure 4.8 and Figure 4.9 can be treated as arsenosugar X and DMAA respectively. Again the relative amount of arsenosugar X to other arsenosugars in the tips (Figure 4.8) is smaller than in the remainder of *Fucus* (Figure 4.9).

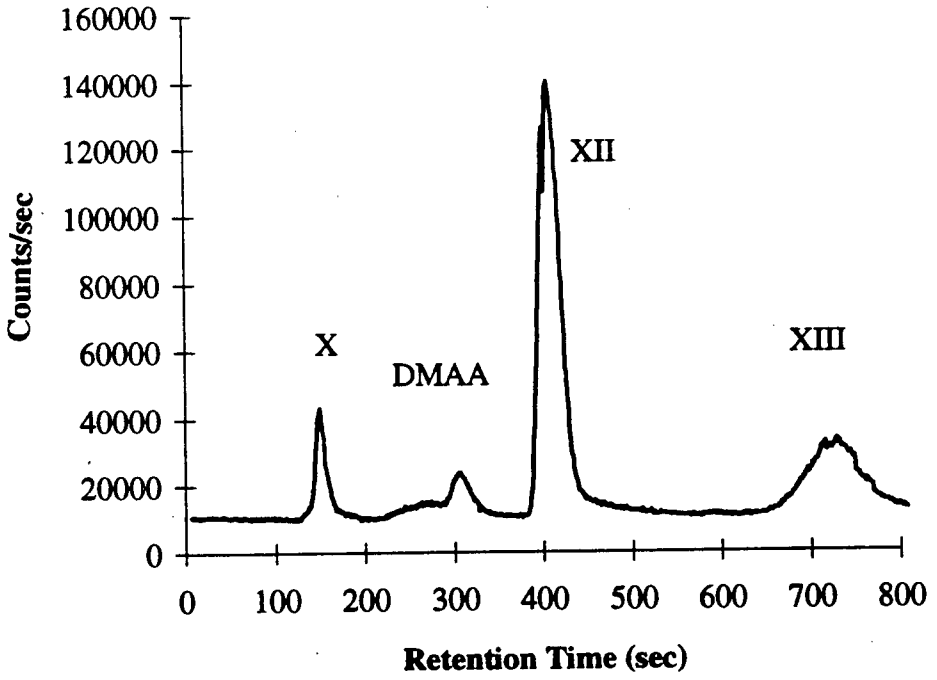


Figure 4.8 A Chromatogram of Freeze-Dried Tips of *Fucus*, condition 2b

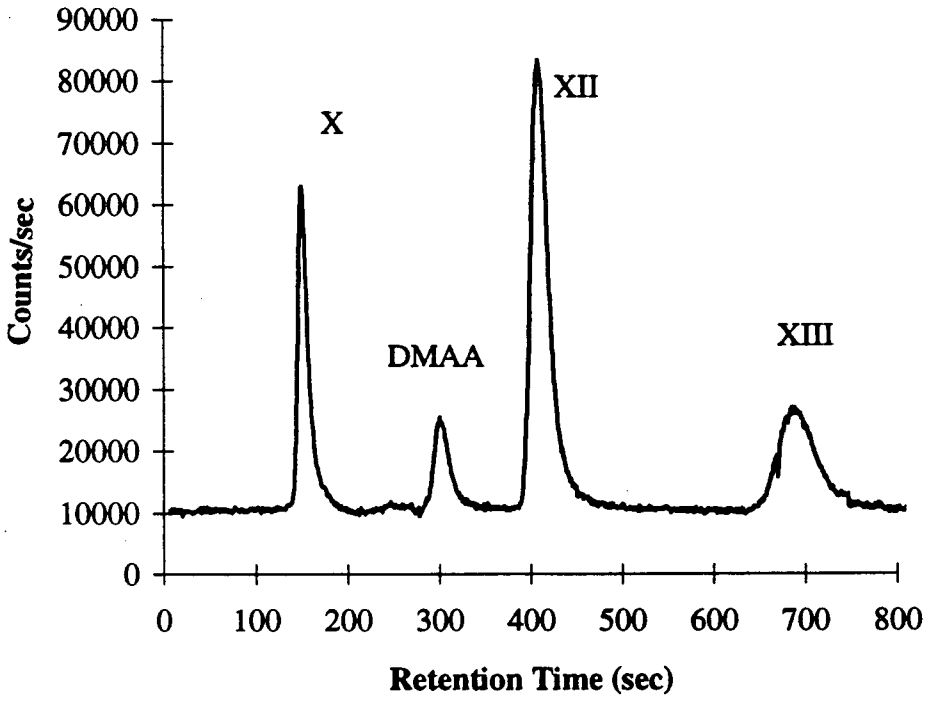
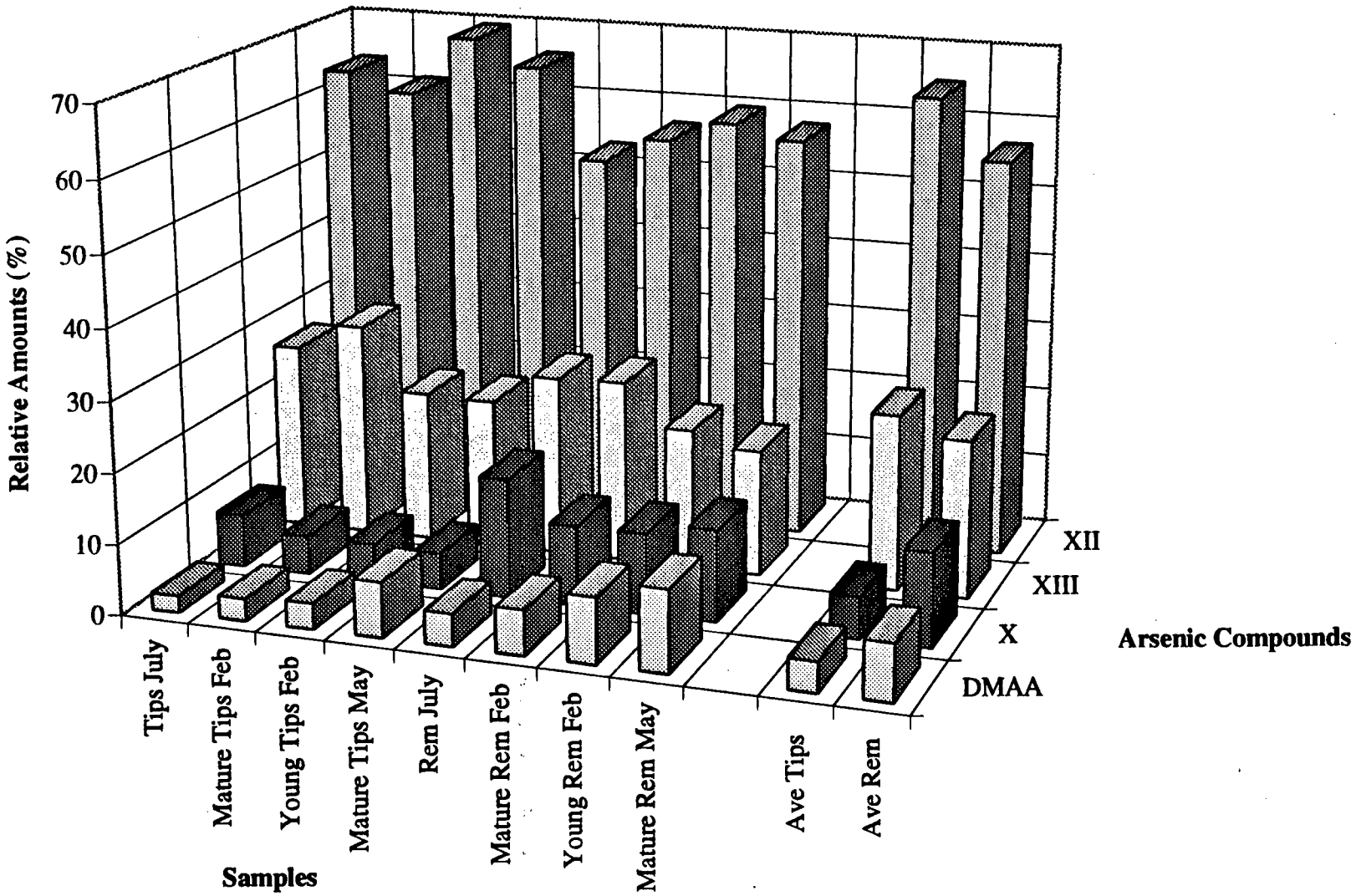


Figure 4.9 A Chromatogram of the Freeze-Dried Remainder of *Fucus*, condition 2b

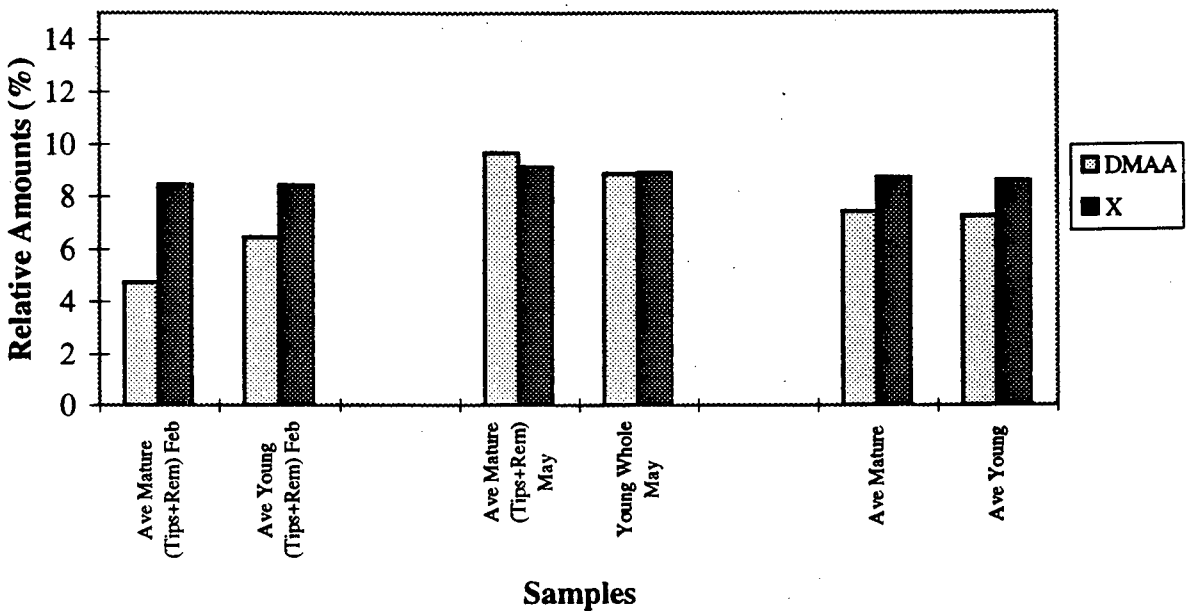
Table 4.2 Relative Amounts of Arsenic Compounds in All *Fucus* Samples

Samples	DMAA	X	XII	XIII
July Collection(Summer)				
1. Tips	2.4%	7.6%	63.4%	26.6%
2. Remainder	4.6%	16.9%	52.8%	25.7%
February Collection (Winter)				
3. Mature Tips	3.2%	5.5%	60.7%	30.6%
4. Mature Remainder	6.4%	11.4%	56.4%	25.8%
5 Young Tips	3.7%	5.5%	69.4%	21.4%
6. Young Remainder	9.3%	11.3%	59.4%	20.0%
May Collection (Spring)				
7. Mature Tips	7.8%	5.2%	65.6%	21.4%
8. Mature Remainder	11.5%	13.0%	57.5%	18.0%
9. Young Whole *Fucus*	8.8%	8.9%	66.5%	15.8%
Averages				
All Tips (1,3,5,7)	4.3%	5.9%	64.8%	25.0%
All Remainders (2,4,6,8)	7.9%	13.2%	56.5%	22.4%
Mature *Fucus* from February (3,4)	4.8%	8.4%	58.5%	28.3%
Young *Fucus* from February (5,6)	6.5%	8.4%	64.3%	20.8%
Mature *Fucus* from May (7,8)	9.7%	9.1%	61.5%	19.7%
All Young *Fucus* (5,6,9)	7.3%	8.5%	65.1%	19.1%
All Mature *Fucus* (3,4,7,8)	7.2%	8.8%	60.0 %	24.0%



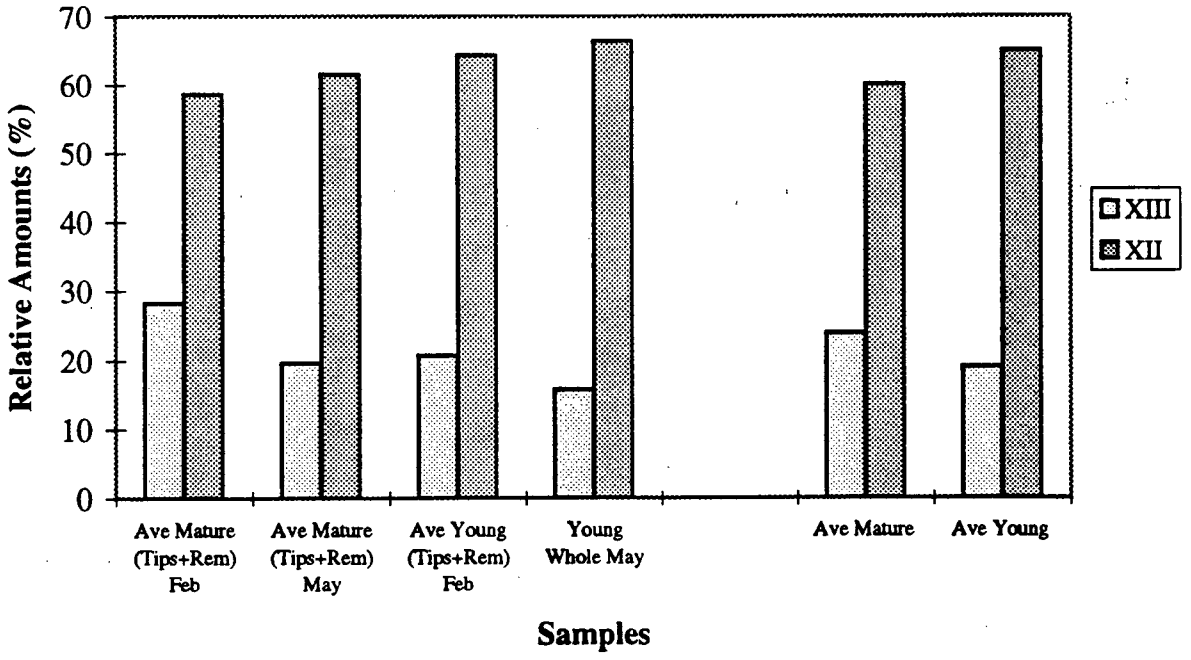
Remarks: Tips-the Tips of *Fucus*, Rem-the Remainder of *Fucus*, Whole-whole plant, July-Summer collection, Feb-Winter collection, May-Spring collection (Table 4.2), Ave-Average

Figure 4.10 Relative Amounts of Arsenic Compounds in the Tips and the Remainder of *Fucus*



Remarks: Tips-the Tips of *Fucus*, Rem-the Remainder of *Fucus*, Whole-whole plant, Feb-Winter collection, May-Spring collection (Table 4.2), Ave-Average

Figure 4.11 Relative Amounts of DMAA and Arsenosugar X in Mature and Young *Fucus* Samples



Remarks: Tips-the Tips of *Fucus*, Rem-the Remainder of *Fucus*, Whole-whole plant, Feb-Winter collection, May-Spring collection (Table 4.2), Ave-Average

Figure 4.12 Relative Amounts of Arsenosugars XII and XIII in Mature and Young *Fucus* Samples

Table 4.2, Figures 4.10 to 4.12 show summaries of the relative peak areas of arsenic compounds found in extracts from all samples collected in July, February and May. In all three seasons, extracts of the tips have relatively lower amounts of DMAA and arsenosugar X and relatively higher amounts of arsenosugars XII and XIII than those in the remainder of *Fucus* (Figure 4.10). The results from the February collection of *Fucus* show that there is no significant difference in the relative amounts of DMAA and arsenosugar X between young *Fucus* and mature *Fucus* (Figure 4.11). When the results from young whole *Fucus* collected in May is compared with the samples from older plants, the relative amount of arsenosugar X in this sample is approximately the average of relative amounts of arsenosugar X in the tips and the remainder of the older specimen (Figure 4.11). These results indicate that the age of *Fucus* is unlikely to be a factor affecting the relative amounts of DMAA and arsenosugar X in *Fucus*.

There are at least two possible explanations to account for the lower amounts of arsenosugar X being present in the tips of *Fucus*: (i) The arsenosugars with larger groups (XII and XIII) might have been formed at the tips of *Fucus* and transported towards the body of the plant and (ii) arsenosugar X might have been formed in the body of the plant and transported towards the tips of *Fucus*. During the transport of arsenosugars inside the *Fucus* plant, some chemical reactions might have taken place causing interconversions between the arsenosugars with larger groups and arsenosugar X.

Arsenosugar X, having an OH group attached to the end carbon, has a relatively simple structure as compared to other arsenosugars (Table 1.1). It has been suggested that arsenosugar XI (Table 1.1) can be decomposed to arsenosugar X and then DMAA. [63,

[67] On the same basis, the conversion of arsenosugars XII, XIII and XIV to arsenosugar X does not seem to be impossible.

Figure 4.12 shows that the mature *Fucus* collected in February contains a slightly higher amount of arsenosugar XIII and a lower amount of arsenosugar XII than is found in the young *Fucus* from the same collection. Similar results are observed from the May collection, in which mature *Fucus* contains a slightly higher amount of arsenosugar XIII and a lower amount of arsenosugar XII than does the young whole *Fucus*. Though the differences in the relative amounts of various arsenosugars are small, they might indicate a trend that the formation of arsenosugar XIII ($-OSO_3H$) is more favored compared to that of arsenosugar XII ($-SO_3H$) when *Fucus* matures.

The present study shows the possibility of conversions between arsenosugars in *Fucus*. However, the actual mechanisms are still unknown.

4.2.3.3 Quantitative Analysis by HGAAS

Fucus samples collected in July and February and the residue after $MeOH/H_2O$ extraction were digested to determine the total arsenic content as previously described in Sections 2.4.2 and 2.5.2. Detailed analytical methods are discussed in Section 3.2.2.

Table 4.3 shows a summary of the total arsenic content as well as the extraction efficiency of the samples. The extraction efficiency is defined as ((([As]$_{\text{before extraction}}$ - [As]$_{\text{after extraction}}$)/[As]$_{\text{before extraction}}$) x 100%) as previously mentioned in Section 3.2.2.

Table 4.3 Arsenic Content in *Fucus gardneri***

Samples	$[As]_{before\ extraction}$ ($\mu g\ g^{-1}$)*	$[As]_{after\ extraction}$ ($\mu g\ g^{-1}$)*	Extraction Efficiency
July Collection			
Tips (dry)	8.9 ± 0.5	1.8 ± 0.1	79.2%
Remainder (dry)	9.0 ± 0.5	0.45 ± 0.08	95.0%
Tips (wet)	1.60 ± 0.04	0.100 ± 0.005	93.8%
Remainder (wet)	3.7 ± 0.3	0.06 ± 0.01	98.3%
February Collection			
Young Tips (dry)	17.1 ± 0.8	16.1 ± 0.2	5.8%
Young Remainder (dry)	16.2 ± 0.9	11.5 ± 0.9	29.3%
Mature Tips (dry)	22.2 ± 0.8	11 ± 1	48.7%
Mature Remainder (dry)	17.0 ± 0.9	8.9 ± 0.2	47.6%

The total arsenic contents in different *Fucus* samples collected in the same season are relatively homogeneous. However, the difference of total arsenic contents in *Fucus* samples collected in different seasons is relatively large (8.9 to 9.0 ppm in July and 16.2 to 22.2 ppm in February on dry-weight basis).

* ± standard deviation of triplicate analyses
** duplicate samples were taken from each batch of *Fucus* samples

It is commonly believed that organoarsenicals in higher trophic levels result from the accumulation of compounds that have been synthesized from arsenate at lower levels.[8] It is reasonable to assume that the concentration of arsenic in marine algae depends on the uptake and efflux of arsenic by the algae. In a previous arsenic-uptake study of the *Fucus* sp., Klumpp[72] found that the intensity of illumination and the temperature might affect the uptake and efflux of arsenic by *Fucus* sp. He also found that only living *Fucus* cells were able to accumulate arsenic and that the uptake of arsenic was reduced in the presence of a respiratory inhibitor. These results suggest that the accumulation of arsenic by *Fucus* is an active process dependent on respiratory energy.[72]

In actual marine environments, some other factors might affect the total arsenic content and the speciation of arsenic in *Fucus*. For example, the arsenic concentration in the seawater and the reproduction of *Fucus* are potential factors. The arsenic concentration in seawater might be changing from time to time so that the exposure of *Fucus* to arsenic might be different at different periods of time. A study of the reproduction of a similar *Fucus* sp. by Ang[73] showed that the reproduction peak of *Fucus* in False Creek, Vancouver, occurred in fall to winter coinciding with observed higher levels of arsenic in *Fucus* collected in February in the present study. This correlation between the reproduction and the total arsenic content of *Fucus* might have scientific importance. However, no definite biochemical role has been proposed for arsenic.[26] Whether there are relationships between the total arsenic content in *Fucus*, the season and the growth of *Fucus* is still a mystery.

The extraction efficiency of the samples collected in July (79.2% to 98.3%) is relatively higher than for the samples collected in February (5.8% to 48.7%). The results indicate that most of the arsenic compounds exist as water-soluble forms in the *Fucus* samples collected in July. The differences in the extraction efficiency of *Fucus* between different seasons show that the chemical forms of arsenic in *Fucus* might change from season to season. From the results of the February collection of *Fucus*, it can be seen that the mature *Fucus* samples contain relatively higher amounts of water-soluble compounds than do the young *Fucus* samples.

It has been suggested that the relative proportion of water-soluble arsenic compounds and lipid-soluble arsenic compounds in marine organisms varies considerably between species.[72] The present study shows that the relative proportion of water-soluble arsenic compounds and lipid-soluble compounds in the same algal species might vary from season to season. The different extraction efficiencies of mature and young *Fucus* samples collected in February indicate that the relative amount of water-soluble arsenic compounds present in *Fucus* might change as the plant matures. However, the actual pattern of the interconversions between water-soluble and lipid-soluble arsenic compounds in algae remains unknown.

The results show that the arsenic speciation in *Fucus* might depend on some environmental and biological factors. Detailed arsenic uptake experiments performed by growing *Fucus* in cultures should be very useful for confirmation of these results and for further information of the arsenic speciation of *Fucus*.

5. Summary and Future Development

The combination of HPLC with ICP-MS has been demonstrated to be very useful for arsenic speciation in algae. The identification of arsenosugars in algae can be confirmed by using two different mobile phase conditions.

Kelp powder has been found to be a potential standard reference material for arsenic speciation because it contains four arsenosugars that are commonly found in a variety of marine algae. The identification of an arsenosugar in a terrestrial organism for the first time is a significant discovery which adds to our knowledge of the arsenic cycle in the terrestrial environment.

The arsenic speciation in *Fucus* sp. varies with the parts of the plant being examined and the season of collection. Thus the uptake of arsenic by *Fucus* sp. may change from season to season. The mechanisms of formation of arsenosugars may also be different in different parts of *Fucus*.

The arsenic speciation of algae is very much dependent on the species being studied. Smaller variations in relative amounts of arsenic compounds could result from environmental variations including geographical differences, food processing procedures and storage.

The modified continuous flow HGAAS has been demonstrated to be effective for the analysis of a large number of samples. Quantitative analysis of algae before and after extraction shows the differences in extraction efficiency between different commercial

algae and between *Fucus* collected in different seasons. These results contribute to our understanding of the forms and functions of arsenic in marine and terrestrial environments.

In the future, the analysis of commercial products should be expanded to other marine algae from different geographical locations. Other terrestrial organisms, such as fungi and other blue-green algal species, should also be investigated.

Experiments involving arsenic uptake by *Fucus* cells at different stages of their lives should be performed. The experiments should be set up by growing *Fucus* cells in cultures such that potential environmental variations can be controlled. Investigations of *Fucus* sp. growing in different locations would also be an interesting project.

Biochemically, the metabolism of arsenic compounds is still largely unknown and the uses of arsenosugars are still a mystery. In order to gain a better understanding of the mechanisms of conversions between different arsenosugars, pure arsenosugars will need to be isolated or synthesized and then used in controlled studies.

Current mobile phase conditions can be used to complement each other for the identification of most arsenosugars which have been found in algae. For further method development, different HPLC columns and different mobile phase conditions should be tried to resolve the partially overlapping peaks in the chromatograms.

The analytical methods adopted in the present study enable effective routine analyses of arsenic compounds in algae. The discoveries made in this project enrich our knowledge of the relationship between arsenic speciation and some environmental factors. The results can be added to the database of the arsenic compounds present in a variety of marine and terrestrial algae. The results are useful as indicators of areas of further studies in arsenic speciation and in the biochemistry of arsenic in the environment.

References:

1. Berhard, M.; Brinckman, F. E.; Irgolic, K. J. 'Why "Speciation"?', in *The Importance of Chemical "Speciation" in Environment Processes* **1986**, 7-14.

2. Tamaki, S.; Frankenberger, W. T. Jr. 'Environmental Biochemistry of Arsenic', in *Reviews of Environmental Contamination and Toxicology* **1992**, Vol. 124, 79-110.

3. Tanaka, T.; *Appl. Organomet. Chem.*, **1988**, 2, 283-295.

4. Boyle, R. W.; Jonasson, I. R. *J. Geochem. Explor.* **1973**, 2, 251-296.

5. Vymazal, J. *Algal and Element Cycling in Wetlands* **1995**, 359-361.

6. Fishbein, L. *Chromatography of Environmental Hazards II* **1973**, 103-116.

7. Lunde, G. *Environ. Health Persp.* **1977**, Vol. 19, 47-52.

8. Cullen, W. R.; Reimer, K. F. *Chem. Rev.* **1989**, 89, 713-764.

9. Shibata, Y.; Morita, M.; Fuwa, K. *Adv. Biophys.* **1992**, Vol. 28, 31-80.

10. Morita, M.; Edmonds, J.S. *Pure & Appl. Chem,* **1992**, Vol. 64, no. 4, 575-590.

11. Ni Dhubhghaill, O. M.; Sadler, P.J. *The Structure & Reactivity of Arsenic Compounds: Biological Activity & Drug Design,* Springer-Verlag Berlin Heidelberg **1991**, 130-190.

12. Challenger, F. *Chem. Rev.* **1945**, 36, 315-361.

13. Challenger, F.; Lisle, D.B.; Dranfield, P.B. *J. Chem. Soc.* **1954**, 1760-1771.

14. Cullen, W. R.; Froese, C. L.; Lui, A.; McBride, B. C.; Patmore, D. J.; Reimer, M. *J. Organomet. Chem.* **1977**, 139, 61-69.

15. NRC of Canada, *Effects of Arsenic in the Canadian Environment* **1978**.

16. Perrose, W.R. CRC *Crit. Rev. Environ. Control* **1974**, 465-482.

17. Fowler, B. A. In *Topics in Environmental Health: Biological and Environmental Effects of Arsenic* **1983**, 233-269.

18. Andreae, M. O. In *Organometallic Compounds in the Environment, Principles and Reactions,* Craig, P. J. Ed.; John Wiley: New York, **1986**, 198-228.

19. Knowles, F. C.; Benson, A. A. In *Trends in Biochemical Science: The Biochemistry of Arsenic* **1983**, 8, 178-180.

20. Yen, K-Y. *The Illustrated Chinese Materia Medica Crude Drugs*, Southern Materials Centre, Inc. **1980**, 243.

21. Hsü, H-Y *A Study of Chinese Mineral Drugs*, National Research Institute of Chinese Medicine: Taiwan, Republic of China, **1975**, 40-44. (*in Chinese*)

22. Ishiguro, S. *Appl. Organomet. Chem.* **1992**, 6, 323-331.

23. Maeda, S. 'Biotransformation of arsenic in Freshwater Organisms', In *Chemical Problems Associated with Old Arsenical and 'Mustard' Munitions;* Bunnett, J. F. and Mikolajczyk M., Ed., Kluwer Academic Publishers, **1996**.

24. Byrne, A. R.; Slejkovec, Z.; Stijve, T.; Fay, L.; Gössler, W.; Gailer, J.; Irgolic, K. J. *Appl. Organomet. Chem.* **1995**, Vol. 9, 305-313.

25. Maeda, S.; Mawatari, K.; Ohki, A.; Naka, K. *Appl. Organomet. Chem.* **1993**, Vol.7, 467-476.

26. Edmonds, J. S.; Francesconi, K. A. *Tetrahedron Lett.* **1977**, No.18, 1543-1546.

27. Le, S. X. C.; Cullen, W. R.; Reimer, K. J. *Environ. Sci. Technol.* **1994**, Vol.28, No. 9, 1598-1604.

28. Edmonds, J. S.; Francesconi, K. A. *Appl. Organomet. Chem.* **1988**, 2, 297-302.

29. Francesconi, K. A.; Edmonds, J. S. *Oceanogr. Mar. Biol. Annu. Rev.* **1993**, 31, 111-151.

30. Craig, P. J. In *The Chemistry of the Metal-Carbon Bond, vol. 5.* Hartley, E. R. Ed.; John Wiley: New York, **1989**, 437-447.

31. Bold, H. C.; Wynne, M. J. *Introduction to the Algae* **1978,** 267-356.

32. Duddington, C. L. *Seaweeds and other Algae* **1966** , 13-20.

33. Raven, P. H.; Johnson, G. B. *Biology* **1984**, 552-566.

34. Keeton, W. T.; Gould, J. L. *Biological Science* **1986**, 1061-1072.

35. Scagel, R. F. *Common Seaweeds of B.C.* **1967**, 158-159.

36. Raven, P. H.; Johnson, G. B. *Biology* **1984**, 606-630.

37. Bold, H. C.; Wynne, M. J. *Introduction to the Algae* **1978**, 451-564.

38. Fay, P. *The Blue-Greens* **1983**, 1-3.

39. Fritsch, F. E. *The Structure and Reproduction of the Algae* **1935**, 836.

40. Harvey, W. H. *'Nereis Boreali-Americana, etc. III. Chlorospermeae', Smithsonian Contrib. Knowledge* **1858**, 10, No.2, 110.

41. Wu, H-C, *Freshwater Algae in China*, Shanghai Science and Technology Publication Ltd., **1980**, 53. (*in Chinese*)

42. Lobban, C. S.; Wynne, M. J. *The Biology of Seaweeds* **1981**, 680-741.

43. Levring, T.; Hoppe, H. A.; Schmid, O. J. *Marine Algae* **1969**, 163-167.

44. Le, X-C; Cullen, W. R.; Reimer, K. J.; Brindle, I. D. *Analytica Chimica Acta* **1992**, 258, 307-315.

45. Willard, H. H.; Merritt, L. L. Jr.; Dean, J. A.; Settle, F. A. Jr. *Instrumental Methods of Analysis* **1981**, 149-150.

46. Aggett, J.; Aspell, A. C. *Analyst* **1976**, Vol. 101, 341-347.

47. Le, X-C; Cullen, W. R.; Reimer, K. J. *Appl. Organomet. Chem.* **1992**, 6, 161-171.

48. Melander, W. R.; Horváth, C. 'Theory of Ion-Pair Chromatography', in *Ion-Pair Chromatography: Theory and Biological and Pharmaceutical Applications* M. T. W. Hearn Ed., **1985**, 27-76.

49. Haddad, P. R.; Jackson, P. E. *Ion Chromatography: Principles and Application* **1990**, Chapter 6.

50. Barnes, R.M. In *Biological Trace Element Research Multidisciplinary Perspectives: Inductively Coupled & Other Plasma Sources* **1991**, 158-180.

51. Blades, M. W.; Banks, P.; Gill, C.; Huang, D.; LeBlanc, C.; Liang, D. *Trans. IEEE Plasma Sci.* **1991**, Vol. 19, No.6, 1090-1113.

52. Gray, A. *Spectrochim. Acta* **1985**, Vol. 40B, No. 10-12, 1525-1537.

53. Date, A. *Spectrochim. Acta* **1991**, Vol. 14, No. 1/2, 3-32.

54. Thompson, M.; Walsh, J. N. *A Handbook of Inductively Coupled Plasma Spectrometry*, 2nd ed.; Chapman and Hall: New York, **1989**.

55. Le, X-C Ph.D. Thesis, Department of Chemistry, University of British Columbia, Sept. **1993**.

56. Shibata, Y.; Morita, M. *Appl. Organomet. Chem.* **1992**, 6, 343-349.

57. Cullen, W. R.; Nelson, J. C. *Appl. Organomet. Chem.* **1993**, Vol. 7, 319-327.

58. Bajo, S.; Suter, U.; Aeschliman, B. *Analytica Chimica Acta* **1983**, 149, 321-355.

59. Cullen, W. R.; Dodd, M. *Appl. Organomet. Chem.* **1989**, 3, 79-88.

60. Ojo, A. Ph.D. Thesis, Department of Chemistry, University of British Columbia, Dec. **1994**.

61. Noda, H. *J. Appl. Phyco.* **1993**, 5, 255-258.

62. Klumpp, D. W.; Peterson, P. J. *Mar. Biol.* **1981**, 62, 297-305.

63. Shibata, Y.; Jin, K.; Morita, M. *Appl. Organomet. Chem.* **1990**, 4, 255-260.

64. Shibata, Y., Personal Communication, 1993.

65. Edmonds, Y., Personal Communication, 1995.

66. Edmonds, J. S.; Francesconi, K. A. *J. Chem. Soc. Perkin Trans. I* **1983**, 2375-2382.

67. Lee, K. E. *Phycology*, 2nd ed. **1989**, 585-595.

68. Sansoni, B.; Iyengar, G. V. 'Sampling and Storage of Biological Materials for Trace Element Analysis', in *Elemental Analysis of Biological Materials* **1980**, 57-71.

69. Robyt, J. F.; White, B. J. *Biochemical Techniques Theory and Practice* **1987**, 88-96.

70. Francesconi, K. A.; Micks, P.; Stockton, R. A.; Irgolic, K. J. *Chemosphere* **1985**, vol.14, no. 10, 1443-1453.

71. Beauchemin, D.; Bednas, M. E.; Berman, S. S.; McLaren, J. W.; Siu, K. N. M.; Sturgeon, R. E. *Anal. Chem.* **1988**, 60, 2209-2212.

72. Klumpp, D. W. *Marine Biology* **1980**, 58, 257-264.

73. Ang, P. O. Jr. Ph.D. Thesis, Department of Botany, University of British Columbia, Sep., **1991**.

Appendix I - The Equation of the Detection Limit of HGAAS

The detection limit is defined as the concentration which gives a signal equivalent to blank + 3 SD_b.

$$y = y_b + 3\ SD_b$$

where :

y = signal of the detection limit

y_b = signal of the blank

SD_b = standard deviation of the blank

This detection limit can be estimated by using :

$$y = a + 3\ S_{y/x}$$

where :

a = calculated y intercept in the regression line

$$S_{y/x}^2 = \frac{\sum_i (y_i - \hat{y}_i)^2}{n - 2}$$

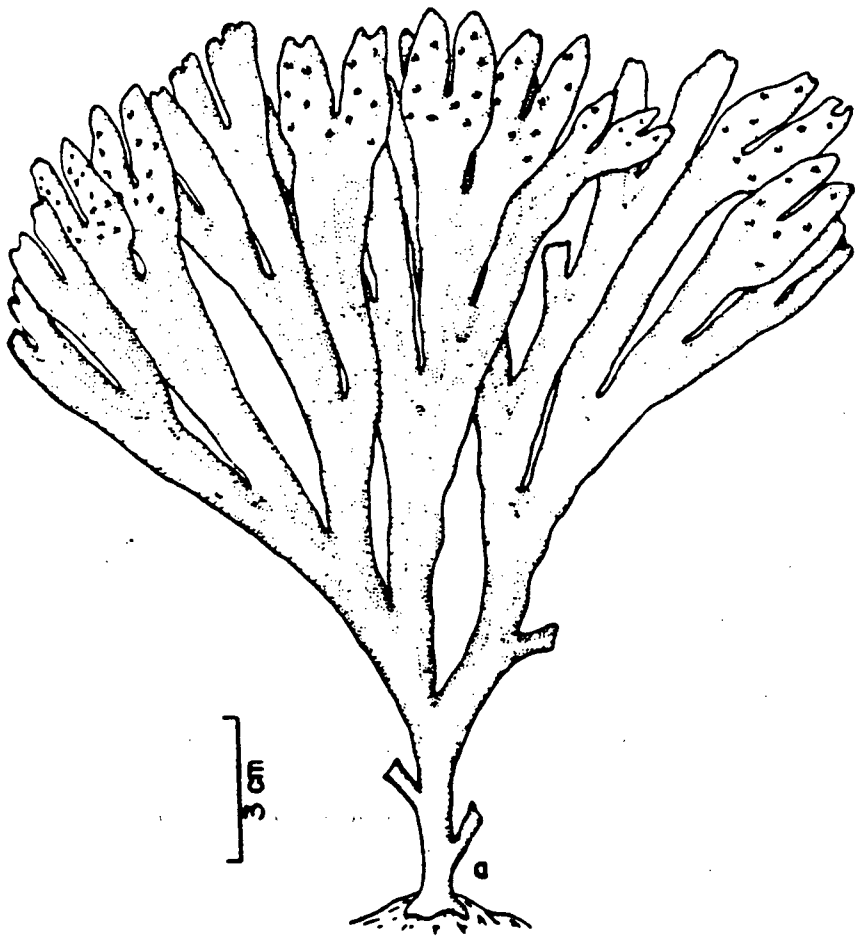
y_i = signal

$\hat{y}_i$ = calculated peak height of the signal

n = number of data points in the calibration curve

Reference : Miller, J. C.; Miller, J. N. *Statistics for Analytical Chemistry*, 2nd ed. **1988**, 110-120.

Appendix II - The Figure of *Fucus gardneri*



Fucus gardneri

Reference : Scagel, R. F. *Common Seaweeds of B.C.* **1967**, 158-159.