SPECIATION OF ARSENICALS

by

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Abstract

Environmentally and biologically important organoarsenicals such as arsenobetaine, arsenocholine, arsenosugars, and the tetramethylarsonium ion do not form volatile hydrides on treatment with sodium borohydride, even though hydride generation is a commonly used analytical technique for arsenic. Consequently, it has been difficult to determine the concentration of these so called "hidden" arsenicals. To this end a method has been developed in which all arsenicals are decomposed completely to arsenate by using microwave oven heated solutions of potassium persulfate and sodium hydroxide. A portable hydride generator is described. Radioactive tracer studies show that fast reactions and high efficiencies (95%) are achieved by using the new generator. A system which combines flow injection analysis, on-line microwave oven digestion, the new hydride generator, and atomic absorption spectrometry (FIA/MD/HGAAS) is developed and shown to be capable of differentiating arsenite, arsenate, monomethylarsonic acid (MMAA), and dimethylarsinic acid (DMAA) from the "hidden" organoarsenic compounds such as arsenobetaine which are usually present in crab, shrimp, and other crustaceans. This system is capable of performing analysis at a sample throughput of 100-120 per hour. Calibration curves are linear from 5 to 200 ng ml^{-1} of arsenic and the detection limit is 0.5 ng ml^{-1} for 100 µl injection (0.05 ng of arsenic). The system can be coupled to a high performance liquid chromatograph (HPLC) and used as a detector for arsenic speciation studies. An alternative system coupling HPLC to an inductively coupled plasma mass spectrometer (ICPMS) is also investigated for the speciation of arsenicals commonly present in the environmental and biological systems.

The analytical methods developed are successfully used to study the arsenicals present in marine animals, seaweeds, and human urine. Following human ingestion of crab and shrimp, which contain arsenobetaine as the major arsenic species, a fast urinary excretion of unchanged arsenobetaine is observed. No difference is found in either the

excretion pattern or the excreted arsenic species in the urine of six volunteers who ingested either crab meat or shrimp. In contrast the arsenosugars present in macroalgae are metabolized and have a longer retention in the human body. When nine volunteers ingested a commercial seaweed product Nori, which contains an arsenosugar as the major arsenic species, both the urinary arsenic excretion pattern and the urinary arsenic species excreted varied from individual to individual. The present work also reveals the presence of arsenosugars in addition to arsenobetaine in marine bivalves. These findings shed light on some important aspects of arsenic biogeochemistry including the toxicological implications of the consumption of seaweed, the metabolism of arsenosugars, the biochemical pathway to arsenobetaine, and ultimately the cycling of arsenic in the marine environment.

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LIST OF ABBREVIATIONS

AA	atomic absorption
AAS	atomic absorption spectrometry and/or spectrometer
AB	arsenobetaine
AC	arsenocholine
AES	atomic emission spectrometry and/or spectrometer
AFS	atomic fluorescence spectrometry and/or spectrometer
As(III)	arsenite and/or arsenious acid
As(V)	arsenate and/or arsenic acid
CCP	capcitively coupled plasma
cm	centimeter
DCP	direct current plasma
DMAA	dimethylarsinic acid and/or dimethylarsinate
FAAS	flame atomic absorption spectrometry
FIA	flow injection analysis
g	gram
GC	gas chromatography
GFAAS	graphite furnace atomic absorption spectrometry
HG	hydride generation and/or generator
HPLC	high performance liquid chromatography and/or chromatograph
hr	hour
ICP	inductively coupled plasma
ICPMS	inductively coupled plasma mass spectrometry and/or spectrometer
kg	kilogram
L	liter
m	meter

M	molar (mol L^{-1})
mA	milliampere
MD	microwave digestion (decomposition)
Me_4As^+	tetramethylarsonium ion
mg	milligram (10^{-3} g)
MHz	megahertz (10^6 Hz)
min	minute
MIP	microwave induced plasma
ml	milliliter (10^{-3} L)
mm	millimeter (10^{-3} m)
mM	millimolar (10^{-3} M)
MMAA	monomethylarsonic acid and/or monomethylarsonate
MS	mass spectrometry and/or spectrometer
m/V	mass-to-volume ratio
ng	nanogram (10^{-9} g)
nm	nanometer (10^{-9} m)
pg	picogram (10^{-12} g)
ppb	parts per billion
RSD	relative standard deviation
s	second
UV	ultraviolet
µg	microgram (10^{-6} g)
µl	microliter (10^{-6} L)
V/V	volume-to-volume ratio
W	watt

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Chapter 1. INTRODUCTION

1.1. GENERAL

Elemental speciation has been defined as the identification and quantitation of the individual physico-chemical forms of an element in a sample (1-4). These physico-chemical forms include inorganic compounds of elements in various oxidation states and organometallic compounds. Speciation studies are very important because the toxicity and availability of an element often depend on its chemical form. Arsenic is a typical example. It is virtually ubiquitous in the environment and living organisms (5-9). Examples of some major arsenic compounds of environmental and biological importance are shown in Table 1.1. Toxicity of these arsenic compounds varies greatly from the more toxic arsenite, to the moderately toxic methylated arsenicals, to the essentially non-toxic arsenobetaine, arsenocholine, and arenosugars (9, 11-16). Traditional approaches based on the determination of total elemental concentration are no longer sufficient to assess environmental impact and health risk.

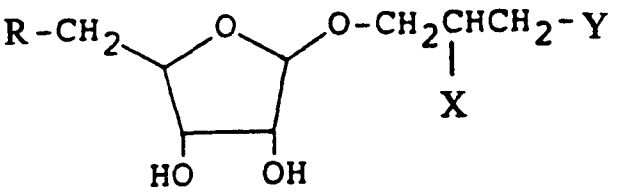
1.2. ARSENIC IN THE ENVIRONMENT

Arsenic is present in the earth's crust at an average concentration of 2-5 mg kg^{-1} and is associated primarily with igneous and sedimentary rocks in the form of inorganic arsenic compounds (5, 9, 17-20). It is found most frequently combined to sulfur, especially as arsenopyrite, FeAsS. Weathering of arsenic-containing rocks, which is considered to be the major natural source (21), liberates arsenic in the form of inorganic compounds.

Table 1.1. Some of the major arsenic compounds present in environmental and biological systems*

	Name	Abbreviation	Formula	pKa value#
I	Arsenite	As(III)	$As(OH)_3$	9.29, 13.5, 14.0
II	Arsenate	As(V)	H_3AsO_4	2.3, 6.8, 11.6
III	Monomethylarsonic acid	MMAA	$CH_3AsO(OH)_2$	3.6, 8.2
IV	Dimethylarsinic acid	DMAA	$(CH_3)_2AsO(OH)$	6.2
V	Trimethylarsine oxide	TMAO	$(CH_3)_3AsO$	
VI	Tetramethylarsonium ion	Me_4As^+	$(CH_3)_4As^+$	
VII	Arsenocholine	AC	$(CH_3)_3As^+CH_2CH_2OH$	
VIII	Arsenobetaine	AB	$(CH_3)_3As^+CH_2COO^-$	2.1^+
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	$(CH_3)_2As(O)$-	$-NH_2$	$-SO_3H$
XIV	$(CH_3)_2As(O)$-	-OH	$-OSO_3H$
XV	$(CH_3)_3As^+$-	-OH	$-OSO_3H$

*: The first 10 arsenic compounds are available in our laboratory and are used as standards for identification purpose.

#: pKa values are taken from reference 10.

+: From Cooney, R. V.; Mumma, R. O.; Benson, A. A. *Proc. Nat. Acad. Sci.* 1978, 74, 4262.

The concentration of arsenic in natural waters of rivers, lakes, and oceans is not constant. In fresh water systems, the arsenic concentration varies considerably with the geological composition of the drainage area and the extent of anthropogenic input (5, 21-23). Andreae *et al.* (22, 23) reported a geometric mean of 1.4 ng ml^{-1}, with a range of 0.1-75 ng ml^{-1}, of dissolved total arsenic in some European and American rivers. Similar large variations of arsenic concentration in fresh water systems have been reported by others (24-26). A concentration range of 0.4-80 ng ml^{-1} is typical (5, 11). As for speciation, arsenite, arsenate, monomethylarsonate (MMAA), and dimethylarsinate (DMAA) have all been found to be present and their proportions vary with the extent of anthropogenic input and biological activities (26-28).

Arsenic concentrations in seawater tend to be less variable than in freshwater, although in surface seawater concentrations may be subject to some seasonal changes due to biological uptake, particularly in highly productive coastal regions (29, 30). An arsenic concentration range of 1.0-1.8 ng ml^{-1} has been reported for deep Pacific and Atlantic waters (29-31). In coastal waters a wider range is observed, but 1-3 ng ml^{-1} is typical. Arsenate is the major arsenic species in seawater (12, 27); arsenite, MMAA, and DMAA are present at lower levels, usually under 10% of the total arsenic concentration.

The concentration of arsenic in marine algae is considerably above that in their surrounding water (5, 6, 12, 32-34). For example, an arsenic concentration of 95-109 $\mu g\ g^{-1}$ was found in a marine alga, *Laminaria digitata* (33). A variation of the total arsenic content between 0.4 and 32 $\mu g\ g^{-1}$ was reported for three main classes of macroalgae (34). Although a high concentration of arsenate (38% of the total arsenic) is present in brown algae, *Sargassum muticum*(35) and *Laminaria digitata* (12), generally only a small portion (usually under 10%) of the total arsenic present in the marine algae is in inorganic form (34, 36). The rest is present as organoarsenicals, both water soluble and lipid soluble (6, 32, 37, 38).

The major arsenic compounds present in marine macroalgae are arsenosugar derivatives (5, 6, 32, 37, 39, 40). Edmonds and Francesconi (32, 37) first identified the water soluble arsenosugar derivatives X, XI, and XII (Table 1.1) from the brown kelp, *Ecklonia radiata*, with XII being the major arsenic component (79%). Subsequently, this arsenosugar has been found to be a major arsenic compound in all sources of brown alga; arsenosugars X and XI appear to be ubiquitous in marine algae (6, 39). All the arsenosugar derivatives shown in Table 1.1, X-XV, have been found in various marine macroalgae (6, 15, 32, 37-42).

The concentration of arsenic species in marine and freshwater animals is also much higher than the background concentrations in the surrounding water. Cullen and Reimer (5) have summarized some typical arsenic concentrations found in marine animals, ranging from 0.3 $\mu g\ g^{-1}$ in salmon to a high of 340 $\mu g\ g^{-1}$ in the midgut gland of the carnivorous gastropod *Charonia sauliae*.

It had been known for many years that arsenic found in marine animals, usually referred to as "seafood arsenic", was chemically and physiologically different from inorganic arsenic (33, 43-45), but the chemical nature of the "seafood arsenic" was not much understood. In 1977, Edmonds *et al.*(46) isolated and identified arsenobetaine (VIII in Table 1.1) from the western rock lobster *Panulirus cygnus*. This compound was characterized by using several spectroscopic and chromatographic techniques and its structure verified by X-ray crystallography (46, 47). Since then arsenobetaine has been shown to be ubiquitous in marine animals, usually as the major arsenic species (5, 6, 9, 11).

In addition to arsenobetaine, the tetramethylarsonium ion (VI in Table 1.1) was first reported in the clam, *Meretrix lusoria,* by Shiomi *et al.*(49) in 1987. Subsequently, this compound has been found in sea hare and sea anemone (50), a gastropod mollusc (51), and five species of clams from British Columbia (52).

Some works claimed that arsenocholine found in shrimps (53-55), while others (56, 57) could not confirm its presence. The claim (53-55) was based mainly on a comparison of chromatographic retention times of a sample extract with that of a standard. Although a fast atom bombardment (FAB) mass spectrum with a peak at m/z 165, the parent ion of arsenocholine, was offered as supporting evidence (55), the rest of the mass spectrum showed little resemblance to that of the standard (55, 56). A study (58) of arsenic speciation in a standard reference material, DORM-1 (dog fish muscle, National Research Council of Canada), revealed that a trace amount of the tetramethylarsonium ion was previously (59) incorrectly identified as arsenocholine. The misidentification resulted because these two compounds showed similar retention time under the chromatographic conditions used (60). This example clearly illustrates the complexities involved in speciation studies.

While marine algae contain arsenosugars as the major arsenic species and do not contain arsenobetaine (6, 15, 32), some marine animals such as bivalves have been recently found to contain arsenosugars in addition to arsenobetaine (61). Originally arsenosugars X and XII were isolated from the kidney of the giant clam *Tridacna maxima,* and their presence was attributed to their formation by symbiotic algae which are also in the giant clam. However, bivalves such as *M. lusoria*, which have been found to contain arsenosugar XI (6), do not contain symbiotic algae, and it has been suggested (6) that bivalve's food source, phytoplankton, may be the origin of the arsenicals

The presence of organoarsenicals in marine organisms is commonly assumed to be due to the accumulation of compounds that have been synthesized from arsenate at low trophic levels. Arsenobetaine is believed to be the end product which is accumulated in higher trophic levels through the food chain (53, 63-66), although its actual metabolic origin is not clear. It is also proposed that arsenosugars present in marine algae are involved in the production of arsenobetaine at some stage in the food chain (5, 7, 9, 12, 67-69). It is unclear, however, how arsenosugars are transformed into arsenobetaine

within the higher trophic levels of the marine environment. The American lobster, *Homarus americanus*, retains arsenic in its muscle tissue as arsenobetaine (70), but is unable to synthesize arsenobetaine from organoarsenic compounds (presumably arsenosugars) obtained from ingested algae (71). Edmonds and Francesconi (72, 73) found dimethylarsinylethanol (IX in Table 1.1) as a major anaerobic decomposition product of the arsenosugar contained in the brown alga, *E. radiata*, and thought this to be an intermediate between arsenosugars in algae and arsenobetaine in marine animals. However, feeding experiments indicated that fish do not retain dimethylarsinylethanol (74). In contrast, when arsenocholine was administered orally to the fish, it was rapidly metabolized to arsenobetaine and efficiently retained in the fish body (74). Recently, Hanaoka *et al.* (75) found that arsenocholine was converted to arsenobetaine by micro-organisms present in sediments. Francesconi *et al.*(76) demonstrated that under conditions of anaerobic microbial activity, a minor constituent of marine algae, a trimethylarsonioriboside, was degraded to arsenocholine. Thus, they proposed that arsenocholine could be a precursor in the biosynthesis of arsenobetaine from trimethylarsonioriboside (76). However, whether or not trimethylarsonioribosides occur in algae in sufficient quantities to account for the high levels of arsenobetaine found in marine animals remains to be evaluated.

Degradation of arsenobetaine to inorganic arsenic is required to complete the biological cycling of arsenic. Microbial degradation has been demonstrated (77, 78) during the incubation of coastal water sediments with arsenobetaine. Under anaerobic conditions, arsenobetaine is degraded to trimethylarsine oxide, which is further broken down into DMAA, MMAA, and inorganic arsenic. However, it was found (79) that arsenobetaine as a sole carbon source could not support the growth of pure cultures responsible for the degradation and thus co-metabolism may be involved.

1.3. ARSENIC IN HUMAN URINE AND URINE ANALYSIS

A background level of 17.2 ng ml^{-1} of arsenic in human urine has been reported (80, 81) based on a survey of 148 subjects who had no occupational exposure to arsenic compounds and who had not eaten seafood within the preceding week. Of this total arsenic measured, inorganic arsenic amounted to 1.9 ng ml^{-1}, MMAA 1.9 ng ml^{-1}, DMAA 2.1 ng ml^{-1}, and the rest (~70%) was present in unidentified forms. Following seafood consumption, however, an elevated total urinary arsenic concentration of 132.2 ng ml^{-1} (mean value) was observed from 12 subjects. The consumption of seafood had little effect on the concentration of inorganic arsenic (2.1 ng ml^{-1}), MMAA (1.7 ng ml^{-1}), and DMAA (1.8 ng ml^{-1}).

Industrial and agricultural uses of arsenic compounds (82) can result in excess human exposure to arsenic, especially in occupations such as mining, smelting, glass making, and pesticide manufacture (83). In the work environment, exposure to As(III) is mainly in the form of As_2O_3 in non ferrous smelters and in glass making. Exposure to arsenate arises from its use in insecticides, cotton desiccants, and wood preservatives. Exposure to MMAA and DMAA is due to their use as selective herbicides. Rapid urinary excretion of these arsenic compounds from humans (83-89), monkeys (90), dogs (91), and hamsters (92) has been reported. Workers who are exposed to airborne arsenic compounds, particularly smelter workers who inhale As_2O_3, seem to eliminate this arsenic in urine, principally in the dimethylated form (93). In high-exposure groups the level of this metabolite can be as high as 6 times that of the control group. Reports of the biotransformation of inorganic arsenic in the body, to DMAA and MMAA, are numerous (84, 88, 92-97).

Chemical analysis of urine is a convenient method to use to assess occupational exposure as urine samples are readily obtained non-invasively. Such analysis can provide important information about exposure to many chemical species as well as information about many of the body's metabolic functions. The importance of urine examination in

health and disease was realized as early as 400 B.C (98-100). Diagnosing disease by visual examination of urine - referred to as uroscopy - was very popular among ancient medical practitioners. Although they lacked sophisticated equipment, physicians were able to obtain diagnostic information from such basic observations as color, volume, turbidity, viscosity, odor, and even sweetness. Interestingly, these same urine characteristics are currently reported in clinical laboratories, although modern urinalysis includes both the physical examination of urine and chemical analysis. In addition to routine chemical analysis, the determination of trace elements in urine has gained much attention (101-103) and the result provides a useful tool in health assessment. Urinary excretion is the major pathway for the elimination of arsenic compounds from the body (43, 84-87), therefore, urinary arsenic determination is important for assessing occupational exposure and for studying the metabolism of arsenic compounds in human body (80, 83, 104).

1.4. SPECIATION OF ARSENIC COMPOUNDS

1.4.1. Selective determination

Two major types of technique have been studied extensively for arsenic speciation: selective determination, and chromatography followed by spectrometric detection. Most early work on arsenic speciation was based on the selective determination of arsenite and arsenate by using a spectrophotometric method. The molybdenum blue method, which involves measuring the absorption of a blue complex (λ= 865 nm) formed between arsenate and a molybdate color-forming reagent, has been commonly used (105-107). Arsenite does not form this complex with the same reagent and its concentration can be obtained by the difference of two measurements made before and after it is oxidized to arsenate. As full color development of this method requires a reaction time of 2-4 hr such methods are consequently slow. The method is also susceptible to interferences from phosphate which forms a similar blue complex with the

molybdate (106, 108-110). Organoarsenic compounds can not be determined by using this method.

1.4.2. Hydride generation

Determination of arsenic species by using hydride generation techniques is based on the formation of volatile arsines upon chemical treatment of a sample with a reducing agent (111, 112). Initially a metal-acid reaction system, usually consisting of zinc and hydrochloric acid, was used to generate arsines (113-115). In this method arsenate has to be reduced to arsenite, by using potassium iodide for example, prior to the formation of arsine (AsH_3). A reaction time of over 20 min is usually required in order to react quantitatively with trace amounts of arsenic.

A better hydride generation system is based on the use of sodium borohydride ($NaBH_4$) as the reducing agent. This was first described in 1972 by Braman *et al.*(116) for spectrometric analysis. A fast and complete reaction can be achieved by using sodium borohydride, resulting in rapid determination. Therefore, the sodium borohydride reaction system has virtually replaced the metal-acid reaction system for analytical methods involving hydride generation (117-119).

Several arsenic species can form arsines upon treatment with sodium borohydride in an acid medium: arsenite and arsenate give AsH_3, MMAA gives CH_3AsH_2, and DMAA gives $(CH_3)_2AsH$ (27, 28, 120). The formation of these arsines is acid dependent (27, 28, 121), and selective determination of arsenite in the presence of arsenate can be carried out by carefully selecting an acid and optimizing the acid concentration for hydride generation (121-123).

A technique involving coupling hydride generation with cryogenic trapping has been developed (27, 28, 120, 124) for arsenic speciation. It is based on the difference in the boiling points (28) of the various arsines as given below (^oC): AsH_3, -55; CH_3AsH_2, 2; $(CH_3)_2AsH$, 35.6; and $(CH_3)_3As$, 70. Arsines produced in a reaction vessel are swept

to and trapped in a U-shaped Teflon tube immersed in liquid nitrogen. After complete trapping of arsines, liquid nitrogen is removed, and the U-tube is warmed up. The hydrides evaporate upon heating, and are transported to a detection system, where they are detected sequentially. Thus the concentration of MMAA, DMAA, and the sum of arsenite and arsenate can be obtained. By regulating the pH of the reaction medium, for example pH 5, arsenite can be selectively determined without interference from arsenate. Modification of this system has been made by incorporating a gas chromatograph between the liquid nitrogen trap and the detector (125, 126). In this way the separation of the arsines is further improved.

Hydride generation techniques have been shown to be very useful for trace analysis. By coupling hydride generation with spectrometric techniques, detection limits of 2-3 orders of magnitude better than the conventional nebulization methods have been achieved (111, 112, 117-119). The major limitation in using hydride generation for arsenic speciation, however, is that some environmentally and biologically important organoarsenicals such as arsenobetaine do not form an arsine upon treatment with sodium borohydride. Thus most studies on arsenic speciation that use hydride generation have dealt primarily with arsenate, arsenite, MMAA, and DMAA (27, 28, 120-128).

A somewhat related thermochemical hydride generation technique has been described recently (129) for the determination of arsenobetaine, arsenocholine and the tetramethylarsonium ion. This technique does not require the use of sodium borohydride. Instead, samples are pyrolyzed in a methanol-oxygen flame, and analytes are derivatized to hydrides in the presence of an excess of hydrogen.

1.4.3. Chromatographic methods

The most commonly used "comprehensive" speciation techniques often involve a combination of chromatographic separation with spectrometric detection. Both gas and liquid chromatography have been popular as separation techniques (4, 130-132). Some

recent studies also involve the use of supercritical fluid chromatography (SFC) (133, 134). Gas chromatography (GC) requires the species or its derivatives to be volatile and thermally stable. For example, the formation of methyl thioglycolates of arsenic compounds makes these compounds amenable to GC analysis (135, 136).

For many organometallic compounds that are not sufficiently volatile for GC separation, high performance liquid chromatography (HPLC) becomes the method of choice (137, 138). Some advantages in using HPLC include (i) its capability of separating non-volatile and thermally labile compounds - no derivatization is required; (ii) minimum sample preparation - aqueous samples can be directly analyzed; and (iii) operational parameters concerning both the stationary and mobile phases can be varied to achieve better separation. Thus HPLC is more suitable for the separation of arsenic compounds in the environment, most of which are water soluble and non-volatile.

Three major HPLC systems, ion exchange, reversed phase ion pair, and gel permeation, have been used for the separation of arsenic compounds. At neutral pH, arsenate, MMAA, DMAA, and arsenosugars are present as anions; arsenobetaine, arsenocholine, and the tetramethylarsonium ion as cations; and arsenious acid as an uncharged species. Thus both anion exchange (139-148) and cation exchange (140, 146-148) chromatography have been commonly used for the separation of ionic arsenic species. Reversed phase ion pair HPLC has also been used (59, 146, 149-152) with appropriate counter ions, e.g. tetramethylammonium cation or heptanesulfonate anion, in the mobile phase. The counter ion forms an ion pair with oppositely charged analyte ions and therefore additional interactions are introduced resulting in better separation. Gel permeation HPLC has been used for the separation of arsenosugars (140, 149). In order to separate both anionic and cationic arsenic species in a single run, a column switching system involving a combination of anion exchange and reversed phase HPLC has been developed (153).

The coupling of chromatography with various sensitive detectors that are used in atomic spectrometry has lead to significant improvements in the case of performing element speciation studies. A major concern in this coupling is the interface. The flow rate of a gas or a liquid through a GC or HPLC column must be matched or adjusted to the gas or liquid uptake rate of a particular detector. A number of recent reviews have dealt with this aspect extensively (1, 130-132, 154-156).

Flame atomic absorption spectrometry (FAAS) (157-160), graphite furnace atomic absorption spectrometry (GFAAS) (55, 129, 146, 150, 161, 162), atomic emission spectrometry (AES) (156, 163, 164) and atomic fluorescence spectrometry (AFS) (165, 166) with various plasma excitation sources, and plasma mass spectrometry (MS) (1, 59, 167, 168) are among the spectrometric detectors commonly used to couple with the HPLC for element speciation studies. Extensive discussions on these HPLC/spectrometry techniques can be found in a number of recent reviews (1, 4, 130-132, 154-156). Thus only a brief discussion on HPLC/ICPMS is given below.

1.4.4. HPLC/inductively coupled plasma mass spectrometry (ICPMS)

A plasma is a gas or a mixture of gases in which a significant fraction of the atoms or molecules is ionized (169-172). Inductively coupled plasma (ICP) (169-173), direct current plasma (DCP) (173-175), microwave induced plasma (MIP) (173, 176, 177), and capacitively coupled plasma (CCP) (178, 179) have all been used for spectrochemical analysis, with the ICP being the most popular choice (169-173, 180, 181). The basic operation of the ICP has been documented extensively in the literature (169-172, 181). Briefly, the ICP is formed in a quartz torch consisting of three concentric tubes where the interaction of a radiofrequency field with a flowing plasma gas, usually argon, occurs. Power (usually 1-2 kw) is applied to the torch via a water-cooled copper load coil by use of a radiofrequency generator that typically operates at 27 or 41 MHz. The plasma is produced when initial "seed" electrons from an external source are

supplied in the region of the induction coil. These electrons ionize the neutral plasma gas. Once the plasma gas conducts, the plasma forms spontaneously and maintains temperatures of 6000-8000 K.

Total sample desolvation, nearly complete solute vaporization, and a high atomization/ionization efficiency can be achieved when a sample is introduced to the ICP, for example as a liquid aerosol. Thus the ICP has been used as an atomization/excitation source for atomic emission spectrometry (AES), allowing simultaneous and multielement analysis (169-172, 181). The efficiency of the ICP in producing singly-charged positive ions for most elements makes it an effective source for atomic mass spectrometry (182-186).

The coupling between the ICP which operates at atmospheric pressure and a mass spectrometer which operates under vacuum (usually below 10^{-6} torr) is accomplished through an evacuated sampling cone and a skimmer cone of small diameter (~1 mm) (182-186). Only a small fraction of the ions present in the ICP is extracted into the mass spectrometer. A quadrupole mass analyzer with unit mass resolution is commonly used in an ICPMS instrument. Typical components of a commercial ICPMS instrument are shown schematically in Figure 1.1.

Solid, gaseous, and liquid samples can be introduced into the ICPMS for analysis, but liquid sample introduction is by far the most common and convenient method (1). Pneumatic nebulizers, including concentric and cross-flow types, are commonly used. A V-groove nebulizer is useful when dealing with samples having high salt content. A spray chamber is used to separate larger droplets produced by the nebulizer and to reduce the solvent load to the plasma.

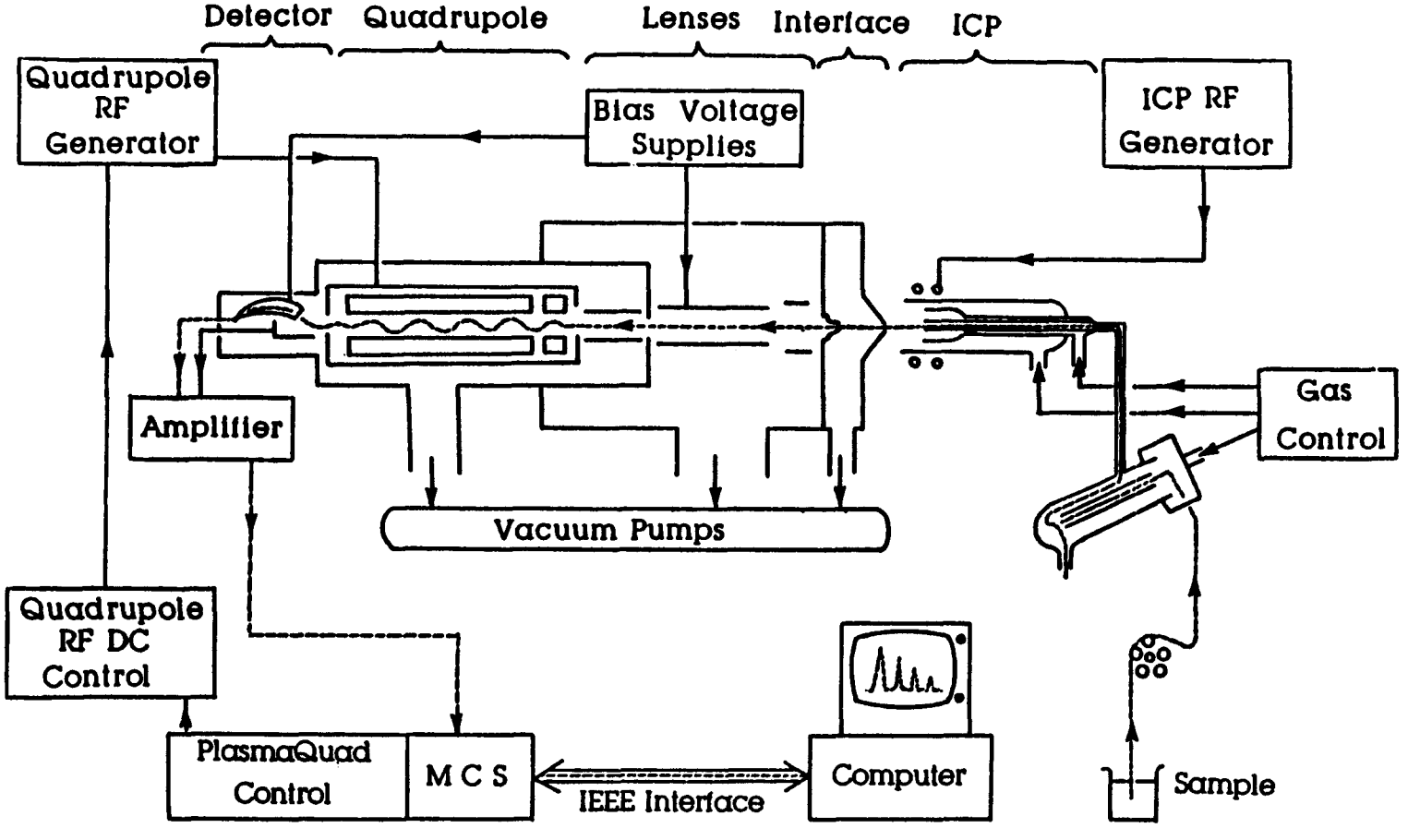


Figure 1.1. A schematic diagram showing the major components of a commercial ICPMS instrument, PlsamaQuad Turbo 2+ (VG Elemental, Fisons Instrument)

Typical HPLC flow rates, on the order of 0.5-2 ml min^{-1}, are within the range usually required for liquid sample introduction to the ICP by using traditional pneumatic nebulization. Therefore the interface between the HPLC and the ICPMS is straightforward. The shortest possible length of narrow-bore tubing of inert material (usually Teflon) connecting appropriate HPLC fittings is often adequate (1, 59, 168).

HPLC/ICPMS has been used to study the speciation of arsenic compounds in a variety of samples (1, 15, 58, 59, 61, 145, 149, 167, 168, 187). Both ion exchange and ion pair chromatography have been used for the separation. The good detection limits obtained, 20-300 pg, suggest that the HPLC/ICPMS is particularly useful for the speciation studies related to environmental and biological systems where extremely low detection limits are often required.

1.5. OBJECTIVES AND OVERVIEW OF THE THESIS

The overall objective of this work was to study arsenic speciation in environmental and biological systems. Both the development of analytical methods and their applications to studies of systems of interest are described. Because arsenic is often present in complex matrices at very low concentrations in these systems selective and sensitive analytical methods are required. The methods should also be reliable and easy to operate.

Investigations into hydride generation atomic absorption spectrometry were carried out by using an AAS instrument available in the laboratory. This lead to the development of new hydride generators, offering improved signal-to-noise ratio and thus a better detection limit (Chapter 2).

During the course of this study, it was found that arsenite, arsenate, MMAA, and DMAA do not give the same response upon hydride generation because the formation of arsines from these arsenic species is pH dependent. Consequently, the commonly used method for the determination of arsenic under a compromized acid concentration is

subject to error. The addition of cysteine into the sample solution was studied to eliminate this error and the effect of cysteine is discussed in Chapter 3.

Arsine generation techniques as usually applied are only useful for the determination of arsenite, arsenate, MMAA, and DMAA. Environmentally and biologically important arsenic species, arsenobetaine, arsenocholine, arsenosugars, and the tetramethylarsonium ion do not form hydrides. In order to determine these "hidden arsenicals", decomposition methods were studied to convert these arsenicals quantitatively to a form amenable to further analysis. A successful microwave assisted digestion system is described in Chapter 4. The methodology was applied to the determination of urinary arsenic and to the differentiation of the more toxic hydride-forming arsenic species from the less toxic non hydride-forming species.

In order to study individual arsenic species, further separation of arsenic compounds was carried out by using HPLC. The on-line microwave assisted digestion HGAAS system which was evaluated in a FIA mode (Chapter 4) was used as an element specific detector for HPLC. An ICPMS instrument was also coupled to the HPLC for arsenic speciation studies. Both HPLC/MD/HGAAS and HPLC/ICPMS systems are discussed in chapter 5.

The methods developed in Chapters 2-5 were then used to study arsenic speciation in seaweed and marine animal samples (Chapter 6). These methods were also used to study the urinary arsenic excretion behavior following human ingestion of arsenosugars (in seaweed product Nori) and arsenobetaine (in crab and shrimp) (Chapter 7).

Chapter 2. DEVELOPMENT OF NEW CONTINUOUS HYDRIDE GENERATORS FOR THE DETERMINATION OF ARSENIC, ANTIMONY, AND TIN BY HYDRIDE GENERATION ATOMIC ABSORPTION SPECTROMETRY

2.1. INTRODUCTION

Hydride generation techniques coupled with atomic spectrometry have found wide application in trace analysis (112, 117). As an efficient sample introduction method, hydride generation enhances sensitivity normally by 10-1000 fold over the more commonly used liquid sample nebulization procedures (111, 112, 117-119). Two major operation modes are used in hydride generation systems, batch mode and continuous mode. In the batch mode, the sample and reducing reagent (usually sodium borohydride) are injected into a reaction vessel and any hydrides produced are then purged and carried by a stream of carrier gas, either directly into a detector, or collected in a trap before being introduced into a detector. Sometimes the hydrides reach the detector via a gas chromatograph to separate the products. With continuous systems, however, sample solution and borohydride solution are continuously delivered to a reaction vessel for the production of hydride. The hydride of an analyte is constantly generated and is separated from the liquid waste with the aid of a gas/liquid separator. Gases generated from the reaction are subsequently introduced into a detector for measurement. The continuous system is simpler to operate than the batch system and easier to automate. It also achieves better precision (119) over the batch system because in the batch system, peak height and peak shape are critically dependent on some factors which are difficult to control, particularly when collection methods such as cold trapping are involved.

Continuous hydride generators developed by Vijan and Wood (190) and Thompson *et al.* (191) have been widely used with very little change in design. In these instances, sample and reagent solutions are introduced by using a proportioning pump

(190) or a peristaltic pump (191) and meet at a T-joint where reaction takes place. In Vijan and Wood's design (190), a glass mixing coil is connected to the T-joint to allow sufficient time for the sample and reagents to mix and to react. Following the reaction coil, a gas/liquid separator is used to separate hydrides from waste solution. Hydrides are subsequently introduced to a flame atomizer for atomic absorption measurement (190) or to an ICP spectrometer for optical emission measurement (191). Although considerable work has been carried out on the application of continuous hydride generators to trace analysis (112, 117, 192), modification of the design, to further improve sensitivity and selectivity, has received little attention (193-196). De Andrade *et al.* (193) used a Teflon membrane phase separator instead of the commonly used reaction flask gas/liquid separators (190, 191, 197, 198) for the determination of mercury by using flow injection analysis (FIA) cold vapor generation AAS. The authors optimized their system but offered no comparison to the conventional system. Pacey *et al.* (194) also examined the use of a membrane gas/liquid separator in the determination of arsenic by FIA/HGAAS; hydrides are more efficiently separated from the waste solution and interferences are reduced. A similar microporous polytetrafluoroethylene (PTFE) tubing gas/liquid separator was also studied by Barnes and Wang (195).

A simpler continuous hydride generator system has been described by Dedina (196), who combined a reaction vessel and a gas/liquid separator in one unit and studied its optimization for the determination of selenium. When a similar system was initially tested in our laboratory for arsenic determination, however, the signals obtained were rather noisy.

In the present work, a new continuous hydride generator in which a Buchner funnel serves as both a reaction chamber and a gas/liquid separator was developed. The carrier gas flow was introduced from the bottom of the funnel to create efficient solution mixing and gas/liquid separation, resulting in an improvement in the signal-to-noise ratio. Radioactive tracer studies were carried out to characterize the hydride

generation/release efficiency of this new system. A similar hydride generator was also developed for FIA system which maintains the high reaction efficiency.

2.2. EXPERIMENTAL

2.2.1. Instrumentation

Atomic absorption measurements were performed with a Varian Model AA 1275 atomic absorption spectrophotometer equipped with a standard Varian air-acetylene flame atomizer. A deuterium background corrector was used. A Hewlett Packard 3390A integrator was connected to the AA spectrometer to record flow injection transient signals, continuous flow steady state signals, and noise levels. Absorbance values were also printed out on a Hewlett Packard 82905A printer. A conventional (119, 190, 199) open-ended T-shaped quartz absorption tube (11.5 cm x 0.8 cm i.d.) was mounted in the air-acetylene flame of the burner. Light from both the arsenic hollow cathode lamp and the deuterium lamp was aligned to pass through the flame heated quartz tube. Hydrides were introduced to the quartz tube from its side arm for atomization and AA measurements.

Continuous flow hydride generation

The hydride generator shown in Figure 2.1 was used for most of the studies. A Gilson Minipuls 2 four-channel peristaltic pump was used to deliver sample, acid, and sodium borohydride solutions into the combined reaction and gas/liquid separation apparatus. This glass apparatus consists of an inner Buchner funnel and an outer glass cylinder. Reactions take place in the funnel where the sample and reagent solutions are mixed. Carrier gas (nitrogen) continuously flows through the fritted disc of the inner funnel and carries hydrides into the quartz absorption tube mounted in the flame for AA

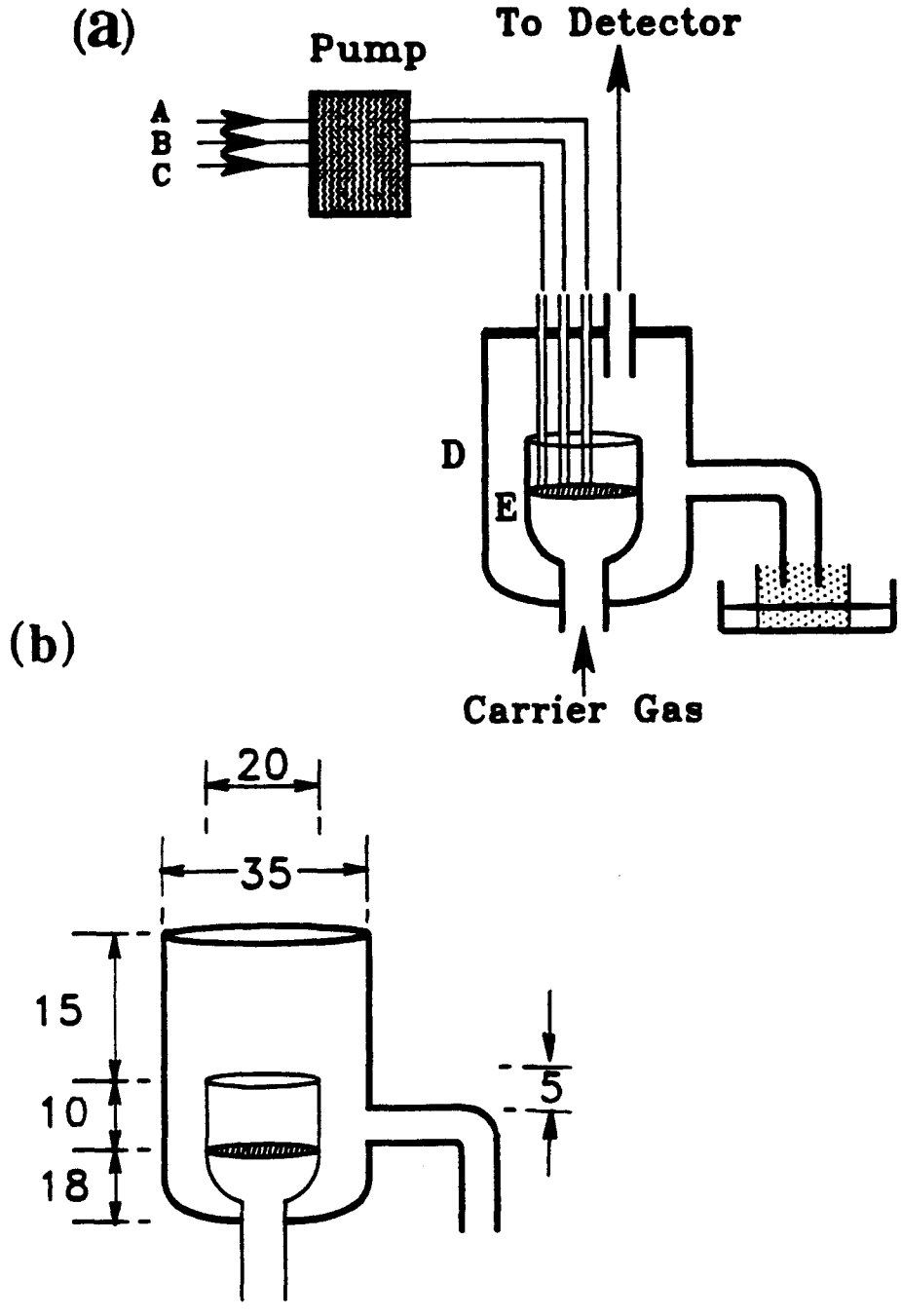


Figure 2.1. Schematic diagram of a new hydride generator.
Dimensions are in mm.
A, B, and C -- sample, $NaBH_4$, and acid solution flow
D -- outer glass cylinder
E -- inner funnel

measurement. Waste solution overflows from the inner Buchner funnel to the outer cylinder. The liquid waste in the outer cylinder drains out through a side arm into an open reservoir that can overflow. This arrangement ensures constant pressure inside the apparatus, maintains constant drainage of liquid waste, and prevents loss of hydride.

Hydride generators made of Buchner funnels (Corning Glass Works, USA) with fine porous glass frits (nominal maximum pore size 4-5 microns) showed lower noise level in hydride generation AAS determination than those with coarse frits (Pore size 40-60 microns) and medium frits (pore size 10-15 microns). Therefore commercially available 15-ml Buchner funnels (20 mm i.d.) with fine porous glass frits were used. The height of the inner funnel above the fritted disc was optimized with respect to signal-to-noise ratio, for arsenic, antimony, and tin determinations. It was found that for all these three elements funnel heights in the range of 10 to 20 mm showed effectively constant signal-to-noise ratio. When this height was decreased to 5 mm, the absorption signal from a mixture of arsenic compounds (containing As(III), As(V), MMAA, and DMAA) was slightly reduced. Therefore, a funnel height of 10 mm was chosen for subsequent work. This corresponds to an inner volume of the funnel of approximately 3 ml and allows a short signal rise time while achieving a maximum signal-to-noise ratio.

The height of the outer glass cylinder between the inner funnel and the stopper of the outer cylinder was also optimized. It is desirable to have a small volume for this outer cylinder in order to minimize the dead volume. As expected when the height of the outer glass cylinder was changed from 45 mm, to 30 mm, to 15 mm, a shorter time for reaching a steady state signal was observed. Within this range there was essentially no difference in the signal-to-noise ratio observed. When the height was further decreased, aerosol from the reaction accumulated in the hydride transport line, causing signal fluctuation. Therefore, a height of 15 mm between the inner funnel and the stopper of the outer cylinder was chosen for further work. These optimized dimensions are shown in Figure 2.1b.

For comparison, a conventional continuous hydride generator similar to that described by Thompson *et al.* (191) was also used. It consisted of a Gilson Minipuls 2 four-channel peristaltic pump, an 18-turn glass mixing coil, and a gas/liquid separator. The peristaltic pump was used to introduce sample, acid and sodium borohydride solutions into the gas/liquid separator via the mixing/reaction coil. The hydrides were stripped with carrier gas (nitrogen) and led into the quartz absorption cell for AA measurement.

FIA/HGAAS

Another hydride generator was developed to couple with flow injection analysis (FIA) as shown in Figure 2.2. Sample injection was accomplished by using a Rheodyne 6-port sample injection valve (V) fitted with a 100 µl sample loop. The injected 100 µl sample, carried by a stream of deionized water, meets with continuous flows of the reagents, hydrochloric acid and sodium borohydride. Hydride generation takes place upon mixing of these solutions and continues in the new reactor-gas/liquid separator apparatus. A medium porosity gas dispersion tube is located in the center of this glass apparatus. As the carrier gas (G) flows through the porous glass tube, fine bubbles are generated. These fine bubbles assist mixing and reaction and create an efficient gas/liquid separation. Hydrides produced are carried to the AAS detector (D). Waste solution from the hydride generator constantly drains out via a side arm arranged to maintain a constant pressure. Compared with the hydride generator described above, this version is smaller, has minimum dead volume, and it is therefore particularly suitable for the FIA system.

A Packard 1900 TR Liquid Scintillation Analyzer (Canberra Packard Canada) was used to measure the radioactivity of tritium (^3H) in radioactive tracer studies with labeled compounds.

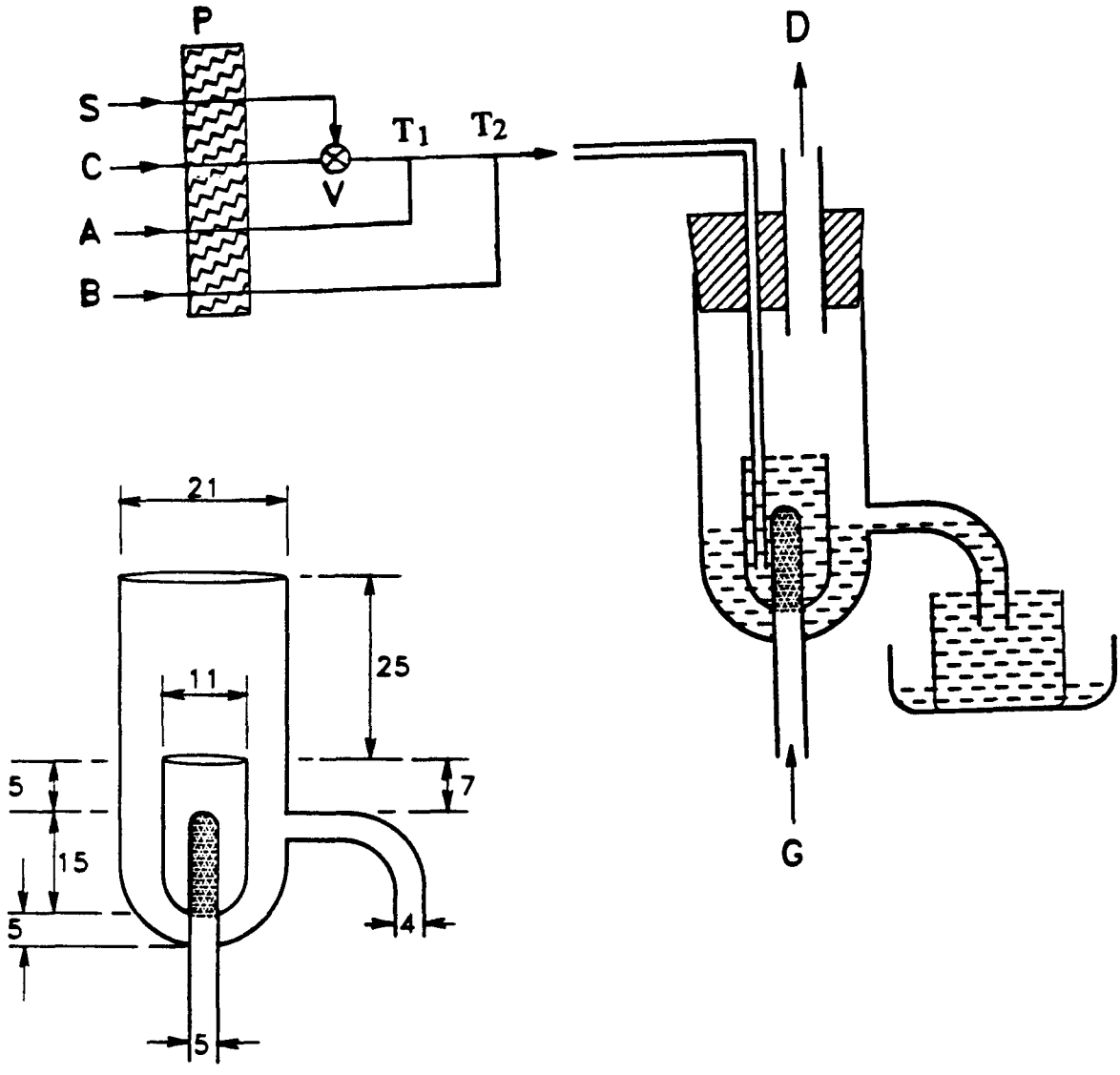


Figure 2.2 Schematic diagram of a flow injection hydride generation system.
Dimensions are in mm.
S, C, A, and B -- sample, carrier, acid and $NaBH_4$ flows, respectively
P -- peristaltic pump
V -- injection valve
T1 and T2 -- T-joints
D -- to detector (AAS)
G -- carrier gas (N_2)

2.2.2. Reagents

All reagents used were of analytical reagent grade or better. Aqueous stock solutions (1000.0 μg ml^{-1} of arsenic) of arsenite, arsenate, MMAA and DMAA were prepared by dissolving appropriate amounts of As_2O_3 (BDH), $Na_2HAsO_4.7H_2O$ (MCIB), $CH_3AsO(ONa)_2.6H_2O$ (Alfa), and $(CH_3)_2AsO(OH)$ (Fisher). The arsenic concentrations in arsenate, MMAA and DMAA solutions were standardized against an arsenite solution by using both ICPAES and flame AAS. A stock antimony solution (1000.0 μg ml^{-1}) was prepared by dissolving $SbCl_3$ (BDH) in 2 M hydrochloric acid and diluting with 1 M hydrochloric acid. A stock solution of tin (1000.0 μg ml^{-1}) was prepared by dissolving granular metal tin (Mallinckrodt) in 4 M hydrochloric acid and diluting with 1 M hydrochloric acid. Standard solutions were prepared by serial dilutions of these stock solutions with 0.01 M hydrochloric acid unless stated otherwise. Sodium borohydride (Aldrich) solutions were made fresh in 0.1 M sodium hydroxide (BDH) and filtered before use. L-cysteine (1%) (BDH) was prepared in the sample solution or in hydrochloric acid solution.

A liquid scintillation counting cocktail (Fisher) was used in radioactive tracer experiments.

2.2.3. Procedures

Except for the interference studies, three channels of the peristaltic pump were used to introduce respectively the sodium borohydride solution, the acid solution, and the standard solutions of arsenic, antimony, or tin in either 0.01 M hydrochloric acid or 0.01 M hydrochloric acid containing 1% L-cysteine. Hydrides and hydrogen produced during the hydride generation process were continuously stripped with nitrogen carrier gas and introduced into the quartz absorption tube for flame AA measurement. Absorbance readings were recorded by using a printer, and continuous steady state signals were recorded by using a plotter/integrator. Signal heights and noise levels were measured to

obtain signal-to-noise ratios. The instrument parameters and optimized hydride generation conditions are listed in Table 2.1.

For interference studies, sodium borohydride solution (in 0.1 M NaOH), the interfering ion solution (Ni^{2+} in 0.01 M hydrochloric acid, Cu^{2+} and Co^{2+} in 0.01 M nitric acid), and standard solution (As and Sb in 2 M hydrochloric acid, Sn in 0.5 M hydrochloric acid) were introduced with the aid of the peristaltic pump. When needed, cysteine (1%) was added to the standard solutions to evaluate its effect on the interference. Relative responses of analyte signals were measured by comparing the absorbance of an analyte in the presence of the interfering ion with that of the standard analyte solution in the absence of any interfering ion.

For radioactive tracer studies, the methyl- group of MMAA ($CH_3AsO(OH)_2$) was labeled with tritium (^3H, $t_{1/2}$=12.3 years). Solutions of the labeled MMAA (16 µM, 160 µM, and 1.6 mM) were used during this study. A liquid scintillation counting (LSC) cocktail (7 ml) was mixed with 100 µl of liquid sample in a measuring vial prior to liquid scintillation counting. A mixture of 100 µl deionized water and 7 ml of LSC solution was used as blank. Each sample was counted for 2 minutes and the radioactivity of ^3H in the sample was measured in counts per minute (CPM) and disintegrations per minute (DPM).

To study the rate of hydride generation, the new system (Figure 2.1) was operated in batch mode as follows. While the stream of carrier gas was maintained through the porous glass of the inner funnel, 2 ml of acidified labeled MMAA solution (in 1 M hydrochloric acid) was injected with a syringe into the Buchner funnel followed by 1 ml of 2% (m/V) sodium borohydride solution. At measured intervals of time after the sodium borohydride solution was added, 100 µl of the reaction mixture was withdrawn from the reaction mixture by using an Eppendorff pipette and placed into a liquid scintillation counting vial which was immediately capped and placed in a liquid scintillation counter for measurement.

Table 2.1. Experimental Conditions *

Parameters	As	Sb	Sn
Wavelength (nm)	193.7	217.6	224.6
Hollow Cathode Lamp Current (mA)	8	8	8
Spectral Bandwidth (nm)	1.0	1.0	1.0
Sample uptake rate (ml min^{-1})	5.1	5.1	5.1
Acid uptake rate (ml min^{-1})	4.7	4.7	4.7
$NaBH_4$ uptake rate (ml min^{-1})	4.5	4.5	4.5
HCl Concentration (M):			
Without cysteine	2.3 (2.0-4.0)	2.2 (1.0-4.0)	0.80 (0.60-0.80)
With 1% cysteine	0.10 (0.050-0.20)	0.20 (0.010-0.50)	0.10 (0.050-0.15)
$NaBH_4$ Concentration (%, m/V):			
Without cysteine	1.8 (1.0-2.0)	1.0 (0.5-1.3)	1.3 (1.0-1.5)
With 1% cysteine	1.8 (1.0-4.0)	1.0 (0.50-6.0)	2.0 (1.3-6.0)
Carrier Gas Flow Rate (ml min^{-1}):			
Without cysteine	90 (70-100)	105 (80-130)	120 (80-130)
With 1% cysteine	90 (70-150)	105 (80-130)	120 (80-130)

* : Numbers in parenthesis indicate optimum ranges for the corresponding parameters.
Values underlined are optimum conditions reached by using the simplex optimization method.

2.3. RESULTS AND DISCUSSION

2.3.1. Hydride generators

Determinations of arsenic, antimony, and tin were carried out to demonstrate the application of the new hydride generator. A conventional hydride generator consisting of a sample delivery system (a four-channel peristaltic pump), an 18-turn mixing/reaction glass coil, and a gas/liquid separator similar to that designed by Thompson *et al.* (191) was constructed and used for comparative measurements. This type of generator has been widely accepted for spectrochemical analysis. Figure 2.3 compares typical signals obtained by using the new generator (Figure 2.3 b1-b3) with those obtained by using the conventional system (Figure 2.3 a1-a3) in the determination of arsenic (a1, b1), antimony (a2, b2), and tin (a3, b3). A significant enhancement in signal-to-noise ratio is achieved when the new generator is used in the determination of all three elements. The improvement is probably due to an improved reaction efficiency and gas/liquid separation efficiency resulting from the fine bubbles of carrier gas flowing through the reaction mixture. This may help both the mixing of the sample and reagents, and the transport of the hydrides to the detector.

2.3.2. Hydride generation efficiency

To evaluate the efficiency of hydride generation and release with the hydride generator, a number of radioactive tracer studies were performed. MMAA ($CH_3AsO(OH)_2$) with a ^3H-labeled methyl group was used in the hydride generation reaction. The apparatus was first used as a batch-type hydride generator to study the rate of reaction. Figure 2.4 shows the radioactivity of the sample in disintegrations per minute (DPM) vs. time after the labeled sample and sodium borohydride were mixed. Since ^3H has a half life of 12.3 years, its natural decay is negligible within the short time frame of this experiment. Thus the change in DPM reflects the change in unreacted MMAA

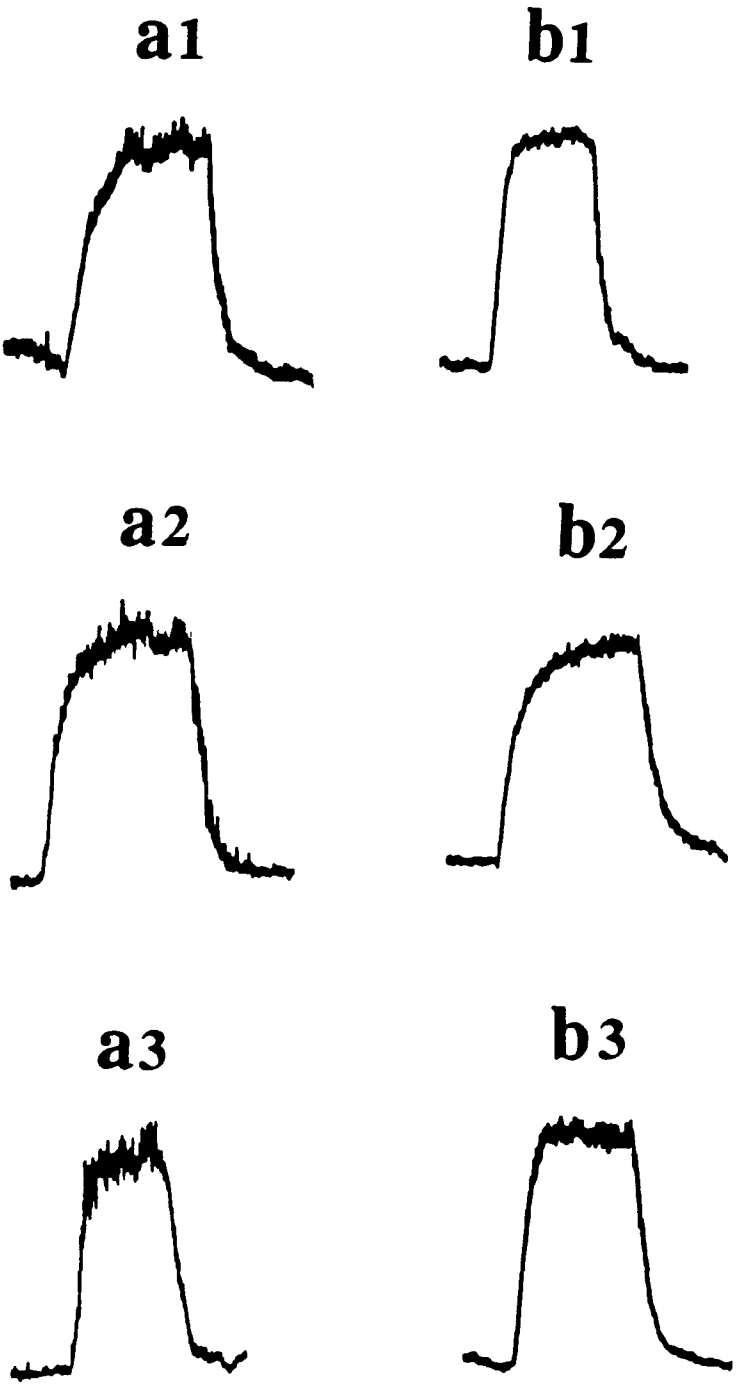


Figure 2.3. Comparison of typical signals obtained by using a conventional hydride generator (a1-a3) and by using the new generator (b1-b3) in the determination of 10 ng ml^{-1} of arsenite (a1 and b1), 20 ng ml^{-1} of antimony (a2 and b2), and 20 ng ml^{-1} of tin (a3 and b3). Note the improvement in the signal-to-noise ratio achieved by using the new hydride generator.

present in the hydride generator. The percentage of MMAA left in the hydride generator calculated from the DPM values is also shown in Figure 2.4. These results are the mean values of three replicate experiments with a relative standard deviation (RSD) of approximately 15%. This high RSD value arises because of the difficulty in taking precise aliquots of the reaction solution while the hydride generation reaction is taking place. The value at time 0 in Figure 2.4 corresponds to the MMAA solution (16 μM) in the hydride generator prior to the addition of sodium borohydride solution. Once sodium borohydride is added, a dramatic decrease of MMAA in the generator to approximately 10% of the original concentration occurs within the first 10 seconds. Thus over 90% of MMAA was released as its hydride, methylarsine (CH_3AsH_2). Similar results were obtained when the MMAA concentration was increased to 160 μM and 1.6 mM. These results indicate that a fast reaction and a high efficiency of hydride generation and release is achieved with the new generator.

When the new hydride generator is used in continuous mode, the efficiency of hydride production can also be studied with a similar radioactive tracer method. Solutions of labeled MMAA (16 μM) in 1 M hydrochloric acid and sodium borohydride (2%) in 0.1 M NaOH were continuously pumped into the generator while the carrier gas flow was maintained, as when the generator is being used for continuous operation. However, in this case, hydride was released to the fume hood instead of being introduced to the flame AAS. An aliquot of 100 μl of the reaction waste solution was taken for liquid scintillation counting. The radioactivity from the waste solution was compared with that of the standard MMAA solution and it was found that only (3 ± 1)% of the original MMAA was present. Thus approximately 97% of the MMAA was converted to hydride.

A higher concentration of MMAA, 160 μM, was also used to study the hydride generation efficiency and similar results were obtained. Less than 5% of the MMAA was found in the waste solution and over 95% of the MMAA was converted to hydride.

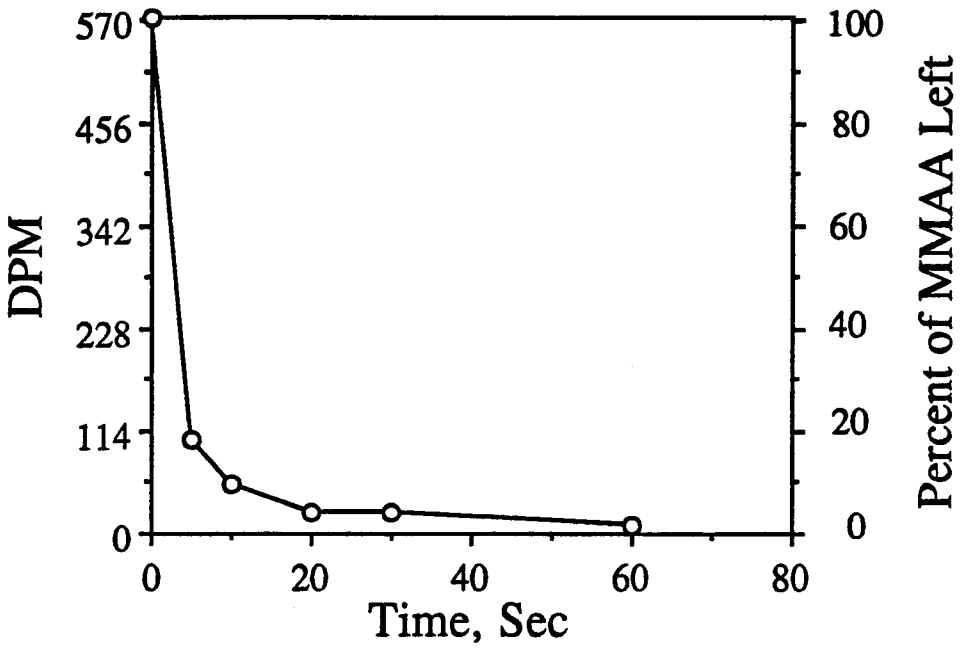


Figure 2.4. Radioactive tracer study showing the release of hydride vs. time after labeled MMAA and $NaBH_4$ were mixed in the new hydride generator.

2.3.3. Optimization

The acid concentration, sodium borohydride concentration and carrier gas flow rate were optimized by using the simplex optimization method (200, 201), while other experimental conditions were kept constant. The signal-to-noise ratio was chosen as the response to be optimized. Optimum conditions under which the maximum signal-to-noise ratios for the determination of arsenic, antimony, and tin were obtained, are listed in Table 2.1. It takes only 15-20 experiments to perform a whole simplex optimization, and an optimum condition may be reached before a whole operation is completed.

For comparison the one-factor-at-a-time method was also used to optimize in turn each of the three factors while all the rest were kept constant. The effect of hydrochloric acid concentration on the determination of arsenic, antimony, and tin is shown in Figure 2.5. The results indicate that the concentration of hydrochloric acid is more critical in the determination of tin than it is for arsenic and antimony. This is consistent with the conclusion reached by using the simplex optimization method. Optimum ranges of hydrochloric acid concentration for maximum sensitivity are 2.0-4.0 M for arsenic, 1.5-4.0 M for antimony, and 0.6-0.8 M for tin, which cover the optimum conditions obtained with the simplex method.

Figure 2.6 shows the effect of the sodium borohydride concentration on the absorbance of arsenic, antimony, and tin. The optimum conditions obtained by using the simplex method summarized in Table 2.1 also fall in the maximum absorbance range shown in Figure 2.6. The decrease of the arsenic and the antimony absorbance at high concentration of sodium borohydride is probably due to the large amount of hydrogen gas produced from the reaction of the sodium borohydride with the hydrochloric acid. A soap bubble flow meter was used to show that approximately 120 ml min^{-1} of hydrogen was generated from the reaction between 2.0 M hydrochloric acid (uptake rate 4.7 ml min^{-1}) and 2% sodium borohydride (uptake rate 4.5 ml min^{-1}). This large flow of hydrogen seems to dilute the hydride and reduce the residence time of the analyte atoms

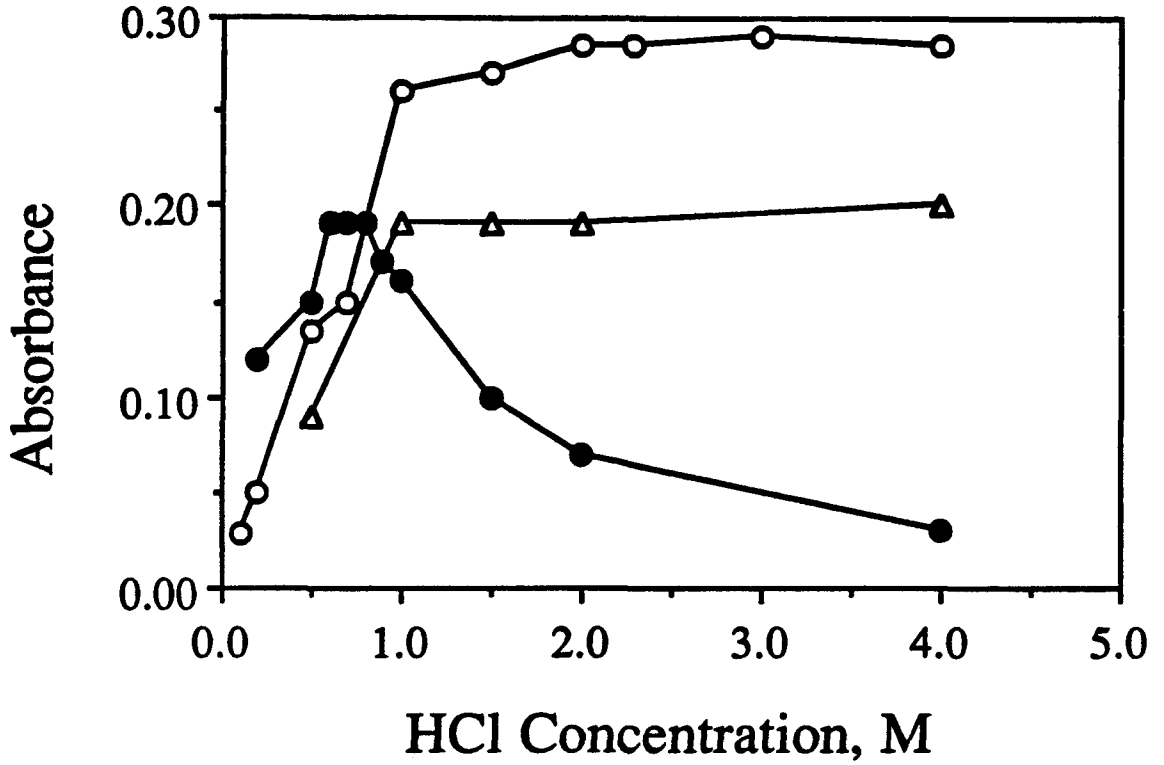


Figure 2.5. Effect of the concentration of hydrochloric acid in the determination of arsenic, antimony and tin.

(O) -- 3.0 ng ml^{-1} of As(III), As(V), MMAA, and DMAA mixture in 0.01 M HCl; 2.0% of $NaBH_4$ in 0.1 M NaOH

(Δ) -- 20.0 ng ml^{-1} of antimony in 0.01 M HCl; 2.0% of $NaBH_4$ in 0.1 M NaOH

(●) -- 20.0 ng ml^{-1} of tin in 0.01 M HCl; 1.5% of $NaBH_4$ in 0.1 M NaOH

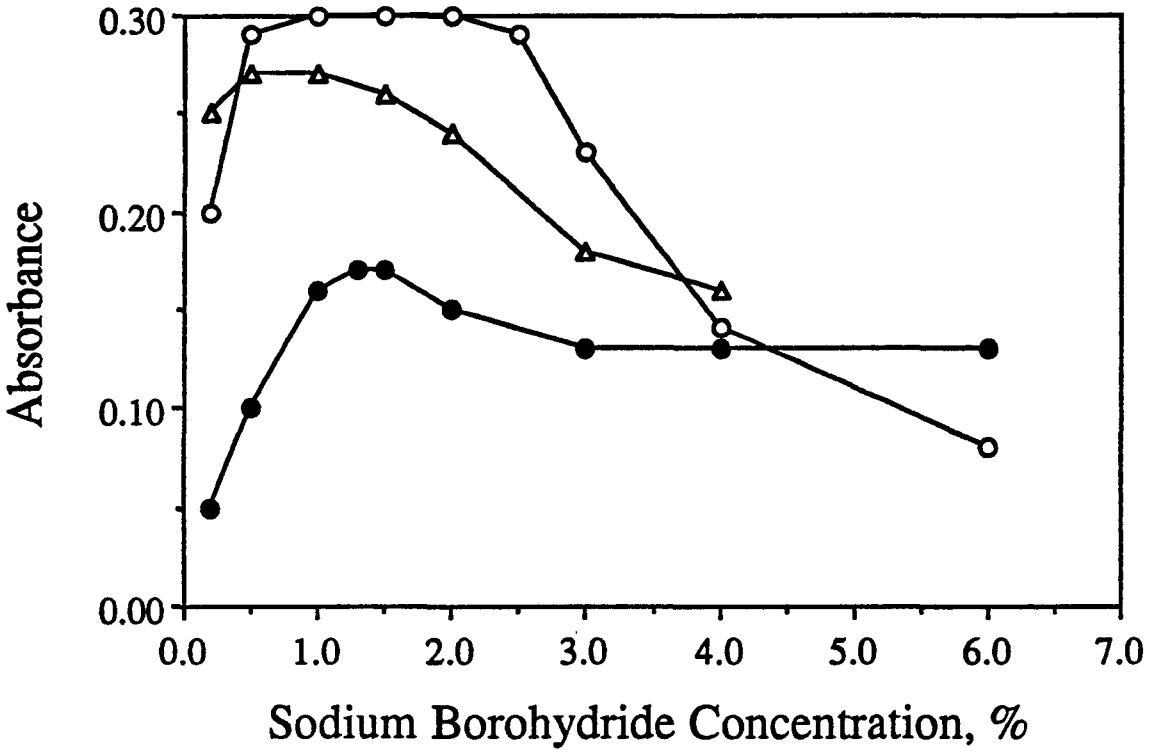


Figure 2.6. Effect of the concentration of $NaBH_4$ on the determination of arsenic, antimony, and tin
(O) -- 3.0 ng ml^{-1} of As(III), As(V), MMAA, and DMAA mixture in 0.01 M HCl
(Δ) -- 20.0 ng ml^{-1} of antimony in 0.01 M HCl
(●) -- 20.0 ng ml^{-1} of tin in 0.01 M HCl

in the quartz tube atomizer, resulting in a lower absorption signal. In the determination of tin, a lower concentration of hydrochloric acid (0.80 M) limited the amount of hydrogen production, hence a concentration of sodium borohydride from 3.0% (0.78 M) to 6.0% (1.5 M) can be tolerated.

The effect of carrier gas flow rate on the absorbance of signals and their noise levels are shown in Figure 2.7. At a low carrier gas flow rate, a higher noise level is observed. When the carrier gas flow is too high, the absorbance of analyte decreases due to a shorter residence time of the analyte atoms in the atomizer while the noise level does not decrease further. Therefore, the maximum signal-to-noise ratio was obtained within an optimum range of carrier gas flow rate. The results in Figure 2.7 are also in good agreement with those obtained with the simplex method.

With both the simplex optimization method and the one-factor-at-a-time method, the same optimum conditions were obtained. The simplex method required for much fewer experiments while the one-factor-at-a-time method gave a possible optimum range under which maximum and constant response can be obtained.

2.3.4. Interference studies

Interference effects in the determination of arsenic, antimony, and tin were studied. Both the conventional and the new hydride generators were used and the results obtained in the presence and absence of cysteine were compared. Ni^{2+} as $NiCl_2$, Co^{2+} as $Co(NO_3)_2$, and Cu^{2+} as $Cu(NO_3)_2$ were chosen as examples to study, since they are known to show severe interference (202-207). Table 2.2 summarizes the results of relative responses when different concentrations of Ni^{2+}, Co^{2+}, and Cu^{2+} are present in the determination of 5.0 ng ml^{-1} of the mixture of arsenic compounds (equal amount of arsenite, arsenate, MMAA and DMAA) and 20.0 ng ml^{-1} of antimony and tin. Relative responses were obtained by comparing absorption signals of the analyte in the presence of interfering ions with signals of the standard analyte in the absence of any interfering

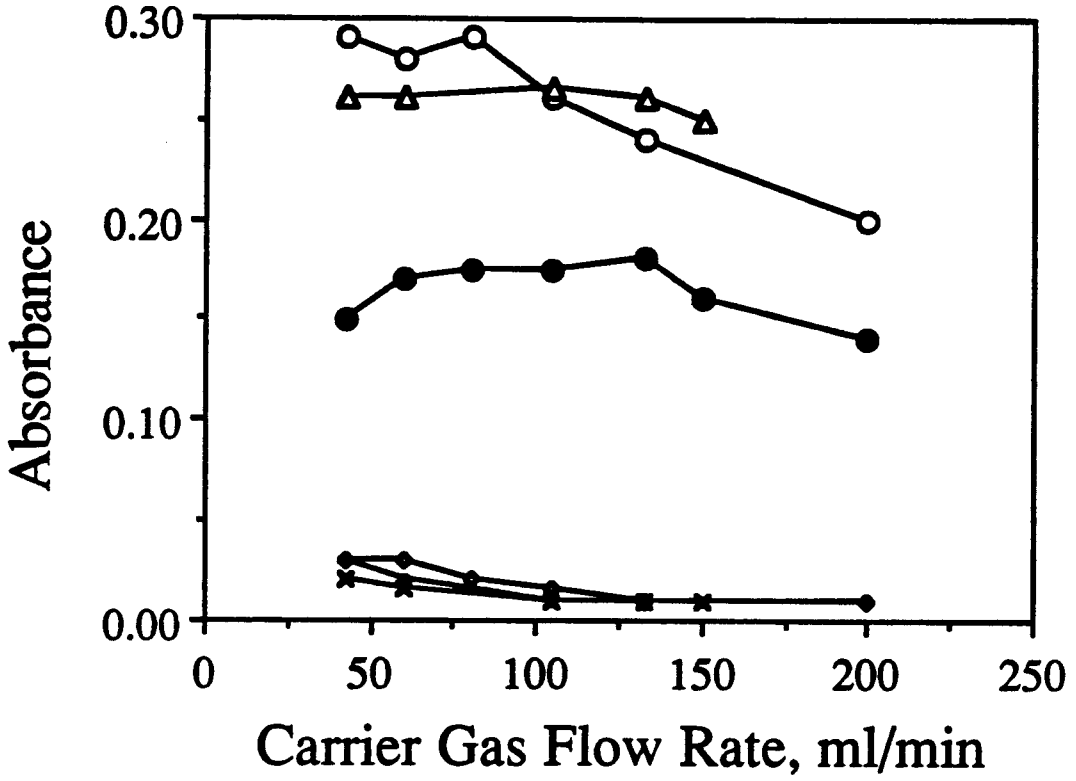


Figure 2.7. Effect of the carrier gas flow rate on the determination of arsenic, antimony, and tin

(O) -- 3.0 ng ml^{-1} of As(III), As(V), MMAA, and DMAA mixture in 0.01 M HCl

(Δ) -- 20.0 ng ml^{-1} of antimony in 0.01 M HCl

(●) -- 20.0 ng ml^{-1} of tin in 0.01 M HCl

(◇) -- noise level in the determination of arsenic

(x) -- noise level in the determination of antimony

(■) -- noise level in the determination of tin

ion. The results in Table 2.2 are the average of three or four determinations (standard deviation of approximately 3%). As is shown in Table 2.2, the ability of the new generator to tolerate interference is comparable to or better than that of the conventional system. When cysteine is used as an interference reducing agent, interferences from nickel, cobalt, and copper are dramatically reduced with both the conventional and the new hydride generators. In the absence of cysteine, severe interferences are observed in the determination of arsenic when 1 $\mu g\ ml^{-1}$ of Ni^{2+}, 10 $\mu g\ ml^{-1}$ of Cu^{2+}, or 100 $\mu g\ ml^{-1}$ of Co^{2+} are present. Similarly 0.5 $\mu g\ ml^{-1}$ of Ni^{2+}, 1 $\mu g\ ml^{-1}$ of Cu^{2+}, or 100 $\mu g\ ml^{-1}$ of Co^{2+} cause suppression of antimony and tin signals by more than 20%. In the presence of 1% cysteine, however, up to 50 $\mu g\ ml^{-1}$ of Ni^{2+}, 1000 $\mu g\ ml^{-1}$ of Cu^{2+}, and 100 $\mu g\ ml^{-1}$ of Co^{2+} have no significant interference with the determination of arsenic, antimony, and tin. Thus 10-1000 fold of these ions can be tolerated without interference. These results are consistent with those found previously (205-207) when a batch type hydride generator was coupled with a direct current plasma atomic emission spectrometer and used for the determination of arsenic (205), tin (206), and germanium (207). The reduction of interferences in the determination of antimony by using cysteine is reported here for the first time. Thus the use of cysteine to reduce interference in the hydride generation process, with both batch mode and continuous mode operations is strongly indicated.

The optimum hydride generation conditions established in the presence of cysteine are different from those in the absence of cysteine. In particular, the acid concentration is lower for the determination of all the three elements, and as a result less hydrogen is produced. Thus, a much wider optimum range of sodium borohydride concentration is available compared with that in the absence of cysteine. The effect of carrier gas flow rate is similar in the presence and absence of cysteine as one might expect. These optimum ranges have been summarized in Table 2.1.

Table 2.2 **Relative Responses of Arsenic, Antimony, and Tin in the presence of interfering ions**

Interfering Ions	Conc. ($\mu g\ ml^{-1}$)	Relative Response, %											
		Arsenic (5.0 ng ml^{-1})				Tin (20.0 ng ml^{-1})				Antimony (20 ng ml^{-1})			
		Conventional System		New System		Conventional System		New System		Conventional System		New System	
		No Cys*	With Cys	No Cys	With Cys	No Cys	With Cys	No Cys	With Cys	No Cys	With Cys	No Cys	With Cys
Ni^{2+}	0.10	99	101	100	100	82	98	94	100	85	101	95	101
	0.50	90	-	92	-	61	-	71	-	56	97	82	100
	1.0	73	100	77	100	56	101	57	98	45	98	60	101
	10	33	84	21	104	24	99	18	100	22	95	39	98
	50	8	58	7	102	10	70	5	95	-	65	15	90
	100	-	55	-	80	-	35	-	86	-	24	-	75
	500	-	26	-	58	-	10	-	30	-	11	-	56
	1000	-	-	-	21	-	-	-	10	-	-	-	-

Table 2.2 continue

Co^{2+}	0.10	-	-	-	-	95	100	97	100	93	100	100	98
	1.0	98	101	100	98	87	100	98	99	95	100	100	100
	10	82	100	92	98	84	103	83	100	80	101	86	99
	100	30	82	35	90	42	81	44	101	40	83	53	100
	500	-	38	14	50	10	29	9	50	18	31	-	65
	1000	-	-	-	31	-	-	-	14	-	20	-	30
Cu^{2+}	0.10	-	-	-	-	97	100	92	99	-	-	-	-
	1.0	95	96	95	100	68	97	67	100	70	98	77	100
	10	80	91	86	100	48	100	51	102	57	100	65	101
	50	48	97	60	92	-	104	29	104	40	100	43	101
	500	20	99	-	100	-	100	-	100	36	99	39	98
	1000	-	85	-	100	-	93	-	97	-	93	-	99
	5000	-	57	-	-	-	43	-	54	-	84	-	80

*: Cys -- cysteine

-: -- not determined

In the presence of 1% cysteine, much lower acid concentrations are necessary for hydride generation. At these low acid concentrations, a high noise level was observed with the conventional hydride generator. However, the noise was dramatically reduced when the new generator was used, resulting in a signal-to-noise ratio enhancement by a factor of three. These results suggest that the utilization of cysteine and the new generator is a good combination to improve the signal-to-noise ratio for the determination of these elements by using HGAAS.

2.3.5. Flow injection coupled to HGAAS

Flow injection analysis (FIA) has advantages of high sample throughput, small sample volume and reduced reagent consumption relative to manual and continuous flow systems (208). For these reasons a FIA/HGAAS system was developed. A new hydride generator which has high efficiency and a reduced dead volume was constructed (Figure 2.2), and it was found to be suitable for operating as a part of an FIA system. The FIA/HGAAS system is capable of performing analysis at a sample throughput of approximately 120 per hour. Calibration curves are linear from 5 to 200 ng ml^{-1} of arsenic and the detection limit is 0.5 ng ml^{-1} for a 100 µl injection of sample (0.05 ng of arsenic). This system was therefore used for the studies described in the following chapters.

Chapter 3.
EFFECT OF CYSTEINE ON THE SPECIATION OF ARSENIC BY USING HYDRIDE GENERATION ATOMIC ABSORPTION SPECTROMETRY

3.1. INTRODUCTION

The hydride generation technique with its major advantages of high sensitivity and relative simplicity of operation has been used widely for arsenic determination at trace levels. Various arsines can be formed. Arsenite and arsenate give AsH_3, MMAA gives CH_3AsH_2, and DMAA gives $(CH_3)_2AsH$, upon reaction with sodium borohydride. Braman *et al.* (27, 28) pointed out that the formation of the arsines was pH dependent and was related to the pKa values of the individual arsenic acids. This pH dependency was further demonstrated by Anderson *et al.* (121). Hinners (209) also emphasized the critical effect of acid concentration on the hydride generation responses from arsenite, arsenate, MMAA and DMAA, and was unable to find a compromised acid concentration under which the same response could be obtained from the four arsenic species. Thus, when 4-10 M hydrochloric acid was used, DMAA gave only 15% (or less) of the signal that was obtained from the same amount of arsenite, whereas at a lower acidity (pH 4.8), arsenate and MMAA were severely underestimated. Similar observations were also reported by Arbab-Zavar and Howard (210). In a method reported by Aggett and Aspell (123), however, total arsenic in environmental water samples was evaluated by using 5M hydrochloric acid as the hydride generation reaction medium and inorganic arsenite as the standard. As clearly indicated by the work of others (209, 210) this method underestimates DMAA, which is often present in natural waters. Similar errors exist in other reports (211, 212) and the problem has not been solved.

In the present study, it is found that when cysteine is added to samples, arsenite, arsenate, MMAA and DMAA give the same response under the same optimum acid concentration when quantified by using hydride generation. Thus the determination error described above can be eliminated. The effect of cysteine and related compounds on the speciation of arsenic by using hydride generation is studied in detail.

3.2. EXPERIMENTAL

3.2.1. Instrumentation

A system coupling flow injection analysis with hydride generation atomic absorption spectrometry (FIA/HGAAS) was used as described in the previous section 2.2.1. A 500-W domestic microwave oven (Toshiba, Japan) was used for the digestion of human urine samples.

A system that employed hydride generation followed by cryogenic trapping, gas chromatographic separation, and atomic absorption detection (HG/GC/AAS) was also used for arsenic speciation. The basic operations used for hydride generation and cryogenic trapping are similar to those reported (28, 120, 125, 126). Some modifications (213, 214) have been made for GC separation and sample introduction. A Jarrell-Ash 810 atomic absorption spectrophotometer and a Hewlett Packard 5830A gas chromatograph were used.

3.2.2. Reagents

Standard solutions of arsenic compounds and sodium borohydride solutions were prepared as described in section 2.2.2. L-cysteine (BDH), methionine, glycine, histidine, thioglycerol (Sigma) and other reagents used were all of analytical reagent grade or better.

3.2.3. Samples

Urine samples were collected from a 30-year old, male both before and after he had eaten crab meat. The subject refrained from eating seafood for at least the 3 day period immediately before the experiment was conducted. Live crabs were purchased from a local seafood market in Vancouver (Canada) and were steam cooked. Urine samples were collected for the next three days following the ingestion of crab by the volunteer. These samples were stored at 4 ^{o}C and were analyzed within 3 days of collection.

3.2.4. Procedures

3.2.4.1. FIA/HGAAS:

Method one: Urine samples were directly analyzed for arsenic by using FIA/HGAAS. A 100-µl sample was injected into a deionized water carrier stream by using a sample injection valve. The injected sample met with a continuously introduced hydrochloric acid stream and a sodium borohydride stream at two T-joints. Arsines were generated and separated from liquid waste in a lab-made gas/liquid separator apparatus as discussed previously in section 2.2.1. The gaseous arsines were then swept by a continuous flow of nitrogen carrier gas into a quartz absorption tube mounted in the air-acetylene flame for AA measurement. Initially both peak height and peak area of signals were measured. It was found that the peak height measurements gave a lower standard deviation and this was therefore used for quantitation. Standard arsenite solutions were used for calibration.

Method two: Cysteine, 0.2 g, was dissolved in each 10 ml urine sample. The solution was left for 10-30 min at room temperature, and the samples were analyzed for arsenic in the same manner as described in method one, except that a lower acid concentration was used (see Table 3.1). Standard arsenite solutions and a blank, treated in the same way, were used for calibration.

Method three: Urine samples were first subjected to batch type microwave digestion as follows. The urine sample or standard (40 ml), potassium persulfate (4.5 g), and sodium hydroxide (2.7 g) were combined in a 125-ml Erlenmeyer flask. Six of such sample-containing flasks were placed in a microwave oven at one time. The microwave oven was then operated at full power for 3 min followed by a 3 min cooling period, and this heating and cooling cycle was repeated for another four times. After these samples were cooled to room temperature, they were each diluted to 50 ml with deionized water. Determination of arsenic in the digested samples was also carried out by using FIA/HGAAS as described above in method one, except that arsenate was employed as standard for calibration.

Table 3.1. Experimental conditions for hydride generation

	Method One	Method Two	Method Three
Microwave Digestion	NO	NO	YES
Cysteine	NO	2%	NO
HCl Conc.	3 M	0.5 M	3 M
HCl flow rate	3.4 ml min^{-1}	3.4 ml min^{-1}	3.4 ml min^{-1}
$NaBH_4$ Conc.	0.65 M	0.65 M	0.65 M
$NaBH_4$ flow rate	3.4 ml min^{-1}	3.4 ml min^{-1}	3.4 ml min^{-1}

3.2.4.2. Hydride generation gas chromatography atomic absorption spectrometry (HG/GC/AAS):

The sample, hydrochloric acid, and sodium borohydride, each 3 ml, were pumped, at a flow rate of 3 ml min^{-1}, into the gas/liquid separator apparatus (Figure 2.2) via a 2-

m reaction coil. Arsines generated from the reaction were separated from waste, passed through an acetone-dry ice bath to remove moisture, and trapped in a Teflon U-tube (30cm x 0.3cm i.d.) cooled in liquid nitrogen. After 6 min of trapping, the liquid nitrogen was removed and the U-tube was warmed by using a hot water bath (70 ^{o}C). The trapped arsines were released and swept with the aid of the helium carrier gas into a Teflon GC column (50 cm x 0.3 cm i.d.) packed with Porapak-PS (80-100 mesh, Waters/Millipore). The GC oven temperature was kept at 50 ^{o}C for the initial 0.5 min and then linearly increased to 150 ^{o}C at a rate of 30 ^{o}C min^{-1}. Individual arsine species were separated on the GC column and were subsequently introduced into a quartz absorption tube heated internally with a hydrogen-air flame for AA measurement (210). Chromatograms were recorded on a Hewlett Packard 3390A integrator capable of both peak height and peak area measurements.

3.3. RESULTS AND DISCUSSION

3.3.1. Effect of acid concentration on arsine generation in the absence of cysteine

Figure 3.1 shows the atomic absorption responses of arsines derived from arsenite, arsenate, MMAA and DMAA in aqueous solution as a function of concentration of hydrochloric acid. It is clear that responses from each arsenic species depend differently on acid concentration and that there is no acid concentration at which an equal response from the four arsenic species can be obtained. Similar effects were observed when nitric acid was used instead of hydrochloric acid. Also, peak area measurement showed the same effect of acid concentration on the responses from these arsenic species. These results along with those reported elsewhere (121, 210, 215), demonstrate that each arsenic species has a different optimum acid concentration required to achieve optimum response by using hydride generation. This is why errors can easily arise in the direct

determination of total arsenic from the use of a compromised acid concentration and a calibration based on a single arsenic species (123, 211, 212).

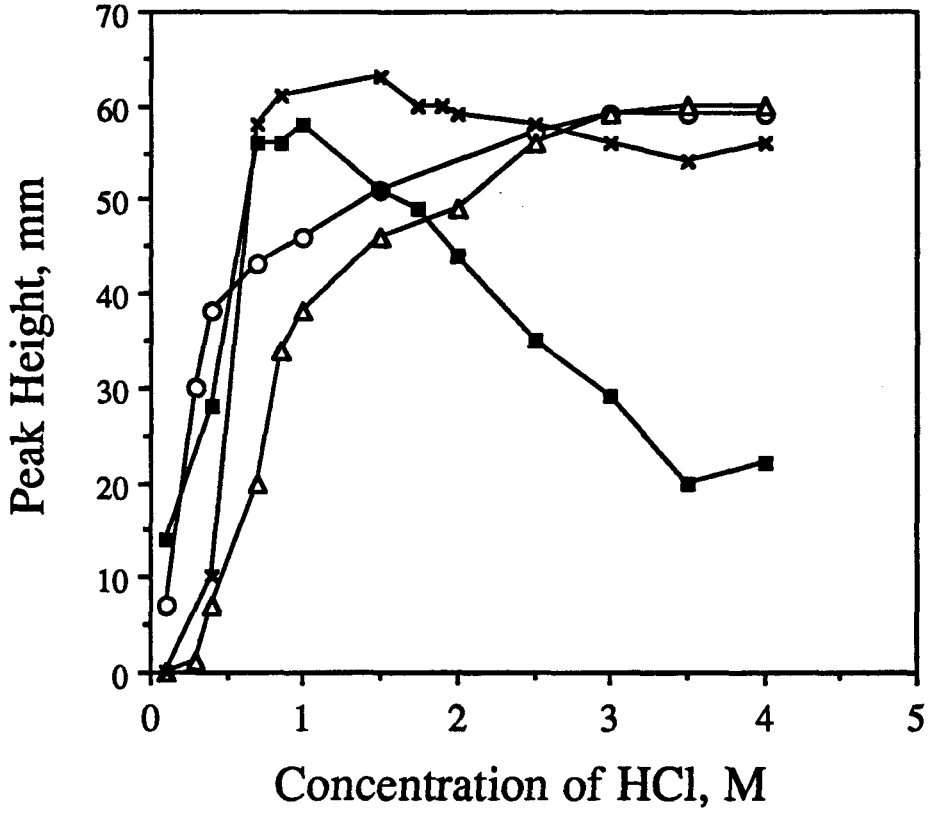


Figure 3.1. Effect of hydrochloric acid concentration on hydride generation responses from 20 ng ml^{-1} of (O) arsenite, (Δ) arsenate, (x) MMAA and (■) DMAA in H_2O.

3.3.2. Effect of cysteine

When 2% cysteine is present in the aqueous arsenic solution, the pH dependence of hydride generation of the same four arsenic compounds is shown in Figure 3.2. As compared to Figure 3.1, the optimum acid concentration for maximum sensitivity is shifted to a much lower level in the presence of cysteine. Also, in the acid concentration range of 0.3 to 0.7 M, maximum and identical responses are obtained from all the four arsenic species. Furthermore, since all four arsenic species give the same response, a single arsenic species can be used for calibration. When thioglycerol, another thiol-containing (-SH) compound was used instead of cysteine, similar effects to those shown in Figure 3.2 were noted.

Anderson *et al.* (121) have obtained similar results when using mercaptoacetic acid. Thus in the absence of any other acid, the optimum concentration of mercaptoacetic acid was found to be 0.1M for the determination of arsenite, arsenate, MMAA and DMAA; but unfortunately, severe interferences from a number of transition metal ions were encountered (121). These interferences can be reduced efficiently (205, 216, 217) by using cysteine. Furthermore, a relatively wider optimum acid concentration range (0.3-0.7M) is available when cysteine is used rather than mercaptoacetic acid (0.1M). An obvious advantage of having a wider optimum concentration range is that it is easier to keep experimental conditions within this range in order to maintain the maximum sensitivity and the minimum deviation.

Other amino acids such as methionine, glycine, and histidine were also studied in the same manner to evaluate how they might affect the optimum acid concentration for arsenic determination. The effect of hydrochloric acid concentration on the responses from arsenic species with 2% methionine present in each arsenic standard solution is shown in Figure 3.3. Very little difference is seen compared with Figure 3.1, indicating that methionine has little effect. The presence of added glycine or histidine likewise, has little effect.

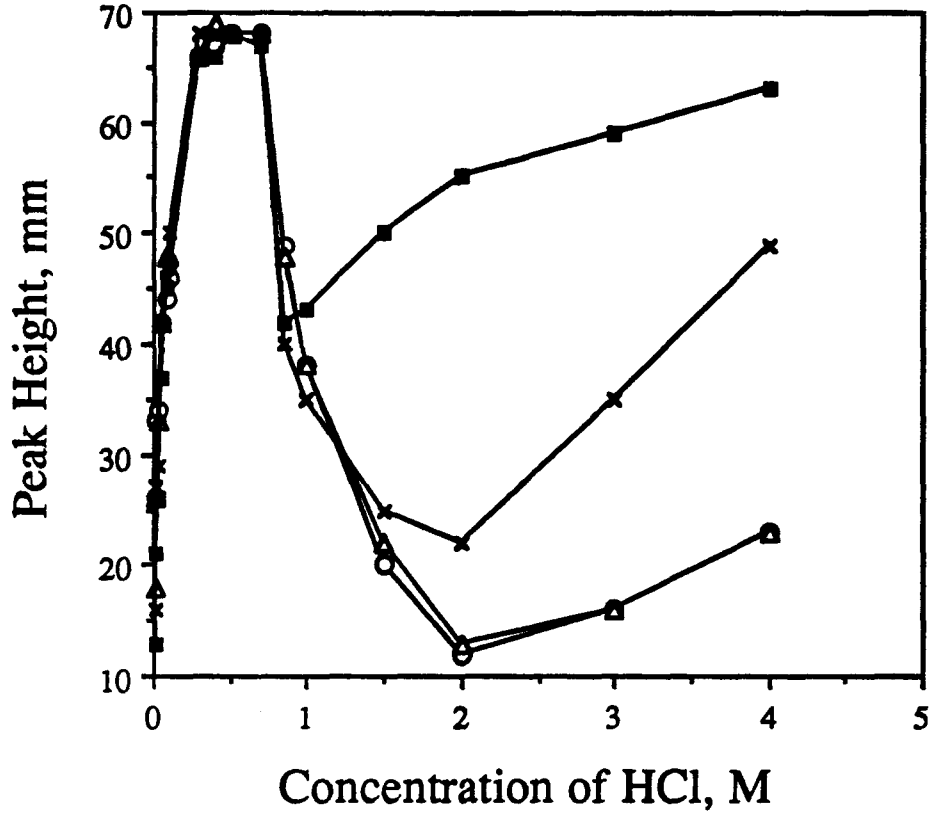


Figure 3.2. Effect of hydrochloric acid concentration on hydride generation responses from 20 ng ml^{-1} of (O) arsenite, (Δ) arsenate, (x) MMAA and (■) DMAA in 2% cysteine aqueous solution.

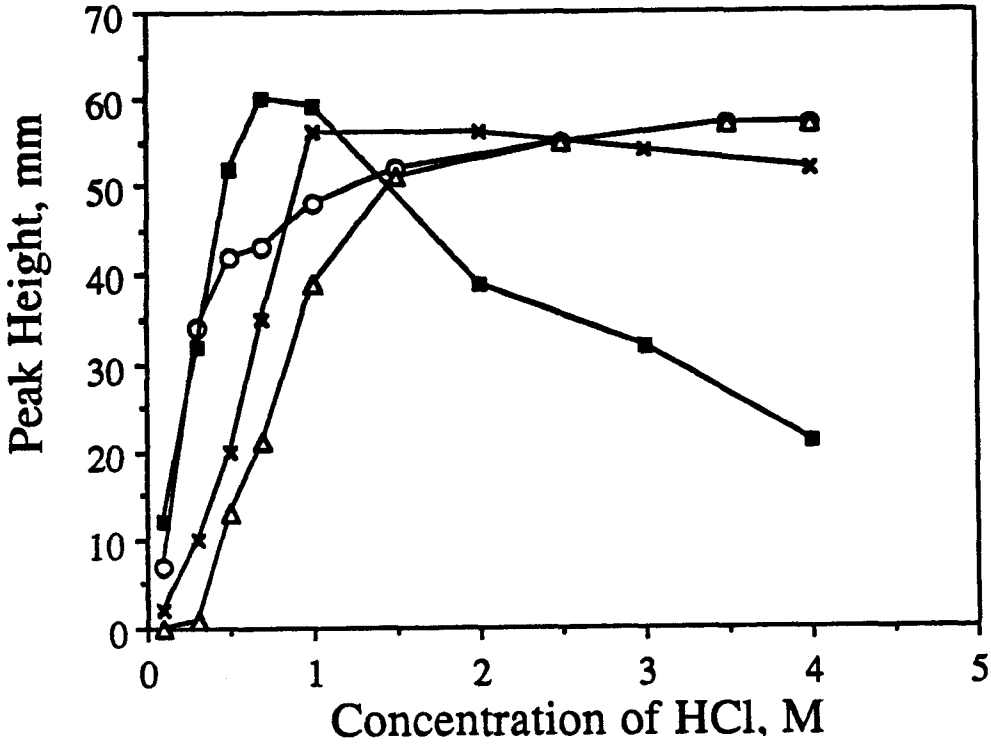


Figure 3.3. Effect of hydrochloric acid concentration on hydride generation responses from 20 ng ml^{-1} of (O) arsenite, (Δ) arsenate, (x) MMAA and (■) DMAA in 2% methionine aqueous solution.

The significant effect by cysteine and thioglycerol and the lack of effect by methionine, glycine and histidine is probably due to the presence of the thiol group (-SH) in cysteine and thioglycerol. Cullen *et al.* (218, 219) have shown that methylarsenicals can be easily reduced by a variety of thiols and dithiols including cysteine, glutathione, dithiothreitol, 2,3-dimercaptopropanol, and lipoic acid. The reactions were found to be quantitative and stoichiometric (218). Chen *et al.* (217) have shown that arsenate is readily reduced to arsenite by cysteine. Therefore, it is reasonable to propose that the following reactions take place in the presence of thiol-compounds, such as cysteine:

$As(OH)_3$		$As(SR)_3$		AsH_3
$AsO(OH)_3$		$As(SR)_3$		AsH_3
	$\xrightarrow{RSH}$		$\xrightarrow{NaBH_4}$	
$CH_3AsO(OH)_2$		$CH_3As(SR)_2$		CH_3AsH_2
$(CH_3)_2AsO(OH)$		$(CH_3)_2As(SR)$		$(CH_3)_2AsH$

where R = $-CH_2CH(NH_2)COOH$ for cysteine and

$-CH_2CH(OH)CH_2OH$ for thioglycerol

Initially the arsenic species react with the thiols to form $As(SR)_3$, $CH_3As(SR)_2$, and $(CH_3)_2As(SR)$ which are all in the As(III) state. These organo sulfur derivatives of arsenic(III) probably require similar conditions to react with borohydride to produce arsines, accounting for the observation that the optimum acid concentration for reduction of the four arsenic species lies in the same range.

The proposed mechanism indicates that although intermediate arsenic sulfur compounds are formed, the final product arsines do not incorporate cysteine. This is proven experimentally by using a modified (213, 214) hydride generation - cold trap - gas chromatograph with atomic absorption detection, a similar technique to that employed by Braman *et al.* (28), Andreae (120), and Vien and Fry (125). Figure 3.4 shows signals from arsenite, arsenate, MMAA and DMAA in the absence (a) and presence (b) of cysteine. In both cases, AsH_3, CH_3AsH_2, and $(CH_3)_2AsH$ are produced

from the hydride generation reaction. The fact that the presence of cysteine has no effect on the arsines produced from individual arsenic species makes it applicable not only for total arsenic determination but also for arsenic speciation studies. In the latter case cysteine may be used to reduce interferences in speciation of arsenic by using hydride generation.

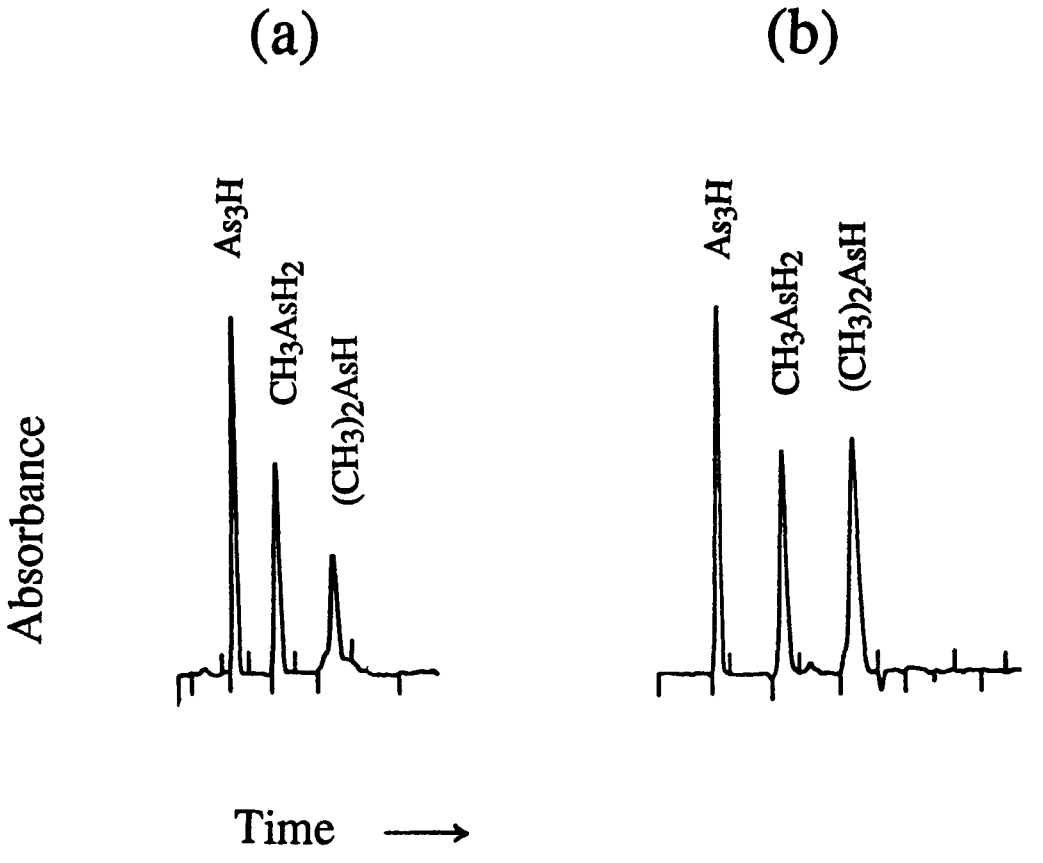


Figure 3.4. Chromatograms obtained by using hydride generation/cold trapping/GCAAS. (a) without cysteine, and (b) with cysteine.

3.3.3. Effect of Sodium borohydride concentration in the presence and absence of cysteine

Figure 3.5 shows the effect of the concentration of sodium borohydride on the HGAAS responses of As(V), As(III)), MMAA, and DMAA in the presence (Figure 3.5a) and absence (Figure 3.5b) of cysteine. In the presence of 2% cysteine in the sample solution (Figure 3.5a), 0.5 M HCl was used for hydride generation. Under these conditions, similar responses are obtained from all four arsenicals, with maximum responses being in the range of 3-6% of sodium borohydride.

When cysteine is absent and 3 M Hydrochloric acid is used for the hydride generation (Figure 3.5b), different responses are observed for DMAA and for As(III), As(V), and MMAA. This is consistent with the results shown previously in Figure 3.1, reconfirming that acid concentration affects the HGAAS signals of these individual arsenicals differently. The effect of sodium borohydride concentration in the absence of cysteine is slightly different from that in the presence of cysteine. This difference is clearly seen at a higher borohydride concentration. In the absence of cysteine, arsenic signals gradually decrease as the borohydride concentration increases from 4 to 6%. This is probably because the larger amount of hydrogen gas produced due to the reaction between 3 M hydrochloric acid and the increased amount of borohydride, dilutes the hydrides and reduces the residence time of arsenic in the quartz absorption tube, resulting in the decreased arsenic signals. However, when 2% cysteine is present in the sample solution, the amount of hydrogen produced is limited by the lower concentration of hydrochloric acid used (0.5 M). Thus constant signals from arsenicals in 2% cysteine are obtained in the same borohydride concentration range.

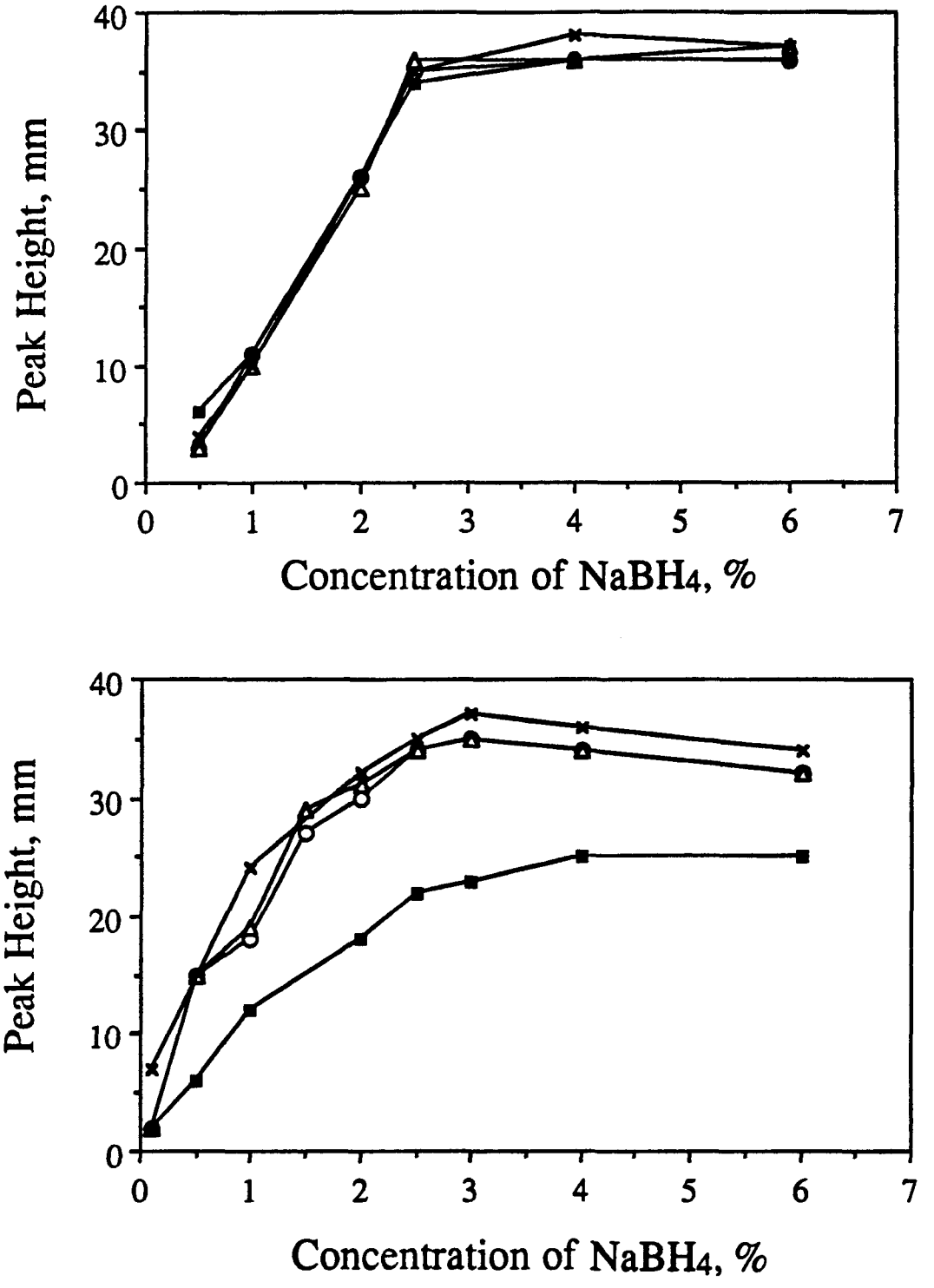


Figure 3.5. Effect of $NaBH_4$ concentration on hydride generation responses from 20 ng ml^{-1} of (O) arsenite, (Δ) arsenate, (x) MMAA and (■) DMAA in the presence (a) and absence (b) of cysteine

3.3.4. Effect of varying the reaction coil length

The effect of reaction time was studied by changing the length of the reaction coil between the gas/liquid separator and the point of mixing of the sample and reagents. Figure 3.6 shows the responses from the four arsenic species when either no reaction coil or a 2-m reaction coil is used. Figures 3.6a was obtained from arsenic compounds in the presence of cysteine and they show that the responses from the arsenicals are approximately the same with or without the reaction coil. This indicates a fast reduction in the presence of cysteine and consequently no reaction coil is required. However, when cysteine is absent from the arsenic solutions, as shown in Figures 3.6b, the responses are not maximized in the absence of the reaction coil, indicating incomplete reaction. When a 2-m reaction coil is added, the reaction is complete and full responses are obtained. Further lengthening the reaction coil up to 5 m did not produce any further increase of the arsenic signals.

It seems that cysteine enhances the rate of arsine generation. The mechanism proposed above suggests that the pentavalent arsenic species, arsenate, MMAA, and DMAA are all reduced to their trivalent oxidation state by cysteine prior to the reaction with sodium borohydride. It seems that faster reactions with borohydride are possible from these pre-reduced arsenic species. It is well established (27, 28, 117, 120, 121, 123, 209, 210, 220-222) that the production of an arsine from arsenite is substantially faster than from the pentavalent arsenicals. In the absence of cysteine or other common reducing agents such as potassium iodide, both the pre-reduction and hydride formation would be carried out by reacting with borohydride.

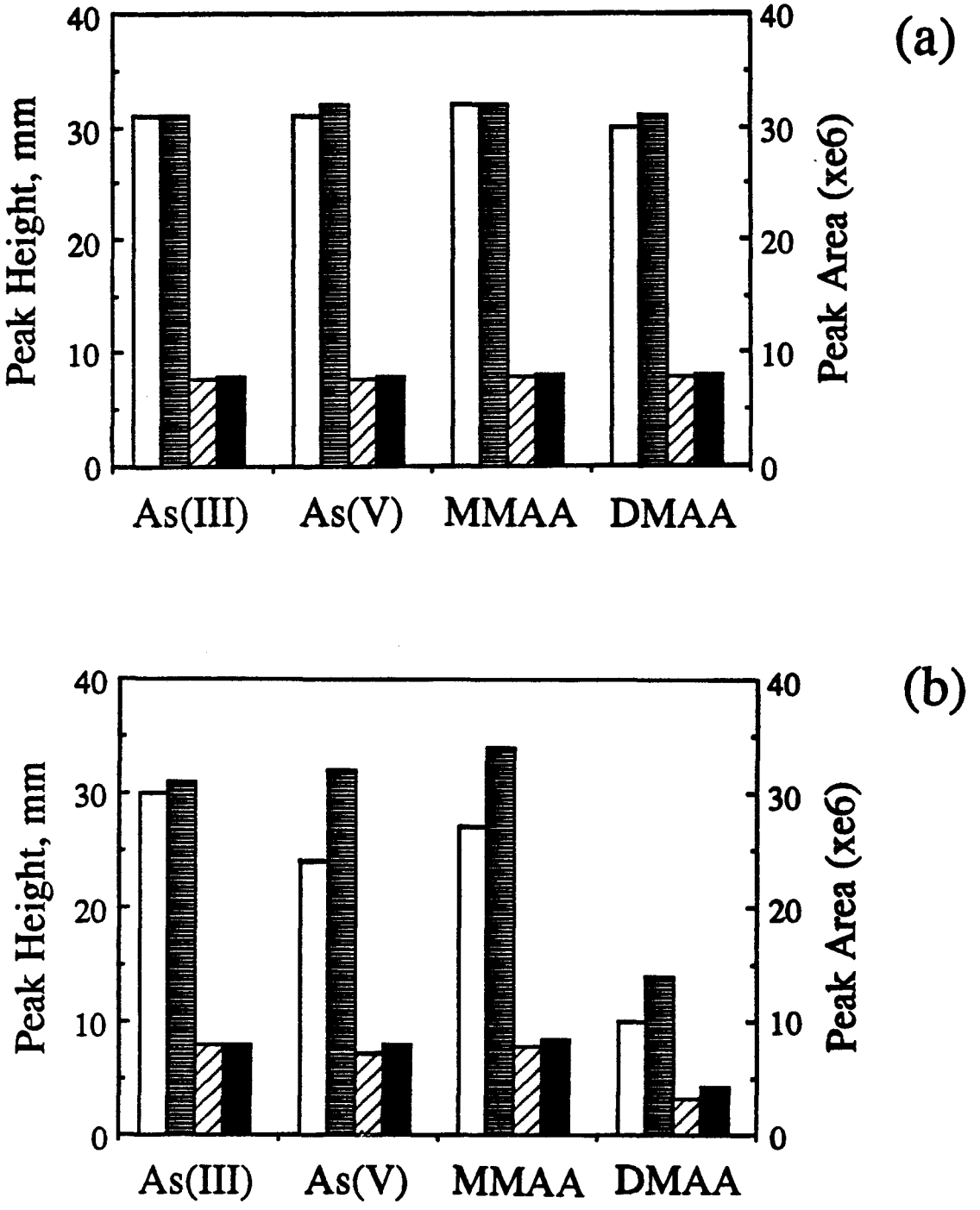


Figure 3.6. Comparison of peak height (□,▤) and peak area (▨,■) of hydride generation signals obtianed with (▤,■) and without (□,▨) a 2-m reation coil.

(a) -- Arsenic species in 2% cysteine

(b) -- Arsenic species in water

3.3.5. Application: Determination of arsenic in human urine

In order to take the effect of the matrix into consideration, optimization of experimental conditions was carried out by maximizing responses from arsenic both in standard aqueous solutions and in a typical urine sample. Concentrations of acid and sodium borohydride, amount of cysteine, and flow rates of sample, reagents, and carrier gas were optimized to achieve maximum sensitivity and signal repeatability. It was found that the effects of these parameters are similar for arsenic in urine samples and in standard solution, and the optimum conditions have been summarized in Table 3.1.

The recovery of arsenic species spiked into a urine sample was studied and compared by using three procedures: (i) direct analysis without the addition of cysteine, (ii) direct analysis in the presence of cysteine, giving total hydride-forming arsenic concentration, and (iii) determination of total arsenic after microwave assisted decomposition. Recovery results are summarized in Table 3.2. It is clearly seen that in the absence of cysteine, when 3M hydrochloric acid is used as reaction medium for all arsenic species, DMAA gives only 57% of the signal as calibrated against arsenite. Consequently when arsenic mixtures containing equal amount of arsenite, arsenate, MMAA and DMAA are spiked into the urine sample, the recovery is also incomplete as calibrated against arsenite. These results support the conclusion of Hinners (209) and Arbab-Zavar and Howard (210) who state that when total arsenic was measured against arsenite calibration, DMAA would be underestimated. However, when cysteine is added to the urine sample, complete recovery from all the arsenicals studied is achieved as shown in Table 3.2. Arsenobetaine (AB), arsenocholine (AC) and the tetramethylarsonium ion (Me_4As^+) do not form a hydride, with or without the added cysteine; however, after microwave assisted decomposition, quantitative recoveries from these organoarsenicals, which have been converted to arsenate, are also obtained (Table 3.2). The decomposition of organoarsenic compounds by using a microwave oven will be discussed further in the next chapter.

Table 3.2. Recovery of arsenic species spiked into a urine sample

Urine Sample Spiked With Arsenicals	Recovery, %		
	Method One	Method Two	Method Three
20 ppb As(III)	98 ± 2	96 ± 6	97 ± 2
20 ppb As(V)	90 ± 2	x	98 ± 3
20 ppb DMAA	57 ± 3	98 ± 3	92 ± 2
10 ppb 4As Mixture*	78 ± 6	97 ± 2	91 ± 2
20 ppb 4As Mixture*	88 ± 5	99 ±3	x
50 ppb AB	0	0	98 ± 6
50 ppb AC	0	0	97 ± 3
50 ppb Me_4As^+	0	0	96 ± 6

*: 4As mixture contains equal amount of arsenite, arsenate, MMAA and DMAA
x: not evaluated.

Determination of arsenic in urine samples collected from an individual who had ingested some crab meat was carried out and compared by using the three methods described in the procedure section. Table 3.3 shows the results of the time course study. In the presence of added cysteine, the mean value of the concentration of total hydride-forming arsenic for the 15 samples analyzed is 11.6 ng ml^{-1}. This represents the sum of arsenite, arsenate, MMAA and DMAA. In the absence of cysteine, however, the arsenic concentration is consistently underestimated in each sample, and the mean value from the 15 samples analyzed is 10.0 ng ml^{-1}. Thus on average a 13.8% error is present. In samples 2 and 8 in particular, the error is as high as 20%. This is not surprising since

urine samples often contain DMAA (80, 86, 88, 104) and DMAA concentration is underestimated in the absence of cysteine.

All arsenic compounds are converted to arsenate by using microwave assisted digestion, and subsequent determination by hydride generation against arsenate calibration gives the total arsenic concentration. The difference in arsenic concentration measured by using method 3 and method 2 is due to the "hidden" organoarsenic compounds which do not form a hydride. The methods 2 and 3 reported here are easy to use for the determination of trace amounts of arsenic and provide a convenient means to differentiate between the more toxic hydride-forming arsenicals and other essentially non-toxic organoarsenic compounds (11-16, 80, 83, 86).

Table 3.3. Concentration of arsenic in urine samples measured by using three methods

Urine Sample No	Collection Time # (hr)	Concentration of Arsenic (ng ml^{-1}) (Mean ± Standard deviation)*		
		Method One	Method Two	Method Three
1	-1	12.8 ± 0.4	14.7 ± 0.4	17.0 ± 0.5
2	1	13.2 ± 0.4	16.7 ± 0.4	28.2 ±0.8
3	4	12.0 ± 0.4	13.0 ± 0.4	64.0 ± 2.0
4	6	11.0 ± 0.4	12.4 ± 0.3	91.3 ± 2.8
5	9	10.0 ± 0.4	11.3 ± 0.3	98.5 ± 3.0
6	11	10.0 ± 0.3	11.0 ± 0.3	95.6 ± 2.9
7	16	6.8 ± 0.3	7.3 ± 0.3	78.3 ± 2.5
8	20	14.0 + 0.4	17.3 ± 0.4	39.0 ± 1.1
9	29	6.0 ± 0.3	7.0 ± 0.3	31.5 ± 1.0
10	32	6.0 ± 0.3	7.0 ± 0.3	29.5 ± 1.0
11	36	7.0 ± 0.3	8.7 ± 0.3	32.0 ± 1.1
12	39	5.5 ± 0.3	6.4 ± 0.3	25.5 ± 0.8
13	41	11.0 ± 0.4	11.3 ± 0.3	21.8 ± 0.6
14	50	12.5 ± 0.4	14.7 ± 0.4	17.9 ± 0.8
15	62	12.8 ± 0.4	15.3 ± 0.4	18.0 ± 0.5

*: From three or four replicate measurements.

#: Time zero is the time of ingestion of the crab meat.

Chapter 4.
MICROWAVE ASSISTED DECOMPOSITION OF ORGANOARSENIC COMPOUNDS AND SUBSEQUENT DETERMINATION OF ARSENIC BY USING FIA/HGAAS

4.1. INTRODUCTION

Organoarsenicals such as arsenobetaine (AB), arsenocholine (AC), arsenosugars, and the tetramethylarsonium ion (Me_4As^+) are widespread in biological systems and presumably play important roles in the cycling of arsenic in the marine environment. Consequently studies of these organoarsenicals in environmental and biological systems are currently receiving much attention (5, 6, 58, 67, 162, 168). Because only trace concentrations of arsenicals are usually encountered in environmental and biological samples, analytical methods with high sensitivity are required. Hydride generation has been recognized as a very useful technique for trace analysis due to its ability to enhance sensitivity. It has been widely used in conjunction with spectrometric detection, for the determination of trace amounts of As(III), As(V), MMAA and DMAA (27, 28, 122, 127, 128). However, arsenobetaine, arsenocholine, and a number of other organoarsenicals do not form volatile hydrides under the commonly used analytical conditions. Therefore it is necessary to convert these organoarsenicals to some hydride-forming arsenic species in order to determine trace amounts of organoarsenicals and/or total arsenic by using hydride generation methodology.

Wet digestion methods utilizing a nitric-sulfuric-perchloric acid mixture (152, 223), or a nitric-perchloric-chloric acid mixture (224) have been reported to decompose samples prior to the determination of total arsenic. Andreae (12) has reported that some organoarsenicals such as arsenobetaine are resistant to acid digestion. Thus heating with magnesium oxide in a muffle furnace was recommended to achieve complete decomposition. Similarly, dry ashing with a mixture of magnesium nitrate and

magnesium oxide has been reported (225). Wet digestion with strong base (32, 53, 226, 227), for example 40% aqueous sodium hydroxide, has also been investigated, although this generally does not result in complete breakdown to species detectable by the hydride generation method (59, 227). Stinger and Attrep (228) subjected arsenicals to ultraviolet (UV) radiation in the presence of hydrogen peroxide and sulfuric acid. A four hour irradiation time was required to photo-decompose disodium methanearsonate, dimethylarsinic acid, and triphenylarsine oxide spiked into a waste water sample. Cullen and Dodd (229) further investigated the photo-oxidation of solutions of organoarsenicals in the presence of different mineral acids. One hour of UV irradiation (1200 W medium pressure lamp) completely oxidized samples to arsenate. Recently, Atallah and Kalman (230) have modified the batch type photo-oxidation procedure to an on-line process. A Teflon tube (5 m x 0.5 mm i.d.) coiled around a mercury lamp, the UV source, was used as a photo-reactor. Decomposed arsenicals, exiting from the photo-reactor, were subsequently determined using hydride generation AAS.

Microwave oven sample digestion (231-233) has been shown to possess advantages over other more commonly used heating methods. In this work the use of the rapid and efficient heating that is possible with a microwave oven is applied to decompose organoarsenicals to a form suitable for arsine generation. The successful on-line coupling of microwave oven decomposition with FIA and HGAAS for the determination of specific organoarsenicals and total arsenic is described. The methodology developed is applied to the determination of urinary arsenic.

4.2. EXPERIMENTAL

4.2.1. Instrumentation

A domestic microwave oven (Toshiba Co., Japan) with a maximum power output of 500 W (variable in 9 steps from 100 to 500 W) and an operating frequency of 2450

MHz was used for digestions. The power outputs at different settings were calibrated by using the literature method (231). A digestion coil (0.5 mm i.d., varied length) and a cooling coil (1 m x 4 mm i.d.), both made of Teflon, were placed inside the microwave oven. Two upper inlet holes and two lower outlet holes were drilled on the same side of the oven and the holes were shielded with proper metal fittings to prevent microwave leakage. A continuous flow of tap water (approximately 10 ml min^{-1}) through the cooling coil was used to prevent damage to the oven through continuous operation.

A schematic of the combined flow injection - microwave decomposition - hydride generation system (FIA/MD/HG) is shown in Figure 4.1. The sample was injected via a Rheodyne 6-port sample injection valve (V) fitted with a 100 µl sample loop. A peristaltic pump (P) (Gilson Minipuls 2) was used to deliver sample and reagent solutions. A continuous flow of digestion reagent (R) carries the sample into the digestion coil located inside the microwave oven (MO) for decomposition. After the decomposition, the solution mixes with the acid flow (A) at the first T-joint (T1), and then sodium borohydride flow (B) at the second T-joint (T2). The evolution of hydrides begins in the second T-joint (T2) and continues in the reactor-gas/liquid separator apparatus. Inside this apparatus a flow of carrier gas (G) assists mixing and reaction and subsequently carries hydrides to the atomic absorption spectrometric detector (D). Waste solution from the hydride generation constantly drains out via a side arm which maintains a constant pressure inside the system.

A Shimadzu UV-2100 UV-Visible Spectrophotometer was used for the photometric determination of arsenate by the molybdenum blue method (105).

4.2.2. Reagents

Standard solutions of arsenite, arsenate, MMAA, and DMAA were prepared as previously described in Section 2.2.2. Stock solutions (1000.0 µg ml^{-1} of arsenic) of arsenobetaine (46), arsenocholine (234), and tetramethylarsonium iodide (162) were

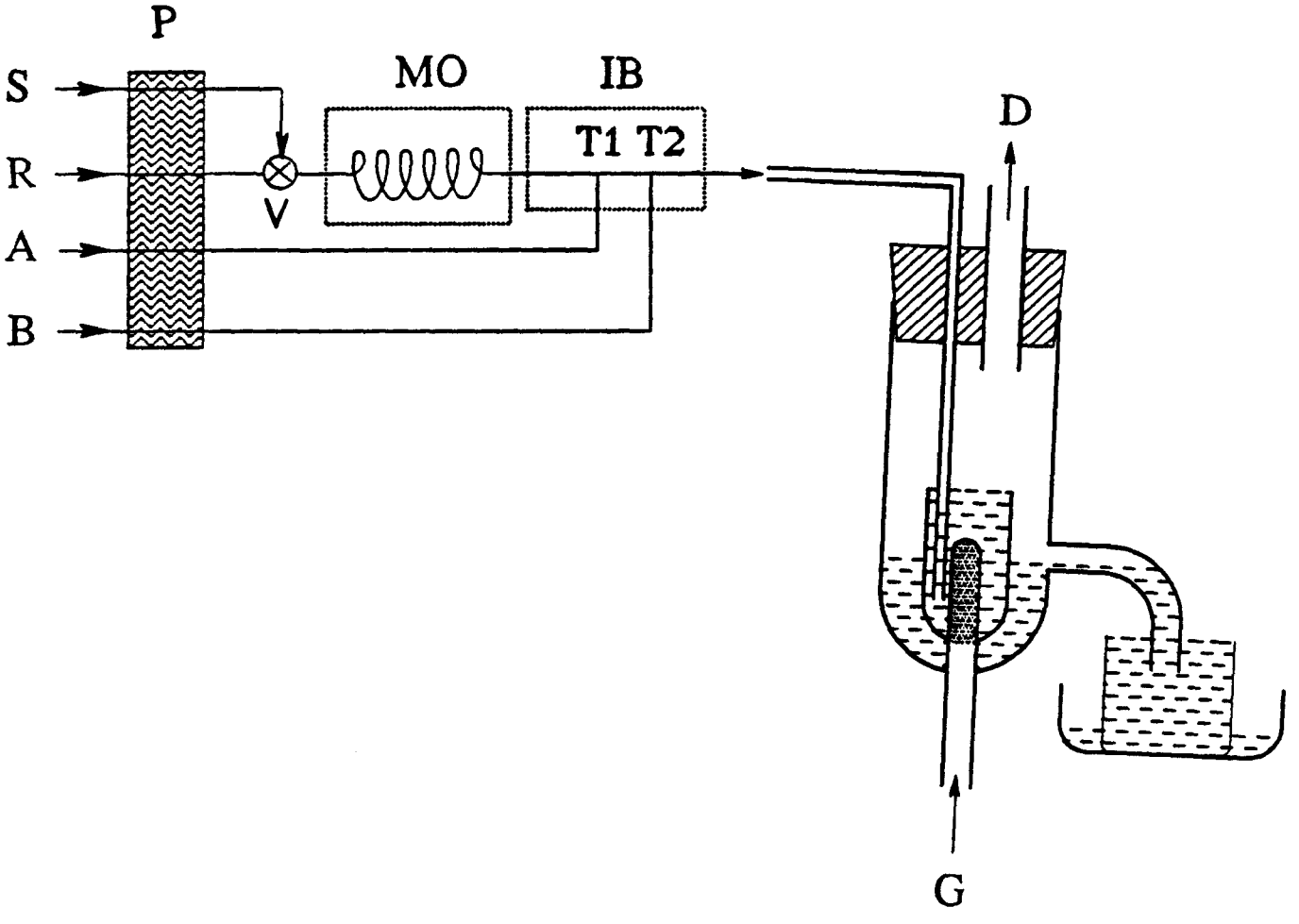


Figure 4.1. A schematic diagram of an on-line coupled flow injection - microwave oven decomposition - hydride generation system (FIA/MD/HGAAS)

S -- Sample flow
A -- Acid flow
V -- Sample injection valve
MO -- Microwave oven
IB -- Ice water batch
D -- To detector (AAS)
R -- Decomposition reagent flow
B -- $NaBH_4$ flow
P -- Peristaltic pump
T1 & T2 -- T-joints
G -- Carrier gas (N_2)

prepared by dissolving appropriate amounts of these compounds in 0.01 M hydrochloric acid. Appropriate amounts of butylarsonic acid ($C_4H_9AsO(OH)_2$), p-arsanilic acid (p-$NH_2C_6H_4AsO(OH)_2$), p-hydroxyphenylarsonic acid (p-$HOC_6H_4AsO(OH)_2$), α-toluenearsonic acid ($C_6H_5CH_2AsO(OH)_2$) (Eastman Kodak), and 4-nitrobenzenearsonic acid (4-$NO_2C_6H_4AsO(OH)_2$) (Aldrich) were dissolved in 0.01 M hydrochloric acid to make individual stock solutions (1000.0 μg ml^{-1} of arsenic). These solutions were standardized against arsenite by using both flame AAS and ICP/AES. Standard solutions were prepared by serial dilutions with 0.01 M hydrochloric acid.

4.2.3. Procedures

4.2.3.1. FIA/HGAAS

The same procedure as described in Section 3.2.4.1 (method two) was used for this operation mode.

4.2.3.2. FIA/on-line microwave decomposition/HGAAS (FIA/MD/HGAAS)

A 100-μl sample was injected into the decomposition reagent stream containing potassium persulfate and sodium hydroxide. The stream carries the sample into a 3 m knotted Teflon coil (0.5 mm i.d.) located in the microwave oven that is operating at full power (500 W). After microwave assisted decomposition, the solution flow mixes with the continuous acid and sodium borohydride feeds. Hydride generation and gas/liquid separation take place and the evolved hydrides are introduced into the flame-heated quartz tube for AA measurement. A peak signal was recorded using an integrator (Hewlett-Packard 3390A) capable of both peak height and peak area measurements. It was found that the peak height measurement gave better reproducibility and lower standard deviation. Thus the peak height of the signal was measured for quantitation unless stated otherwise. The experimental conditions for this mode of operation are summarized in Table 4.1.

Table 4.1. Experimental conditions for microwave assisted digestion and hydride generation

$NaBH_4$ Concentration:	0.65 M in 0.1 M NaOH
$NaBH_4$ Flow Rate:	3.4 ml min^{-1}
HCl Flow Rate:	3.4 ml min^{-1}
Carrier Gas Flow Rate:	160 ml min^{-1}

	FIA/HGAAS Direct Analysis	FIA/MD/HGAAS	Batch Type Microwave Digestion FIA/HGAAS
Digestion	no	microwave	microwave
$K_2S_2O_8$	none	0.1 M (2.7%)	4.5 g
NaOH	none	0.1-2 M	2.7 g
Urine Sample	100 µl	100 µl	40 ml
Digestion Reagent Flow Rate	none	5 ml min^{-1}	none
Digestion Time	none	continuous	5x3 min (for 6 samples)
Digestion Coil	none	3-10m x0.5mm	none
Cysteine	1-2%	none	none
HCl Concentration	0.50 M	3.0 M	3.0 M

4.2.3.3. Continuous microwave decomposition/HGAAS

In the continuous operation mode, the sample injection valve (V) in Figure 4.1 is replaced by a T-joint. Sample and reagent solutions are continuously taken up by the peristaltic pump and meet at the T-joint before flowing into the digestion coil. As a result

of continuous introduction of the sample, a continuous steady state signal is observed and recorded on the integrator.

4.2.3.4. Batch type microwave decomposition

Initially a sample solution (1.0 ml) and the digestion reagent solution (5.0 ml) were combined in a polyethylene bottle. The bottle was then loosely capped and placed in the microwave oven. The microwave oven was operated at the full power setting (500 w) for two minutes followed by a five minute cooling period and finally another two minutes of microwave heating. After the sample was cooled, it was diluted to 10.0 ml. Determination of the decomposition product, arsenate, in the sample was carried out by using FIA/HGAAS.

For the determination of total arsenic in urine, a urine sample or standard solution (40 ml) and the appropriate amounts of potassium persulfate and sodium hydroxide (as shown previously in Table 4.1) were combined in a 125-ml Erlenmeyer flask. Six of such sample-containing flasks were placed in the microwave oven at one time. The microwave oven was then operated at full power for 3 minutes followed by a 2-3 minute cooling period. This heating and cooling sequence was repeated for another four cycles. The samples were each diluted to 50 ml with deionized water after they were cooled to room temperature. The determination of arsenic was carried out by using FIA/HGAAS as described above. Note that excess potassium persulfate was used. This is not completely soluble in the urine sample prior to the digestion, however, the excess potassium persulfate decomposes in the presence of sodium hydroxide under microwave heating and there is no solid residue present after the digestion is complete.

4.2.3.5. Analysis of the decomposed product

A photometric method (105) based on the formation of arsenomolybdenum blue was used to measure the concentration of arsenate in the digested solution. Arsenate is

the only arsenic species to form the blue complex, the absorption of which can be measured for quantitative purposes.

A 10-ml digested solution was collected from the outlet of the digestion coil after four replicate injections of 100 µl of a sample (containing 10 µg ml^{-1} of arsenic). A 5-ml aliquot of this solution was pipetted into a clean and dry 50-ml Erlenmeyer flask. To the flask 1 ml of 1 M hydrochloric acid and 1 ml of a mixed reagent containing 0.6% (m/V) ammonium molybdate, 1.1% (m/V) ascorbic acid, 0.014% (m/V) potassium antimonyl tartrate, and 1.2 M sulfuric acid were added. A blue complex slowly formed and the absorbance at 860 nm was measured after three hours. Distilled water containing potassium persulfate and the same amount of color formation reagents was used as blank. Arsenate solution containing the same amount of potassium persulfate as in the digested solution was used as standard.

4.3. RESULTS AND DISCUSSION

4.3.1. Batch type digestion -- Preliminary studies

In a preliminary study, the microwave oven was used for open vessel batch type digestion to investigate the possibility of decomposing organoarsenicals and to search for appropriate reagents. A four minute microwave heating time was applied to each sample and reagents studied include 1-6 M nitric acid, sulfuric acid, sodium hydroxide, hydrogen peroxide, and 2.5% (m/V) potassium persulfate in 0.1 M sodium hydroxide. It was found that when the first four reagents were used, the decomposition efficiencies for arsenobetaine, arsenocholine and tetramethylarsonium were all less than 30%. When 2.5% (m/V) potassium persulfate in 0.1 M sodium hydroxide aqueous solution was used, complete conversion of arsenobetaine, arsenocholine and tetramethylarsonium to arsenate was achieved. Therefore, potassium persulfate and sodium hydroxide were chosen as the digestion reagents for further studies.

The effect of microwave assisted digestion time on the decomposition efficiency of three organoarsenicals is shown in Figure 4.2. The relative peak height was obtained by comparing signals obtained from the organoarsenicals with those obtained from arsenate upon hydride generation AAS measurements. Only a 30, 20, and 45 second microwave oven heating time is needed to completely decompose 100 µl of 200 ng ml^{-1} of AB, AC, and Me_4As^+, respectively, in 5 ml solution containing 2.5% potassium persulfate in 0.1 M sodium hydroxide. This high efficiency of decomposition within such a short period of time seemed suitable for development into an on-line decomposition system.

4.3.2. FIA/microwave decomposition/HGAAS

Figure 4.3 shows signals obtained for aqueous solutions of arsenobetaine, arsenocholine, tetramethylarsonium ion, arsenite, arsenate, MMAA, and DMAA under the following operating conditions: no digestion (Figure 4.3a), digestion with the aid of a 50 ^oC water bath (Figure 4.3b), and microwave assisted digestion (Figure 4.3c). It is clear from Figure 4.3a that AB, AC, and Me_4As^+ do not form hydrides in the absence of prior digestion; whereas As(III), As(V), MMAA, and DMAA give quantitative signals. This is consistent with literature reports (59, 223, 227, 229, 230). When a warm water heating bath is used along with 0.1 M potassium persulfate and 0.1 M sodium hydroxide as digestion reagents, only a small portion (5-25%) of these organoarsenicals is decomposed to form hydrides (Figure 4.3b). Increasing the temperature of the water bath did not result in complete decomposition of these organoarsenicals although at 90 ^oC the decomposition efficiency was increased to approximately 50%. However, microwave assisted digestion in combination with 0.1 M potassium persulfate and 0.1 M sodium hydroxide resulted in the decomposition of all arsenicals studied, and quantitative signals were obtained with HGAAS measurements (Figure 4.3c).

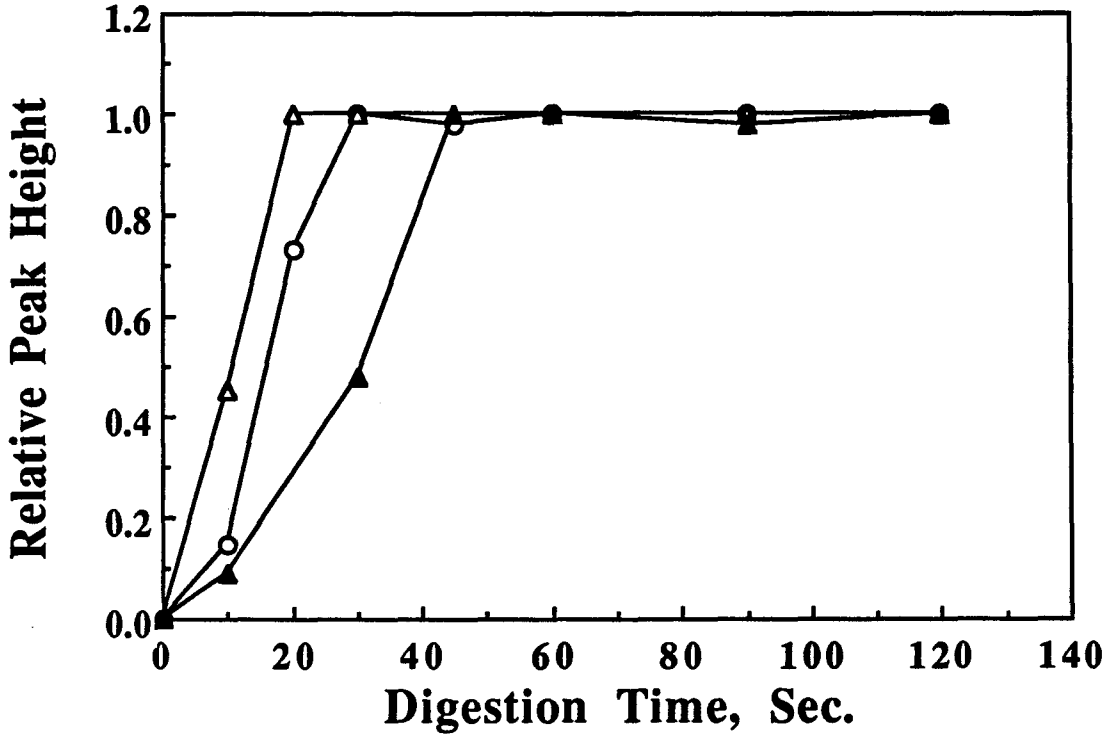


Figure 4.2. Effect of digestion time on the decomposition efficiency with a batch type digestion procedure
(O) -- AB, (Δ) -- AC, and (▲) -- Me_4As^+

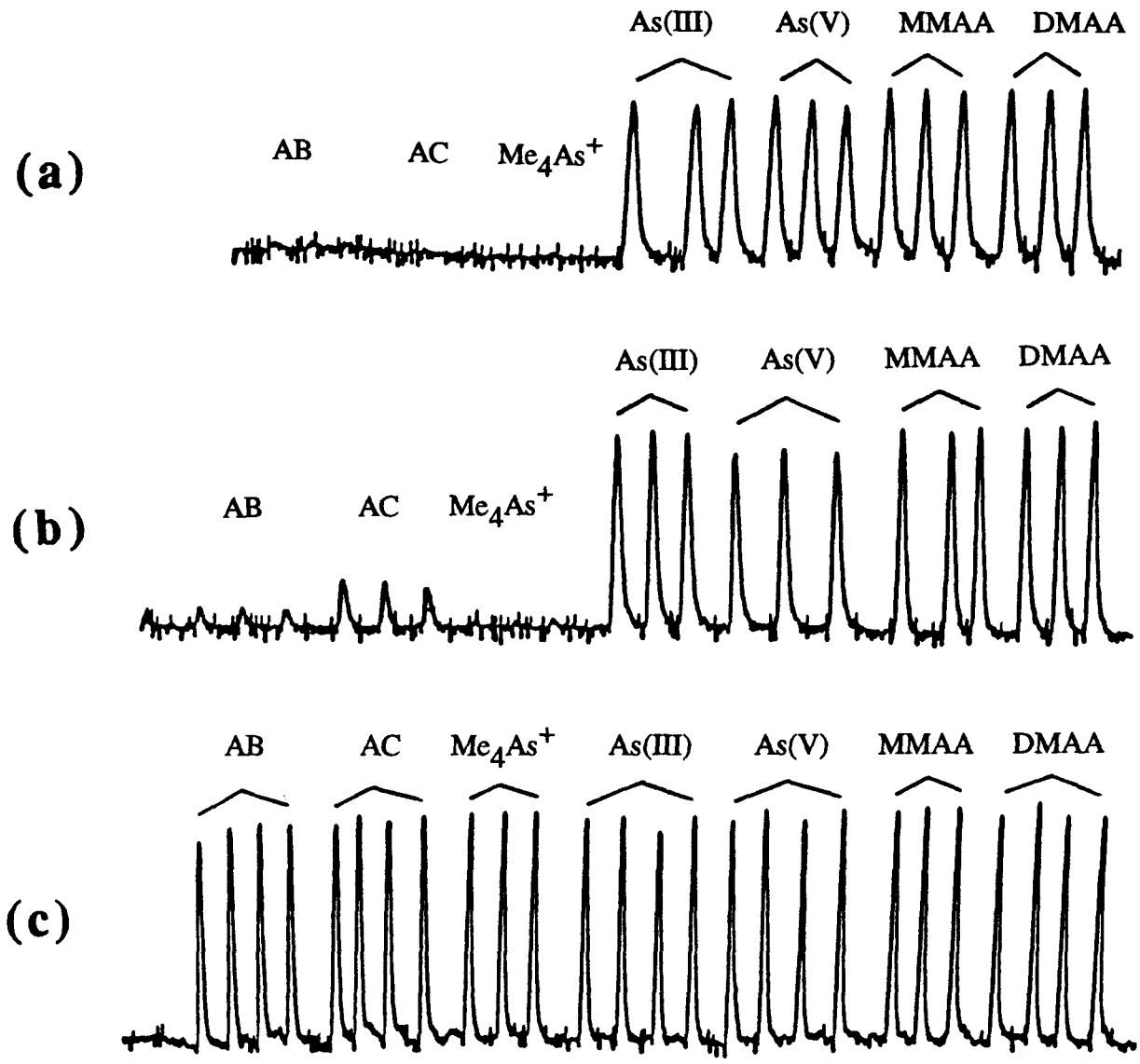


Figure 4.3. Comparison of signals from 100 µl of 200 ng ml^{-1} (as arsenic) solution of seven arsenic compounds obtained by using HGAAS and different on-line digestion methods
(a) -- No digestion; deionized water as carrier
(b) -- With a 50 °C water bath; 0.1M $K_2S_2O_8$ and 0.1M NaOH as carrier
(c) -- With microwave oven digestion; 0.1M $K_2S_2O_8$ and 0.1M NaOH as carrier

Digestion coil

Teflon coils (3 m long) with various inner diameters, 0.5, 0.8, 1.2, and 2.5 mm, were evaluated. When a 2.5 mm or a 1.2 mm i.d. coil was used, the signals observed were broad and sometimes split. This is probably due to dispersion of the analyte. The use of a Teflon coil with an inner diameter of 0.5 mm results in sharp, narrow, and reproducible signals.

The effect on digestion efficiency of sample residence time in the microwave oven was investigated by varying the length of the Teflon coil in the oven that was operating at full power. AB, AC, Me_4As^+ and arsenate were chosen as examples to study the general decomposition efficiency, and the signals obtained under the identical conditions from these arsenicals were compared. The relative signals from these organoarsenicals with respect to those from arsenate are shown in Figure 4.4. As the coil length is increased from 0.2 to 0.5 m, the relative peak height of all the three organoarsenicals increase dramatically. Within the coil length range of 2.2 to 5 m, the three organoarsenicals give signals of the same peak height as that from As(V), indicating complete conversion to hydride-forming arsenicals. On this basis a 3 m Teflon coil (0.5 mm i.d.) was chosen as optimum resulting in a residence time of each sample in the microwave oven of 15 sec under the usual operating conditions.

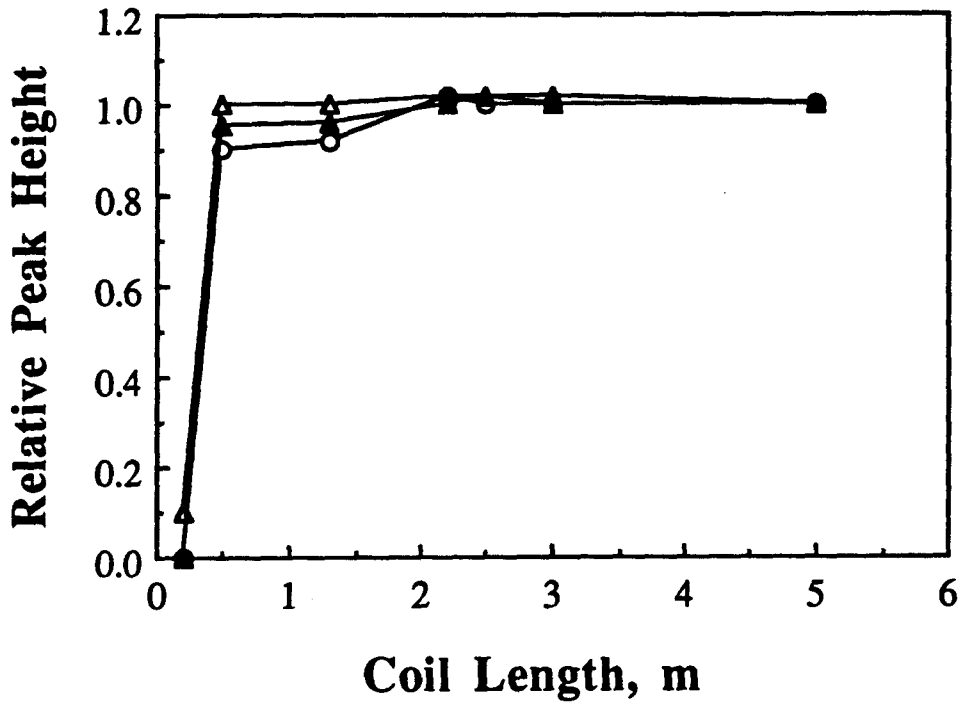


Figure 4.4. Effect of digestion coil length on decomposition efficiency. Samples of 100 µl of 200 ng ml^{-1} solution of (O) AB, (Δ) AC, and (▲)Me_4As^+

Microwave oven power level and digestion reagents

The power level of the microwave oven and the concentration of the digestion reagents are two other obvious important factors that affect the quantitative decomposition of organoarsenicals. A series of experiments were designed to study the effect of these two factors and to optimize them simultaneously. At each of the nine microwave oven power settings available from 100 to 500 W, the concentration of potassium persulfate (made in 0.1 M sodium hydroxide) was varied through the range 3.7, 18, 37, 55, 74, 110, and 150 mM. Determinations of arsenobetaine and arsenate were carried out under each of these conditions. Figure 4.5 shows the relative peak height of signals obtained from 100 µl of a 200 ng ml^{-1} solution of AB with respect to those from the same amount of As(V) at various levels of microwave oven power and concentrations of persulfate. It demonstrates that increasing both microwave oven power and persulfate concentration within the ranges of study achieves maximum and constant signals from AB. A maximum signal region lies in the persulfate concentration range 60-150 mM and microwave oven power range 300-500 W. The relative peak height of 1.0 with respect to As(V) obtained within this region indicates a complete decomposition of arsenobetaine.

A number of other decomposition reagents were investigated. No signal was observed from AB, AC, or Me_4As^+ with any of the following reagents: 0.5-4 M nitric acid and sulfuric acid, 0.07 and 0.15 M of $KClO_4$, $K_2Cr_2O_7$ and KIO_3 in either 1 M H_2SO_4, H_2O or 1 M NaOH. The relative intensities of signals from AB, AC, and Me_4As^+ digested with H_2O_2, $KMnO_4$ and $K_2S_2O_8$ in either acid, neutral, or alkaline solution were obtained and are listed in Table 4.2. Partial decomposition of these organoarsenicals takes place when $KMnO_4$ and H_2O_2 are used along with the strong alkaline condition.

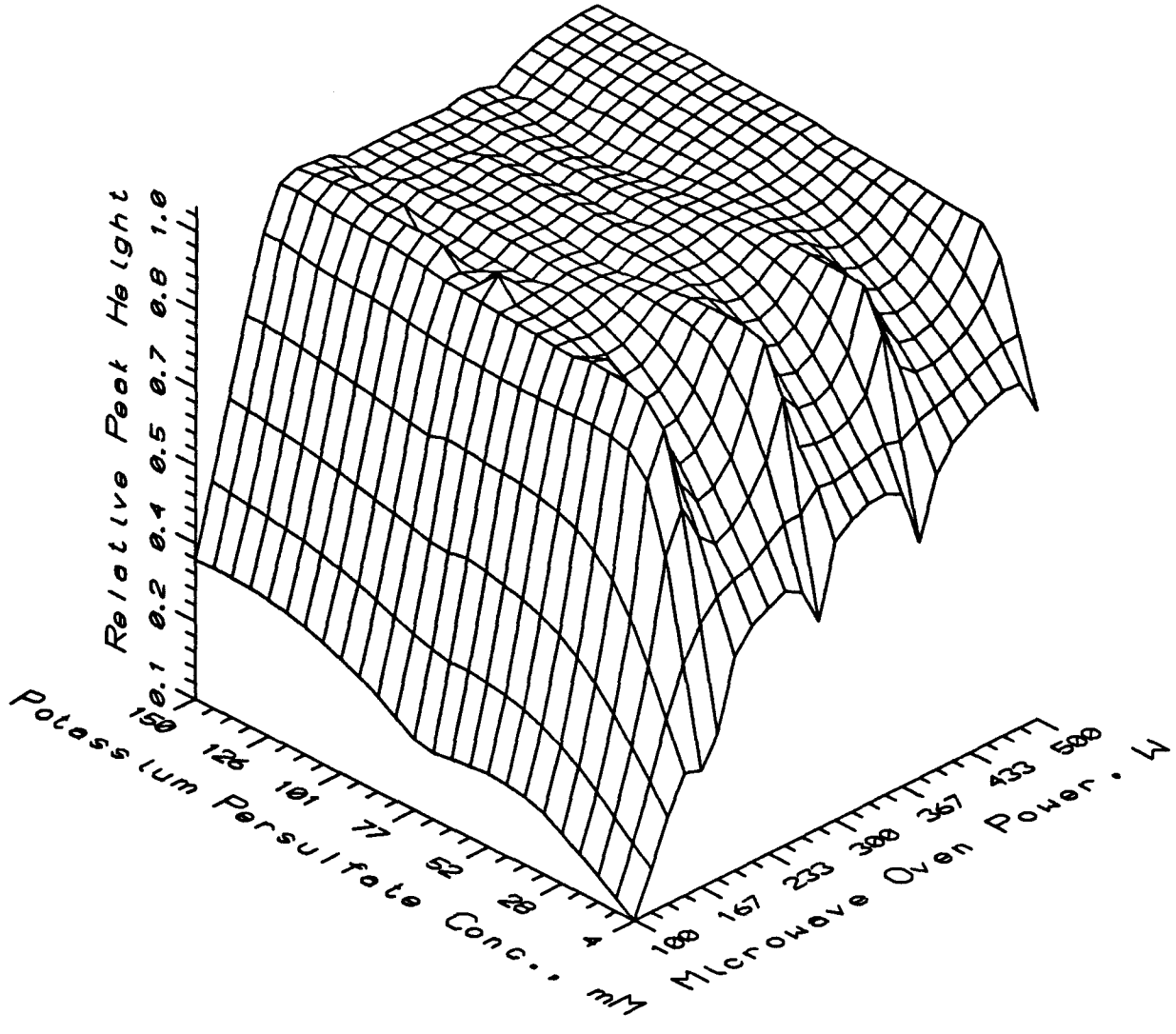


Figure 4.5. A response surface showing the effect of potassium persulfate concentration and microwave oven power on the decomposition efficiency of 100 µl 200 ng ml^{-1} of arsenobetaine

Table 4.2. Intensity of AB, AC, and Me_4As^+ signals relative to that of As(V) obtained when different decomposition reagents were used.

Decomposition Reagent		AB	AC	Me_4As^+
3% H_2O_2	in 1M NaOH	0.10	0.10	0.30
	in water	0	0	0
	in 1M H_2SO_4	0	0	0
0.11M $KMnO_4$	in 1M NaOH	0.33	0.22	0.10
	in water	0.05	0.10	0
	in H_2SO_4	0.05	0.1	0
0.11M $K_2S_2O_8$	in 1M NaOH	1.00	1.00	1.00
	in water	0.68	1.00	0.68
	in H_2SO_4	0.18	0.18	0.15
4M NaOH	in water	0	0	0

It is interesting that these organoarsenicals are not decomposed in the presence of sodium hydroxide alone. However, the addition of sodium hydroxide to potassium persulfate promoted the complete digestion of AB and Me_4As^+. Figure 4.6 shows that the digestion of AB and Me_4As^+ is not complete when 0.1 M potassium persulfate aqueous solution alone is used and microwave oven power is applied. Whereas with 0.1 M potassium persulfate in 0.1 M sodium hydroxide solution, a complete digestion of AB and Me_4As^+ is achieved as it has been shown earlier in Figure 4.3c.

The effect of the sodium hydroxide concentration and microwave oven power level on the decomposition efficiency is shown in Figure 4.7. Various concentrations of sodium hydroxide were prepared in 0.1 M potassium persulfate solution. As can be seen

from Figure 4.7, AB is not completely decomposed to arsenate in the absence of sodium hydroxide, at any microwave oven power setting. As the concentration of sodium hydroxide is increased, less microwave energy is needed to completely decompose AB, and the relative peak height of signals from AB approaches unity. A contour diagram also shows that at full power (500 W), the optimum concentration of sodium hydroxide for achieving complete digestion of AB is in the range of 0.1 to 1.0 M. If the concentration of sodium hydroxide is increased further, the hydride generation reaction is likely to be affected. Thus a solution containing 0.3 M sodium hydroxide and 0.1 M potassium persulfate was chosen as the digestion reagent.

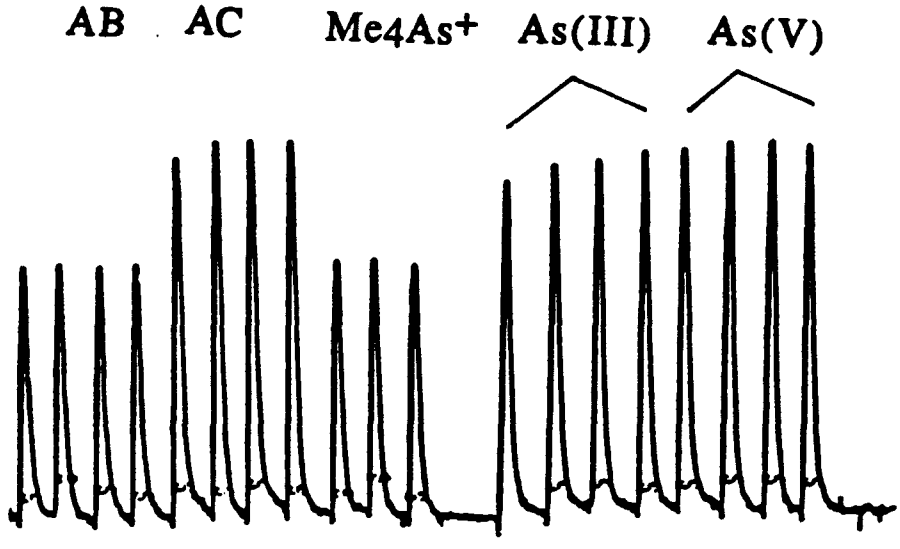


Figure 4.6. Signals obtained by using FIA/MD/HGAAS when the digestion reagent was 0.1 M $K_2S_2O_8$ in deionized waer (No NaOH added).

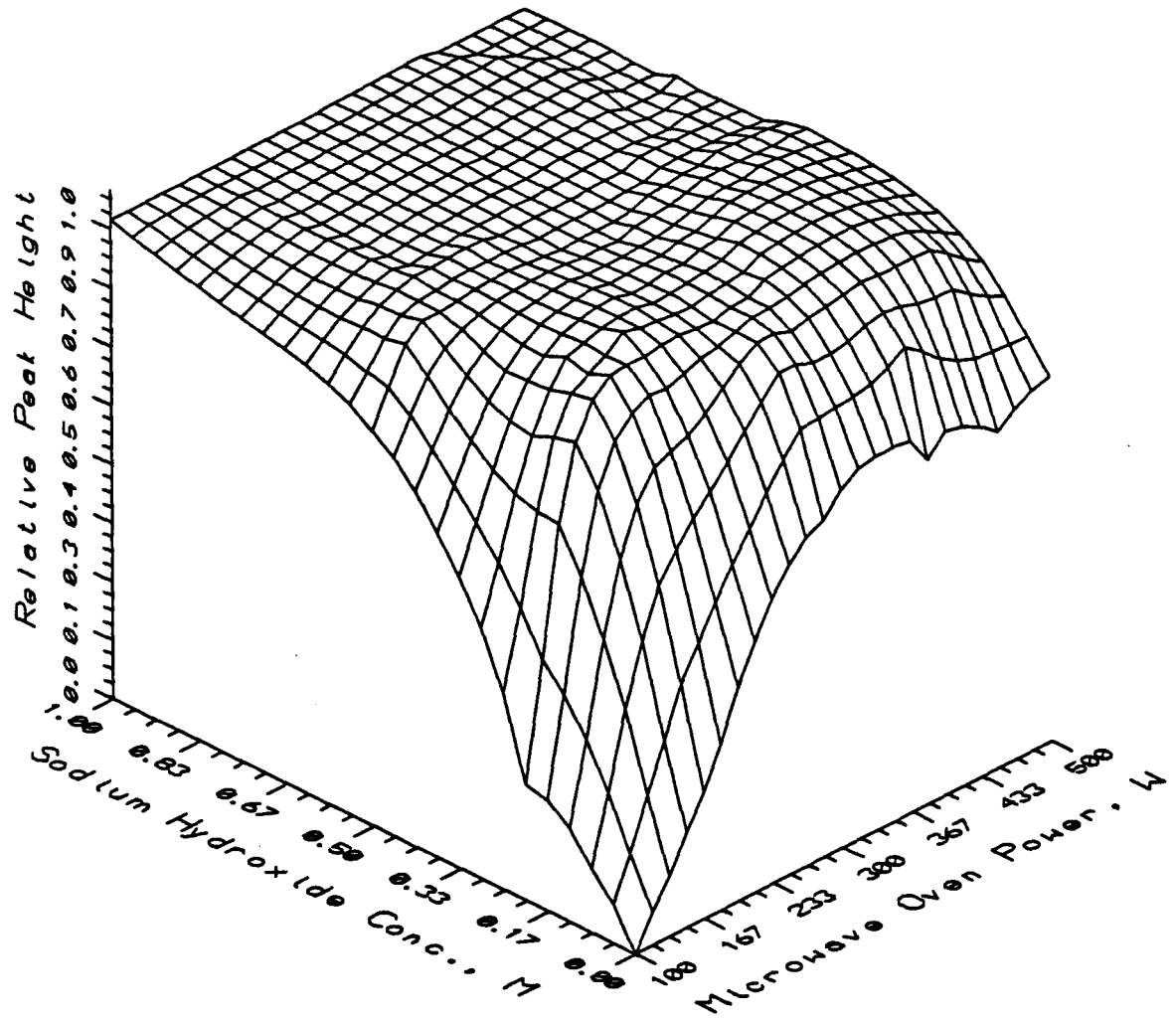


Figure 4.7. A response surface showing the effect of sodium hydroxide concentration and microwave oven power on the decomposition efficiency of 100 µl 200 ng ml^{-1} of arsenobetaine

Concentration of sodium borohydride and hydrochloric acid

Both sodium borohydride ($NaBH_4$) and acid are necessary for the hydride generation reaction. Thus their concentrations need to be optimized for use. In order to examine the possible interdependence between sodium borohydride and potassium persulfate ($K_2S_2O_8$) concentrations, both concentrations were varied and As(V) and AB were chosen as analytes. The response surfaces so obtained revealed that there was no apparent interdependence between these two reagents within the concentration ranges studied, namely 1.8-150 mM potassium persulfate and 0.13-1.6 M sodium borohydride. This is understandable considering the small molar concentration of potassium persulfate relative to that of sodium borohydride.

The effect of the concentration of hydrochloric acid and sodium borohydride on the determination of arsenic was also studied, using AB and As(V) as examples. Response surfaces and contour diagrams of peak height from AB and As(V) vs. concentrations of sodium borohydride and hydrochloric acid were obtained. The optimum concentration ranges were 2.5-5 M hydrochloric acid and 0.5-1.3 M (2.5-6.5%) sodium borohydride in 0.1 M sodium hydroxide. From these optimum ranges, 3 M hydrochloric acid and 0.65 M (3%) sodium borohydride in 0.1 M sodium hydroxide were chosen as operating condition for hydride generation.

Response surfaces clearly show the effect of two variables. However, a large number of experiments is usually required to construct such a response surface. A sample throughput of 100-120 per hour is possible with the present system permitting optimization of two factors to be achieved within 1 hr.

Other organoarsenicals

Relative intensities with respect to those of As(V) of HGAAS signals from p-arsanilic acid, p-hydroxyphenylarsonic acid, 4-nitrobenzenearsonic acid, and α-toluenearsonic acid are shown in Table 4.3. By using microwave oven digestion in the

presence of 0.1 M potassium persulfate and 0.3 M sodium hydroxide, all these compounds are completely decomposed and essentially 100% relative peak intensities are obtained from the HGAAS measurement. Interestingly when the microwave oven is turned off and the same decomposition reagents are used as a carrier to mix with the 100 μl sample injected through the FIA mode, complete decomposition of these compounds is also achieved, indicating that these compounds are easily decomposed in the presence of potassium persulfate and sodium hydroxide.

Table 4.3. Comparison of relative intensities of arsenic signals obtained by using HGAAS with and without microwave assisted decomposition.

Arsenicals	No Digestion H_2O as Carrier	No Digestion 0.1M $K_2S_2O_8$ & 0.3M NaOH as Carrier	Microwave Digestion 0.1M $K_2S_2O_8$ & 0.3M NaOH as Carrier
Arsenate	1.00	1.00	1.00
p-arsanilic acid	0.16	1.00	1.00
p-hydroxyphenylarsonic acid	0.18	0.99	1.02
4-nitrobenzene arsonic acid	0.06	1.00	0.98
α-toluenearsonic acid	0.76	1.02	1.00

Decomposition product

In order to identify the decomposition product from the microwave assisted digestion, the solution was collected from the outlet of the digestion coil. The molybdenum blue method (105), in which only arsenate among arsenic species forms a

blue compound, was used to measure the arsenate content in the digested solution. It was found that all the arsenicals studied above were oxidized to arsenate, with recoveries of 90-110%.

Calibration

Figure 4.8 shows 3-point calibration HGAAS signals from AB following microwave oven digestion (Figure 4.8a), As(V) following microwave oven digestion (Figure 4.8b), and As(V) without microwave oven digestion (Figure 4.8c). It is clear that same degree of response is obtained from the same amounts of As(V) and AB. Similar responses were also obtained from the other arsenic species as is to be expected since these different arsenicals are also oxidized to As(V), as discussed above. Therefore, it is possible to use a calibration curve from a single arsenical for the determination of all the arsenic species. A detailed calibration using AB as standard demonstrated a linear response over the concentration range 5 to 200 ng ml^{-1} (r^2=0.999).

The detection limit, defined as three times the standard deviation of blank , is 0.5 ng ml^{-1} for 100 µl sample injection or 0.05 ng of arsenic.

Interference

A known amount of AB was spiked into a seawater and urine sample matrices and recoveries were evaluated in order to study possible interference in analyzing environmental and biological samples. No interference was encountered from the seawater matrix in the determination of AB and a recovery of 90-100% was obtained. However, when AB was spiked into a urine sample, only approximately 40% of the spiked AB was recovered based on the HGAAS measurement. As(V) spiked into both seawater and urine samples was quantitatively recovered. Thus the interference from the urine matrix in the determination of AB is probably the result of incomplete digestion of AB.

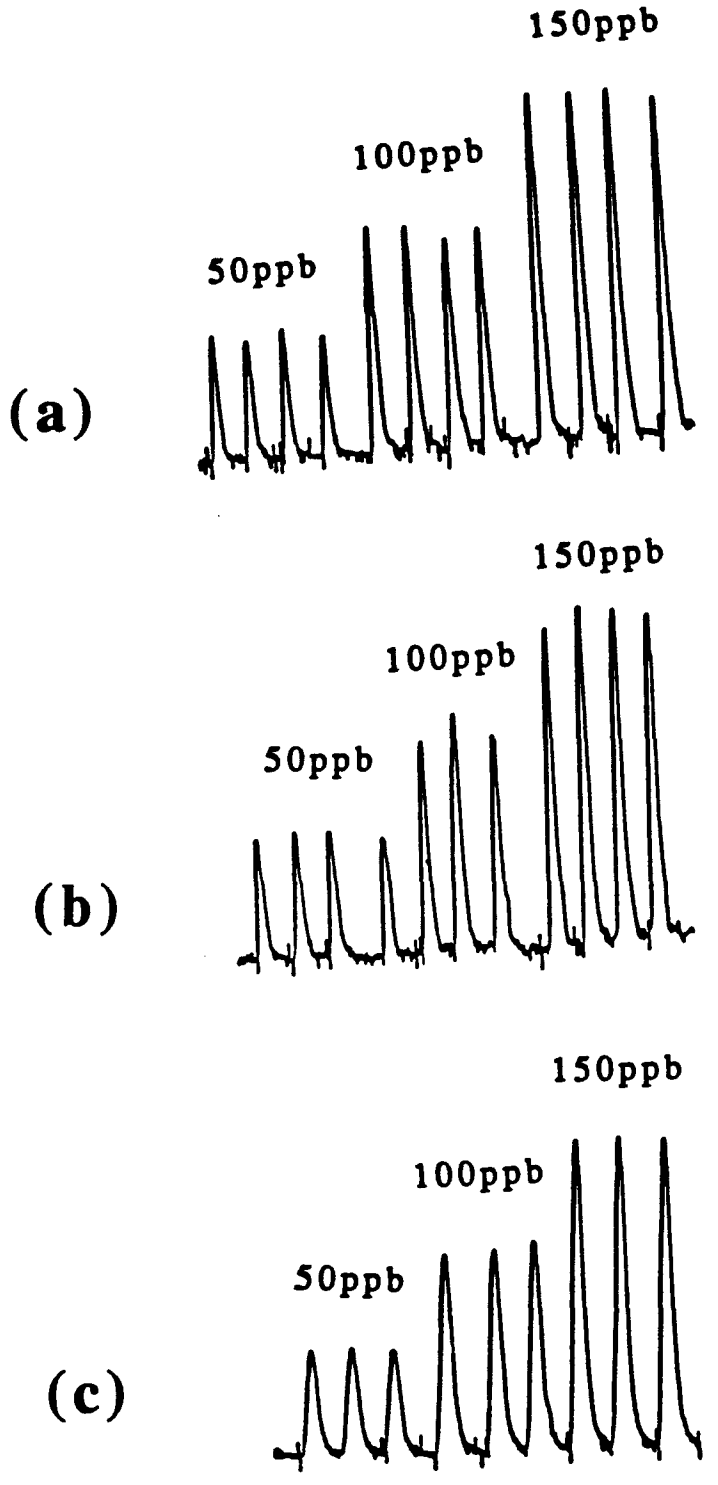


Figure 4.8. Comparison of signals obtained from 100 µl of 50, 100, and 150 ng ml^{-1} solutions of arsenic compounds (as arsenic)

(a) -- AB; with microwave assisted digestion
(b) -- As(V); with microwave assisted digestion
(c) -- As(V); without digestion

Table 4.4. Recovery of arsenobetaine in the presence of possible matrix components.

Compound	Concentration ($mg\ ml^{-1}$)	Recovery (%)	Compound	Concentration (%, V/V)	Recovery (%)
Urea	2	100	Methanol	0.2	95
	20	93		0.3	60
	30	87		0.5	36
	50	83		1	18
	70	80		5	11
	100	57	Acetonitrile	0.2	99
NH_4Cl	20	100		0.3	89
	40	90		0.5	62
	60	88		1	44
	100	76		5	15
NaCl	10	100		10	11
	50	100	Acetone	0.5	58
	110	90		1	46
	190	82	Propanol	0.5	54
K_2HPO_4	100	100	Acetic acid	0.5	67
Malonic acid	20 mM	90		1	48

The recovery of AB in the presence of other compounds such as urea and NaCl was studied and some results are summarized in Table 4.4. There is little interference from sodium chloride (NaCl) and potassium hydrogen phosphate (K_2HPO_4) at a concentration of up to 100 $mg\ ml^{-1}$ on the decomposition and determination of AB. The presence of the same amounts of urea and ammonium chloride (NH_4Cl) results in an

incomplete decomposition of AB and consequently a lower recovery. Severe interference is observed when organic solvents such as alcohol and acetonitrile are present, at levels as low as 0.5%. However, urine samples from healthy subjects are not expected to contain high concentrations of organic solvents (98, 99). Urea, chloride, and sodium are among the most abundant constituents in urine. Their concentrations are in the order of 20, 6, and 4 mg ml^{-1}, respectively, as the average daily urine volume is 1.2-1.5 L and the average daily excretion of urea, chloride, and sodium are 24, 7, and 4.2 g, respectively (99). The interference from urine sample matrix in the decomposition of AB is probably not associated with the presence of a single component but rather with the presence of a number of substances in significant concentrations.

4.3.3. On-line decomposition of organoarsenicals in the presence of urine sample matrix

Initial attempts to completely decompose organoarsenicals in a urine sample matrix were made by optimizing concentrations of the decomposition reagents, potassium persulfate and sodium hydroxide. Figures 4.9 and 4.10 show the recoveries of spiked arsenobetaine (AB) into a urine sample, determined by HGAAS after the on-line microwave decomposition using persulfate and sodium hydroxide of various concentrations. Recovery values were obtained by comparing signals from spiked AB with those of the same amount of an As(V) standard solution. As demonstrated in Figure 4.9, increasing the concentration of potassium persulfate from 0.1% to 4% results in a continuous improvement of recoveries with a maximum recovery of 45% when the potassium persulfate concentration is 4%. Further increase in the persulfate concentration was not studied because of the limited solubility of potassium persulfate in water. Similarly, increasing the sodium hydroxide concentration from 0.1 M to 2 M leads to higher recoveries in the determination of the spiked AB in the urine sample as shown in Figure 4.10. Although recoveries are improved at these higher reagent concentrations, the

desired quantitative recoveries are not achieved because of incomplete decomposition of AB. Therefore, in addition to higher concentrations of decomposition reagents, a longer decomposition time appears to be necessary in order to achieve complete decomposition of AB and thereby a quantitative recovery.

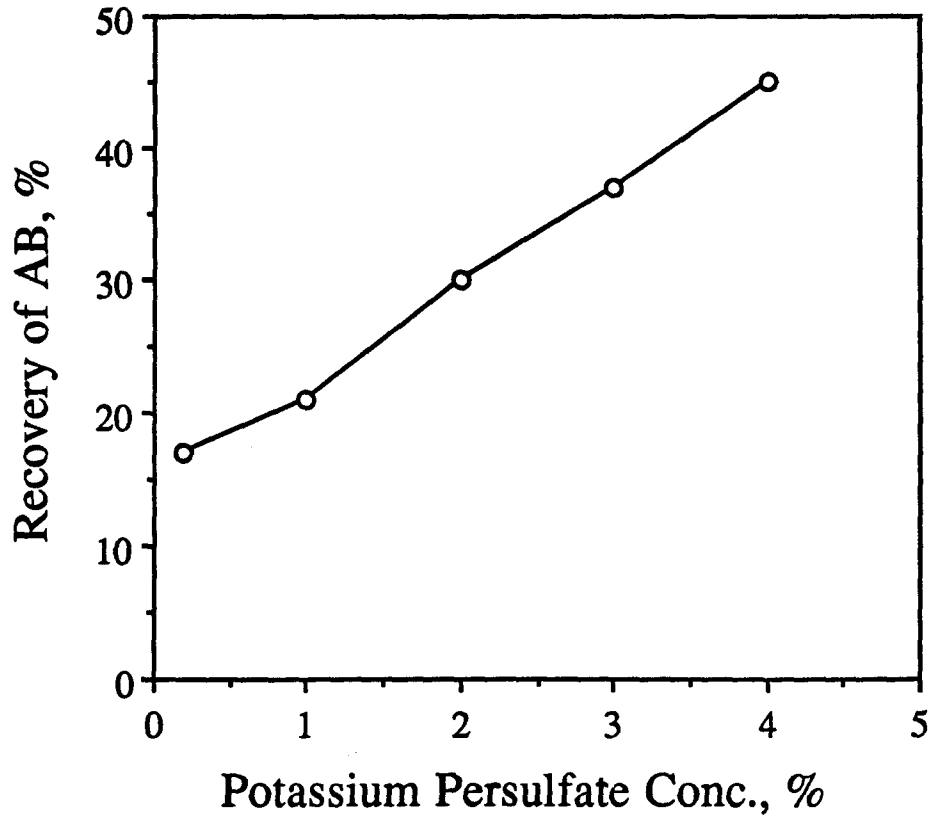


Figure 4.9. Effect of $K_2S_2O_8$ concentration on recovery of arsenobetaine in urine by using FIA/MD/HGAAS measurement.

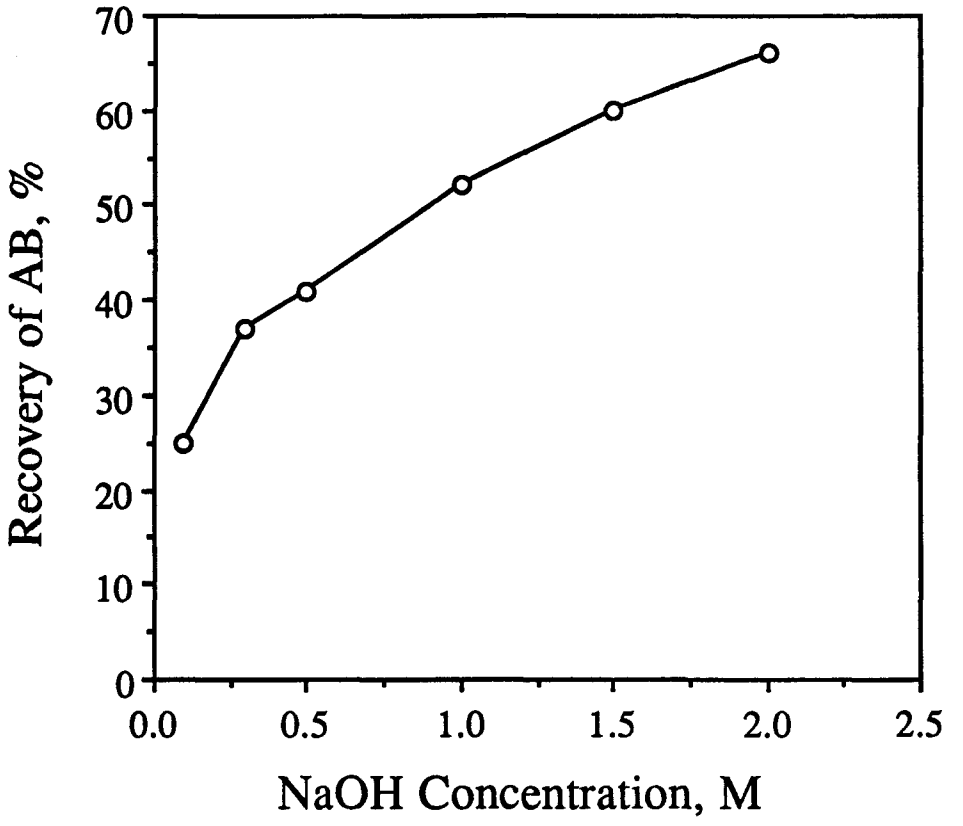


Figure 4.10. Effect of NaOH concentration on recovery of arsenobetaine in urine by using FIA/MD/HGAAS measurement.

Table 4.5 summarizes results of AB recovery when the length of the digestion coil is varied from 3 to 10 m, and it is seen that the recovery of AB spiked into a urine sample is improved by using a longer digestion coil. This is because the sample is digested for a longer time, resulting in a more complete decomposition. The results in Table 4.5 also indicate that improved recoveries are obtained when additional digestion reagents are added to the urine samples before the samples are analyzed by using FIA/on-line microwave decomposition/HGAAS. A recovery of 98% is achieved when the above two approaches are incorporated, i.e. the use of a longer digestion coil and the addition of the digestion reagents into the urine sample that is diluted with equal volume of water. Similarly, quantitative recoveries of AC and Me_4As^+ spiked into the urine sample can be obtained. The use of a longer decomposition coil, however, also results in a higher noise level and poorer signal stability. In order to solve this problem, an alternative approach involving a batch mode microwave oven digestion followed by determination using FIA/HGAAS was studied.

4.3.4. Batch type microwave assisted digestion and FIA/HGAAS for the determination of urinary arsenic

Arsenobetaine (AB) is the major arsenic compound found in most seafood, and it is one of the organoarsenic compounds that is most difficult to decompose to a form amenable to hydride generation. Thus AB was chosen as a model compound for studies on the decomposition of organoarsenicals in urine. A measured amount of AB was spiked into a urine sample and the arsenic concentration was measured using FIA/HGAAS after batch type microwave assisted digestion of the sample in open vessels. Recovery was measured by comparing signals obtained from the spiked AB with those of the same amounts of an As(V) standard solution. For this optimization study, two samples were placed in the microwave oven at a time.

Table 4.5. Effect of digestion coil length and the presence of additional digestion reagent on the recovery of organoarsenicals *

Coil Length	Arsenic Spiked	Spiked Into (Matrix)	Additional Digestion Reagents #	Recovery (%)
3 m	AB	H_2O	none	100 ± 4
	AB	H_2O	3% $K_2S_2O_8$ & 2 M NaOH	97 ± 5
	AB	Urine	none	37 ± 3
	AB	Urine	3% $K_2S_2O_8$ & 2 M NaOH	55 ± 4
6 m	AB	H_2O	none	100 ± 11
	AB	H_2O	3% $K_2S_2O_8$ & 2 M NaOH	98 ± 8
	AB	Urine	none	45 ± 4
	AB	Urine	3% $K_2S_2O_8$ & 2 M NaOH	70 ± 12
10 m	AB	H_2O	none	95 ± 8
	AB	H_2O	3% $K_2S_2O_8$ & 2 M NaOH	97 ± 15
	AB	Urine	none	66 ± 14
	AB	Urine	3% $K_2S_2O_8$ & 2 M NaOH	86 ± 9
	AB	1:1 Diluted Urine	3% $K_2S_2O_8$ & 2 M NaOH	98 ± 9
	AC	Urine	3% $K_2S_2O_8$ & 2 M NaOH	95 ± 6
	Me_4As^+	Urine	3% $K_2S_2O_8$ & 2 M NaOH	92 ± 10

*: All analyses were carried out by using FIA/on-line MD/HGAAS with 3% $K_2S_2O_8$ and 1.5 M NaOH as decomposition reagents.

#: Additional digestion reagents were prepared in urine samples prior to analysis.

Effect of decomposition time and concentrations of potassium persulfate and sodium hydroxide

The effect of decomposition time and the amount of potassium persulfate on the recovery of AB as arsenic spiked into a urine sample is shown in Figure 4.11. Numbers shown on the contour lines represents the recovery of AB. A possible dependence between these two factors should be revealed by studying the two together. As the decomposition time and the concentration of potassium persulfate increase, recoveries of AB approach unity. This is presumably because longer decomposition times and higher reagent concentrations result in complete decomposition of AB in the urine sample. The contour diagram (Figure 4.11) clearly shows that quantitative recoveries of AB are achieved when the amount of potassium persulfate added is in the range of 10-16 g per 100 ml of urine and the decomposition time is longer than 5 minutes.

The effect of the concentration of sodium hydroxide and the decomposition time was also studied, and the results for AB recovery are presented in Figure 4.12. Recovery of spiked AB improves with an increase of the sodium hydroxide concentration from 0.1 to 1.5 M and the decomposition time from 0.5 min to 4 min. Complete recovery is achieved with a sodium hydroxide concentration greater than 1.5 M and a microwave decomposition time of longer than 4 min.

A six minute microwave decomposition time, 1.7 M sodium hydroxide and 11% potassium persulfate were chosen as optimum for digesting two samples at one time to ensure complete decomposition and quantitative recoveries of organoarsenicals in urine samples. For a batch of 6 samples a total of 15 min proved to be sufficient and this was adopted for further applications.

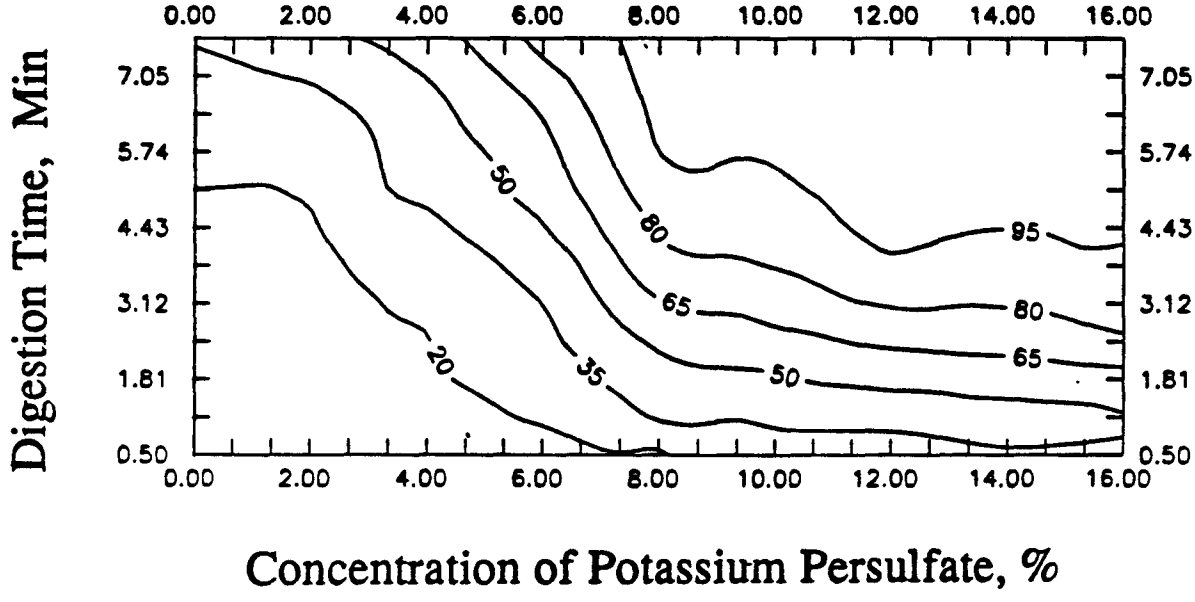


Figure 4.11. Effect of microwave digestion time and $K_2S_2O_8$ concentration on recovery of arsenobetaine in urine by using batch type microwave digestion and FIA/HGAAS determination. (Numbers on contour lines represent recoveries).

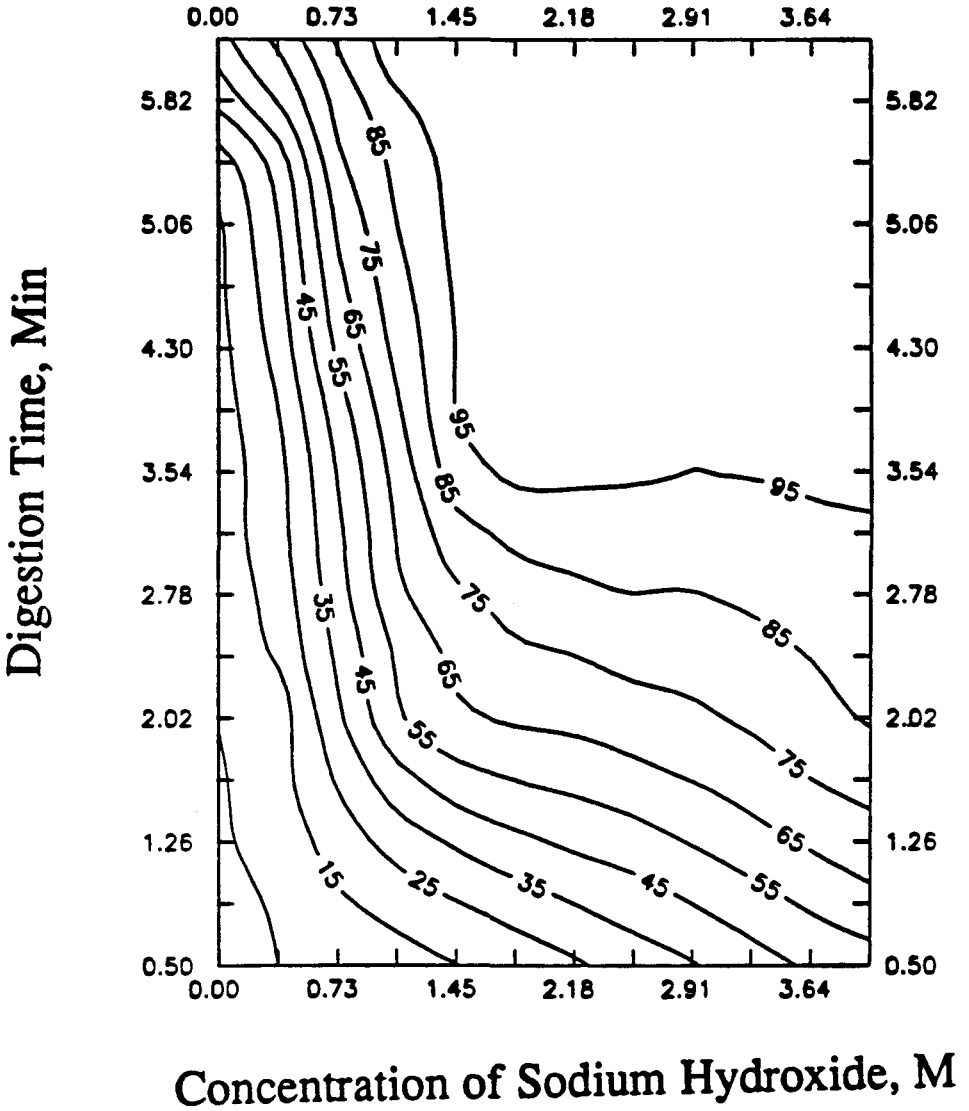


Figure 4.12. Effect of microwave digestion time and NaOH concentration on recovery of arsenobetaine in urine by using batch type microwave digestion and FIA/HGAAS determination. (Numbers on contour lines represent recoveries).

Calibration and standard addition

For comparison, both As(V) and AB were used as standards in obtaining calibration curves. As shown in Figure 4.13, calibration curves obtained from As(V) and AB are superimposable, indicating that AB is completely decomposed to arsenate which subsequently forms arsine upon treatment with sodium borohydride in the same way as does As(V). Therefore, either compound could be used as standard for quantitative analysis. For convenience, As(V) was chosen for routine work.

To estimate any possible interference from the sample matrix, a standard addition study was conducted by separately spiking standard As(V) and AB of various concentrations into a urine sample. Samples spiked with As(V) were directly analyzed for arsenic by FIA/HGAAS, whereas the samples spiked with AB underwent microwave assisted decomposition before being analyzed by the same FIA/HGAAS method. The two standard addition curves are also shown in Figure 4.13 and they are essentially parallel to the calibration curves. These results demonstrate that there is no interference from the urine sample matrix in the determination of As(V) and AB. When As(III), MMAA, DMAA, and arsenocholine were spiked into the urine sample, quantitative recoveries were also obtained, indicating that there is no interference in the determination of these arsenic species in urine.

Analysis of a reference urine sample, UriChem (Urine Chemistry Control Level 1, Fisher Scientific), gives arsenic concentrations of 16 ± 1 ng ml^{-1} in the absence of digestion (cysteine added) and 26 ± 1 ng ml^{-1} following microwave assisted digestion. No information on the arsenic species is available for comparison although the value for total arsenic concentration is given as 25 ± 19 ng ml^{-1}. Our result for total arsenic, 26 ± 1 ng ml^{-1}, is in agreement with this value.

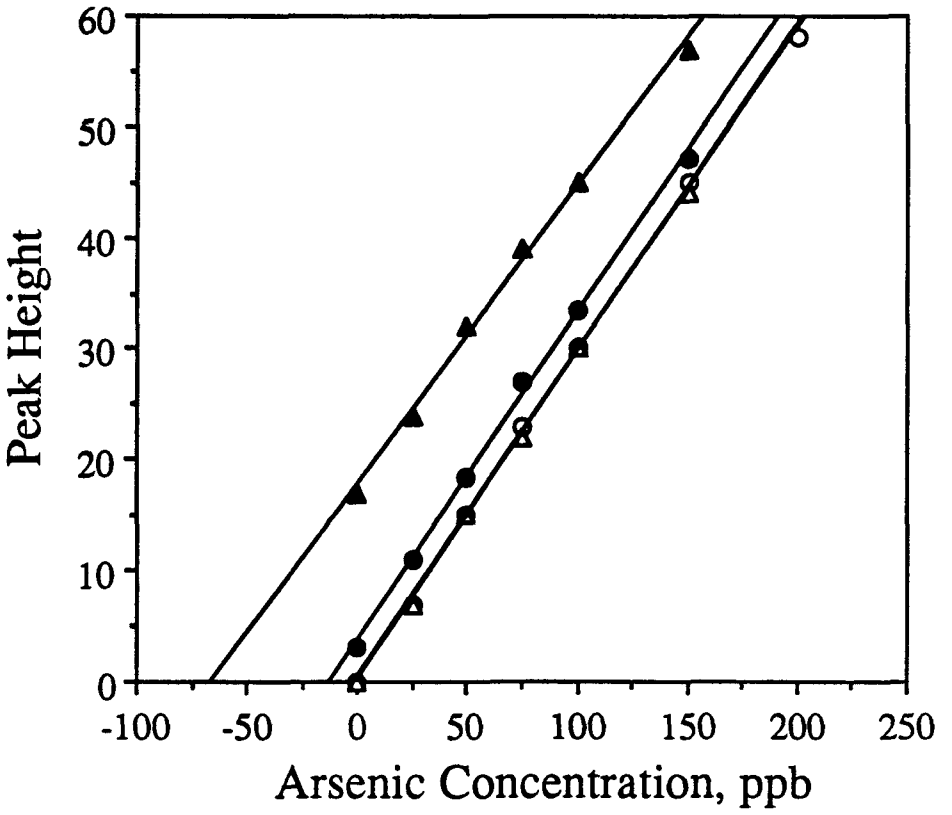


Figure 4.13. Comparison of calibration curves of As(V) (O) and AB (Δ) and standard addition curves of As(V) (●) and AB (▲) spiked into a urine sample.
For AB determination, both standards and spiked samples underwent microwave digestion; whereas no digestion was needed for As(V) determination.

Standard Deviation

Eleven aliquots (40 ml each) of a urine sample (collected approximately 5 hours after the ingestion of some crab meat) were analyzed for the sum of As(III), As(V), MMAA, and DMAA without digestion and total arsenic concentration following microwave assisted decomposition. Each decomposed sample was measured three times. The relative standard deviation (RSD) from the 11 replicate determinations of arsenic in the urine sample (mean arsenic concentration of 63.4 ng $ml^{-1)}$ is 3.0% with peak height measurement and 4.9% with peak area measurement. The sum of As(III), As(V), MMAA, and DMAA measured without digestion of the same urine sample is 10.1 ng ml^{-1}.

Application

The method described above shows simplicity, accuracy, and is free from interference. Because the ingestion of seafood can result in the urinary excretion of increased levels of "hidden" organoarsenic compounds, and the method developed is capable of differentiating these marine organoarsenicals from As(III), As(V), MMAA and DMAA, the method was used to analyze urine samples collected from a person following the consumption of some crab meat.

Figure 4.14 shows the arsenic concentration in urine samples collected at various times encompassing the ingestion of crab meat from a 500 g steam cooked crab. The sum of As(III), As(V), MMAA and DMAA concentrations measured by using FIA/HGAAS without digestion and the total arsenic concentration measured after microwave assisted decomposition of the sample are shown. The difference corresponds to the hidden arsenicals, those that normally do not form hydrides upon treatment with sodium borohydride. As shown in Figure 4.14, ingestion of crab meat has very little effect on the concentrations of As(III), As(V), MMAA and DMAA in urine. However, up to a 10 fold increase in the total arsenic concentration is observed in urine samples collected after

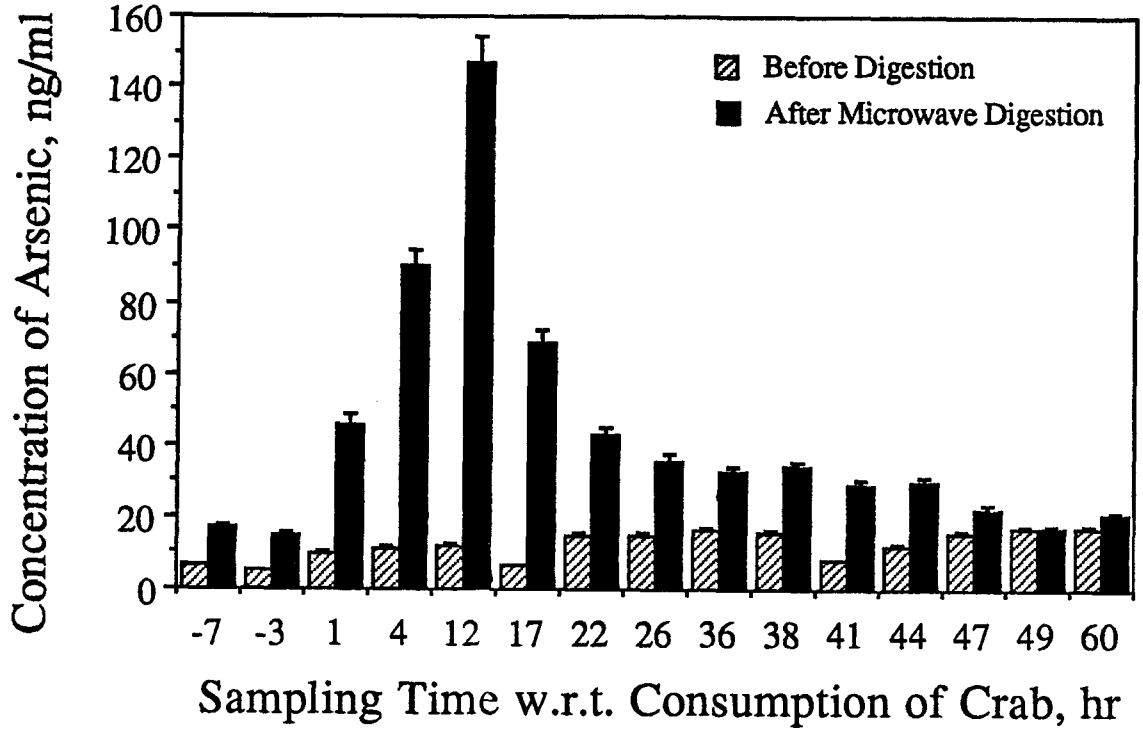


Figure 4.14. Concentration of arsenic in urine samples collected from a person at various times with respect to his consumption of crab meat.

(▨) -- Total hydride-forming arsenic concentration obtained directly by using FIA/HGAAS, without digestion of urine samples

(■) -- Total arsenic concentration obtained by using microwave assisted digestion and FIA/HGAAS determination.

eating crab meat. This result is not unexpected because high concentrations of organoarsenicals such as arsenobetaine have been found in much seafood including crab (86, 94, 152), and these organoarsenic compounds are eliminated by the kidneys and excreted in urine. Obviously, measuring total urinary arsenic concentrations is not acceptable for assessing occupational exposure when the subject has eaten seafood prior to the sampling. Roy (235) stated that the ingestion of seafood 48-72 hours prior to sampling would invalidate the use of urinary arsenic as a measure of occupational exposure if the dietary arsenic was not taken into account. In studies by Buchet *et al.* (88, 89) the subjects were restricted from eating seafood before their urine samples were collected. Foa *et al.* (80) and Chana and Smith (104) have also discussed the interference from dietary arsenic on the biological monitoring of occupational exposure to arsenic. By using the analytical method developed here, however, we are able to differentiate the organoarsenicals due to dietary intake from species such as As(III), As(V), MMAA and DMAA whose concentration would be expected to vary with occupational exposure to arsenic compounds. By using this new methodology, occupational exposure can be assessed easily by measuring urinary arsenic concentration without interference resulting from the consumption of crustacean type of seafood which often contains high levels of arsenobetaine.

Figure 4.14 shows that rapid urinary excretion of these organoarsenicals occurs as early as one hour after eating crab. The excretion of arsenic continues for about two days, and maximum arsenic concentration in the urine appears between 4 and 17 hours after eating crab. Reproducible excretion patterns were obtained from several replicate feeding experiments: the ingestion of some other seafood such as shrimp and salmon. The present results showing rapid urinary excretion of organoarsenic compounds are in agreement with Buchet *et al.* (94) who calculated that the half-time for urinary excretion following crab meat consumption was 18 hours. Freeman *et al.* (87) and Charbonneau *et al.* (90) have reported that most of the arsenic ingested is excreted in the urine from both

men (87) and monkeys (90) within two days following eating arsenic-containing fish (flounder fillets and Altantic grey sole, respectively).

Urinary arsenic excretion pattern and the excretion rate vary with the different arsenic compounds ingested. While the excretion of organoarsenicals present in marine organisms is very fast as can be seen from this work and that of others (87, 90,236), the elimination of inorganic arsenic by the kidney is relatively slow. Buchet *et al.* (94) calculated a biological half-time of 30 hours for the urinary excretion of arsenic following a single ingestion of 3000 μg arsenic as sodium arsenite. They also reported (88) that after the ingestion of 500 μg arsenic in the form of arsenite, 45% of the dose was excreted within 4 days, of which 24% was as DMAA, 10% as MMAA and 11% as inorganic arsenic. Following ingestion of the same amount of arsenic as either MMAA or DMAA, however, approximately 75% of the dose was excreted in the urine within 4 days. Tam *et al.* (84) reported that in six human subjects who ingested 0.01 μg arsenic as ^{74}As labelled arsenic acid, 38% of the dose was excreted in the urine within 48 hours and 58% (30% DMAA, 12% MMAA and 16% inorganic arsenic) within 5 days. The difference in excretion rate between inorganic arsenic and seafood organoarsenic may be due to the fact that inorganic arsenic is metabolized by human (84, 88, 93, 94, 96) whereas organoarsenic such as arsenobetaine is excreted unchanged in urine (86).

It is interesting to note that a trace amount of "hidden" organoarsenicals is found in urine samples collected before the ingestion of seafood as is shown in Figure 4.14. In another set of experiments where urine samples were collected for 60 hours from the same person under a regular diet, which excluded seafood, the arsenic concentration in every urine sample measured following microwave assisted digestion is equal to or higher than that obtained without the digestion. This confirms the presence of "hidden" organoarsenic compounds in the urine of people who do not regularly eat seafood. These results support the findings of Foa *et al.* (80) who reported that unidentified (and non-hydride-forming) organoarsenicals represented up to 70% of the total arsenic in urine

samples for a reference population that did not have occupational exposure and did not ingest large amounts of seafood.

The speciation of arsenic compounds was studied by using HPLC with inductively coupled plasma mass spectrometry detection (HPLC/ICPMS), a technique which will be discussed later in detail. Figure 4.15 shows chromatograms obtained from a crab extract (Figure 4.15a) and urine samples (Figure 4.15b-d) from a volunteer who ingested some crab meat. A high level of AB (approximately 120 ng ml^{-1}) is found in the urine sample collected 12 hr after the ingestion of crab. This concentration is rapidly decreased to approximately 25 ng ml^{-1} in the urine sample collected 38 hr after crab consumption. Very little change in concentration of other arsenic compounds (such as arsenate and DMAA) is observed. These results confirm that the ingested arsenobetaine is rapidly excreted unchanged into urine.

4.3.5. Continuous system

The microwave oven digestion procedure was also adapted for continuous mode operation. Solutions of sample and digestion reagent were continuously taken up and mixed at a T-joint before being introduced into the microwave oven operating at its full power. Steady state signals are obtained as shown in Figure 4.16. No signal is observed from AB, AC, or Me_4As^+ prior to microwave oven digestion (Figure 4.16a). Quantitative decomposition of AB, AC, and Me_4As^+ is achieved by using microwave oven digestion, and successful determinations can be carried out by using HGAAS (Figure 4.16b).

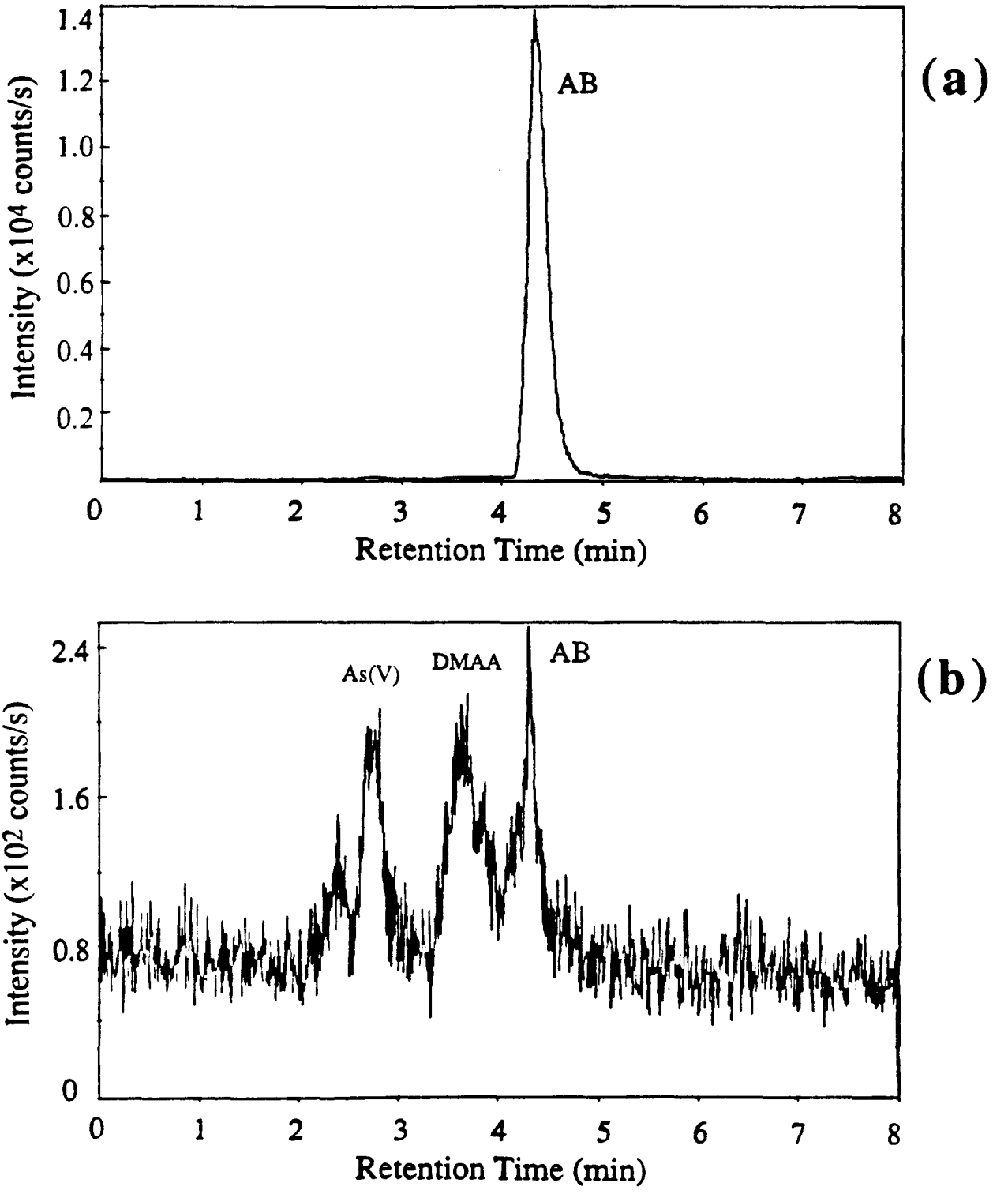
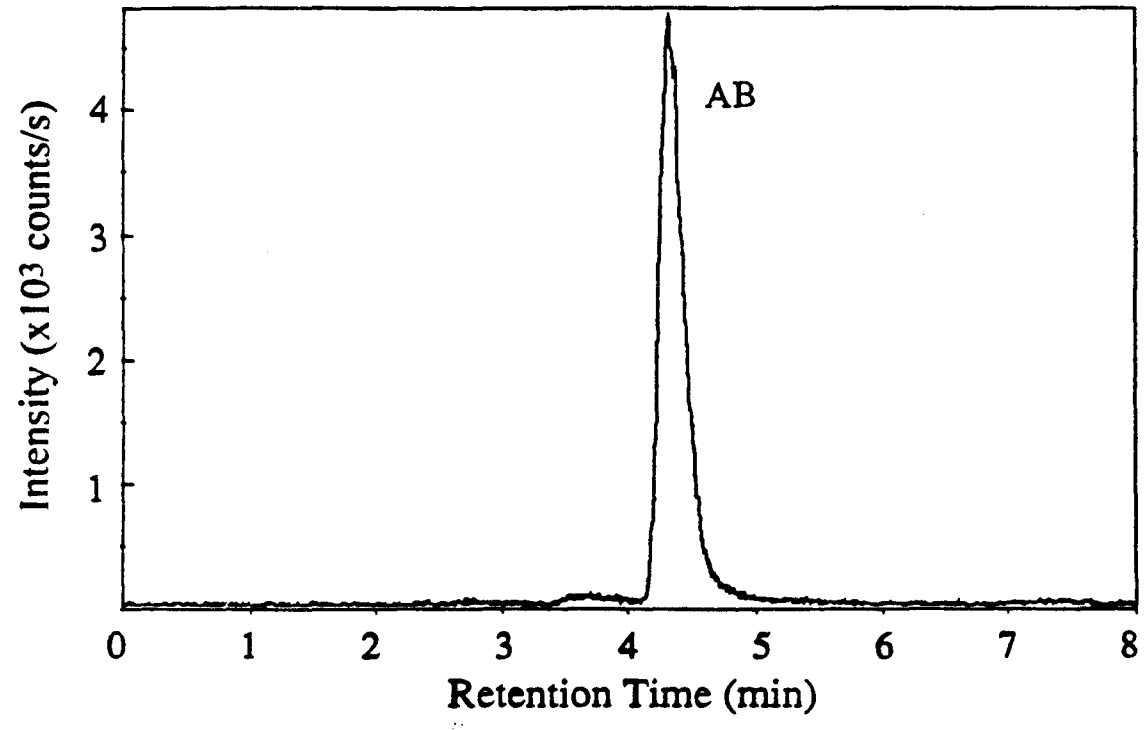
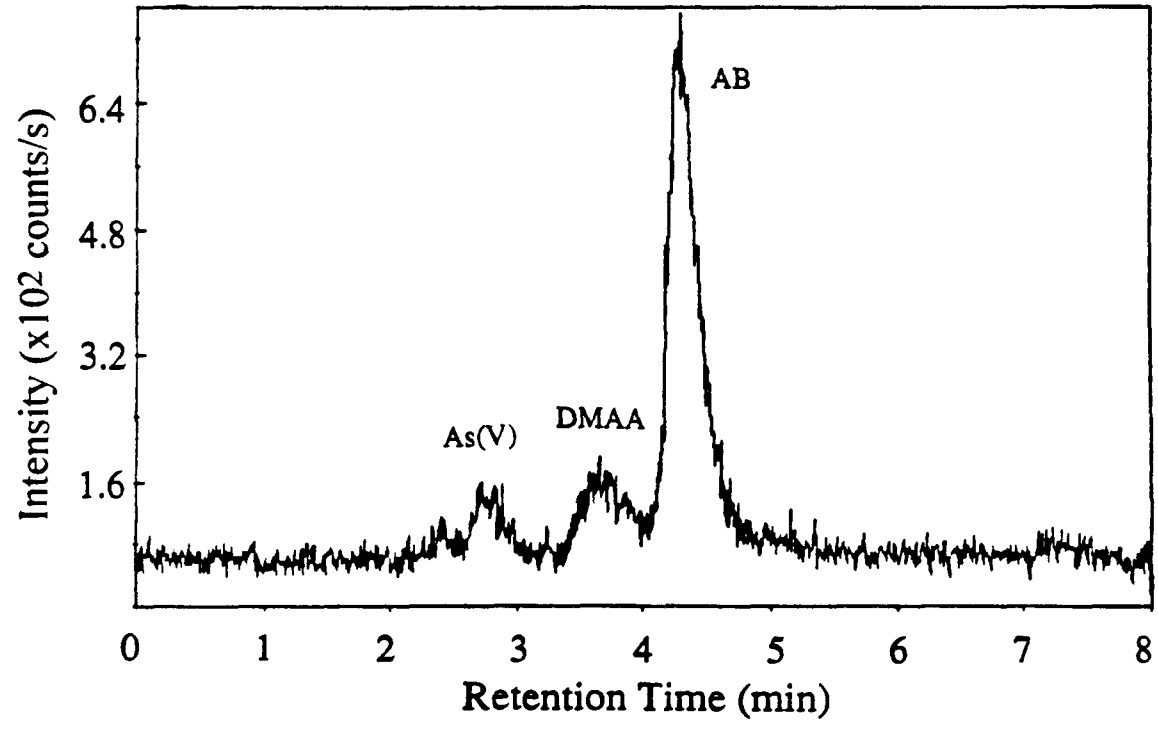


Figure 4.15. HPLC analyses of a crab extract (a) and urine samples collected from a volunteer at 3 hr before (b), 12 hr (c) and 38 hr (d) after the ingestion of crab meat.
HPLC column: Phenomenex μBondclone 10 C18 (300 mm x 3.9 mm)
Eluent: 10 mM butanesulfonate and 4 mM malonic acid, 0.1% methanol, pH 3.5
Eluent flow rate: 1 ml min^{-1} isocratic


AB
(c)
Intensity (x10³ counts/s)
4
3
2
1
0
1
2
3
4
5
6
7
8
Retention Time (min)


AB
(d)
Intensity (x10² counts/s)
6.4
4.8
3.2
1.6
As(V)
DMAA
0
1
2
3
4
5
6
7
8
Retention Time (min)

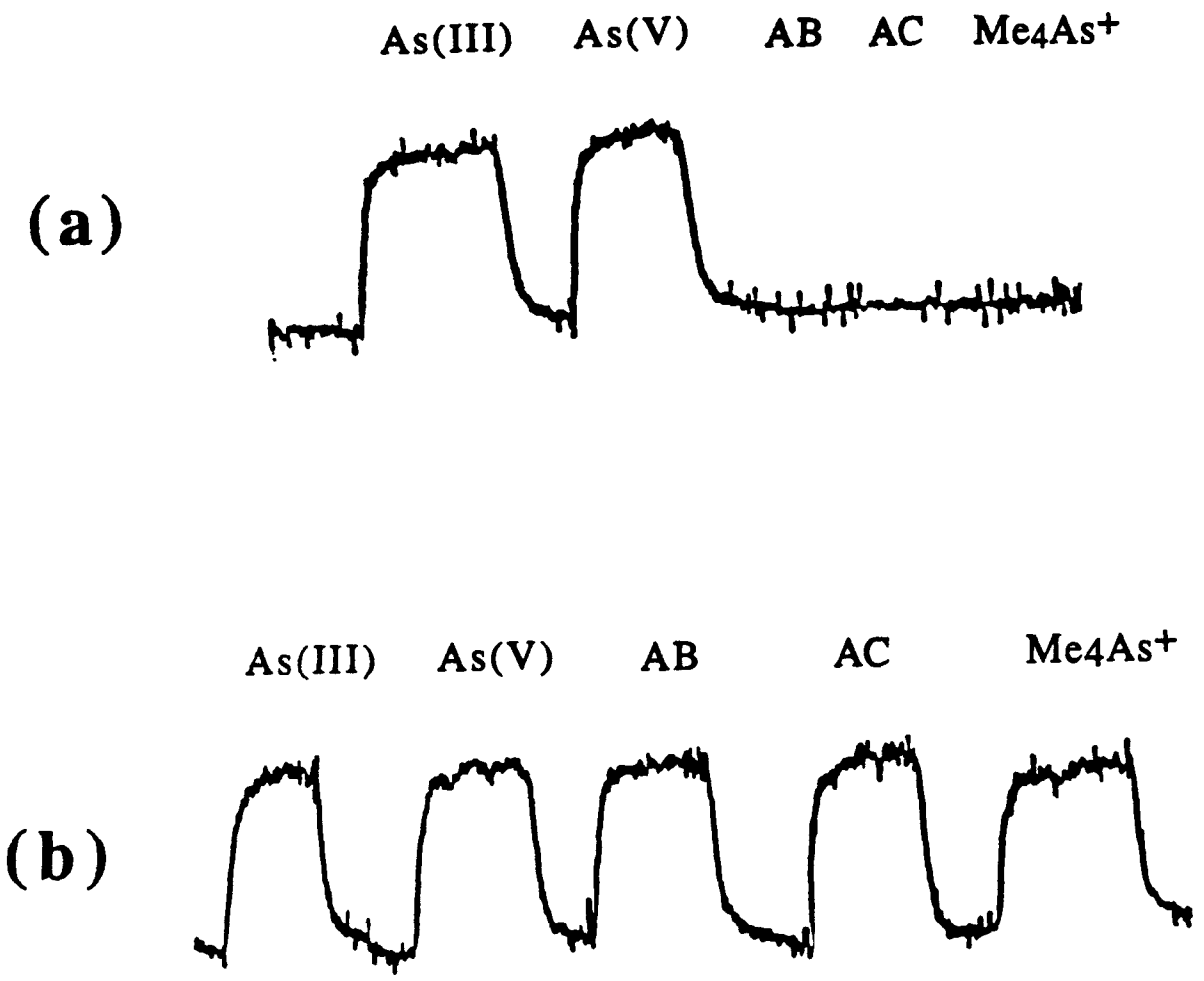


Figure 4.16. Comparison of signals from 20 ng ml^{-1} of arsenic compounds obtained by using continuous HGAAS system
(a) -- Without digestion
(b) -- With microwave assisted digestion; 0.1M $K_2S_2O_8$ and 0.3M NaOH as digestion reagents

Chapter 5.
SPECIATION OF ARSENIC COMPOUNDS BY USING HPLC WITH HGAAS AND ICPMS DETECTION

5.1. INTRODUCTION

It has been demonstrated (5, 11, 13, 80, 94, 104) that total arsenic concentration is not an appropriate measure for assessing toxicity, environmental impact, and effect of occupational exposure. However, because of a lack of techniques for the efficient separation and sensitive detection of particular species, most environmental and clinical studies are based on the measurement of total arsenic concentration. The speciation of arsenic presents an analytical challenge particularly when environmental and biological systems are concerned, where trace amounts of arsenic compounds are often present in complex matrices.

The coupling of high performance liquid chromatography (HPLC) with various spectrometries has proven very useful for chemical speciation studies because the combination takes advantage of both the separation power offered by HPLC and good selectivity and sensitivity of detection obtainable by using modern spectrometry. Thus many HPLC detection systems employed for speciation studies make use of atomic spectrometry and mass spectrometry. Flame atomic absorption spectrometry (FAAS) has been used as a HPLC detector for speciating a number of metal compounds (155, 237, 238). But because FAAS suffers from low sensitivity and high background noise for arsenic determination, many workers (55, 142, 146, 150, 239-241) have used graphite furnace atomic absorption spectrometry (GFAAS) for HPLC detection of this element. It is very difficult to couple HPLC directly to a graphite furnace because it is necessary to use a long analytical cycle including drying and ashing the sample prior to atomization. Thus tedious procedures involving collection of chromatographic fractions followed by

batch analysis of each fraction by using GFAAS have often been utilized (55, 162, 239, 241). Recently, a thermospray interface between HPLC and GFAAS was reported (242). It consists of a thermospray micro-atomizer operated at 700-1000 ^{o}C, where the HPLC effluent is rapidly evaporated to an aerosol before entering the furnace. In another method (161) the HPLC effluent, which runs at 0.2-0.3 ml min^{-1}, is volatilized to an aerosol in a heated silica capillary and enters the furnace through a vitreous graphite tube. However, the use of organic solvents and buffer solutions in the eluent often results in an elevated baseline noise.

Inductively coupled plasma atomic emission spectrometry (ICPAES) has been successfully coupled to HPLC for use in arsenic speciation (140, 147, 163, 243). The coupling is straightforward because the usual flow rate under which a HPLC operates, typically 1 ml min^{-1}, is compatible with the uptake flow rate of an ICPAES system. However, HPLC/ICPAES does not have the high sensitivity that some trace analysis applications require: for example, the speciation of arsenic in human urine requires working with samples where the total concentration of all arsenic species is often at the 10-50 ng ml^{-1} level.

The coupling of HPLC with ICPMS offers advantages because of the extremely high sensitivity, large dynamic range, and isotope ratio measurement capability that an ICPMS instrument can offer. Application of HPLC/ICPMS to arsenic speciation (58, 61, 145, 148, 149, 167, 168, 187, 188) has demonstrated the usefulness of this technique. However, wide utilization of ICPMS as a HPLC detector in routine analysis probably requires a substantial reduction of the current high cost of this instrument.

Hydride generation sample introduction is one of the most inexpensive and convenient methods that can be used to improve analytical sensitivity. By incorporating a hydride generation system as a post-column derivatization method into a HPLC system, the detection limit for arsenic speciation can be improved, regardless of whether the

detection system is flame AAS (141, 143, 230, 244-246), electrothermal AAS (144, 247), or ICPAES (151, 153, 248-250).

One major limitation in using the usual hydride generation techniques is that arsenobetaine, arsenocholine, the tetramethylarsonium ion, and arsenosugars cannot be detected. To solve this problem, an appropriate on-line decomposition procedure is required. In the previous chapter, a decomposition method was discussed that involved microwave heating. It rapidly and completely decomposes these organoarsenic compounds to arsenate, and arsenate can readily form a hydride, AsH_3. The on-line decomposition capability of the method, which was successfully tested for use in flow injection analysis, is now applied to the detection of arsenic species following HPLC separation. Speciation by using HPLC/ICPMS is also described.

5.2. EXPERIMENTAL

5.2.1. Instrument

The HPLC system used consists of a Waters Model 510 or Model M45 solvent delivery pump, a Waters U6K injector or a Reodyne 6-port injector (a sample loop volume of 25 µl), and an appropriate column. The columns used include a polymer-based anion exchange column (BDH PolySpher SAW, 120 mm x 4.6 mm) and three reversed phase C18 columns (Phenomenex 10µBondclone C18, 300 mm x 3.9 mm; Waters 10µBondapak C18, 300 mm x 3.9 mm; and GL Sciences Inertsil ODS-2, 250 mm x 4.6 mm). A guard column packed with the same material was always used preceding the analytical column. The detectors for the HPLC were either a hydride generation atomic absorption spectrometer (HGAAS) or an inductively coupled plasma mass spectrometer (ICPMS).

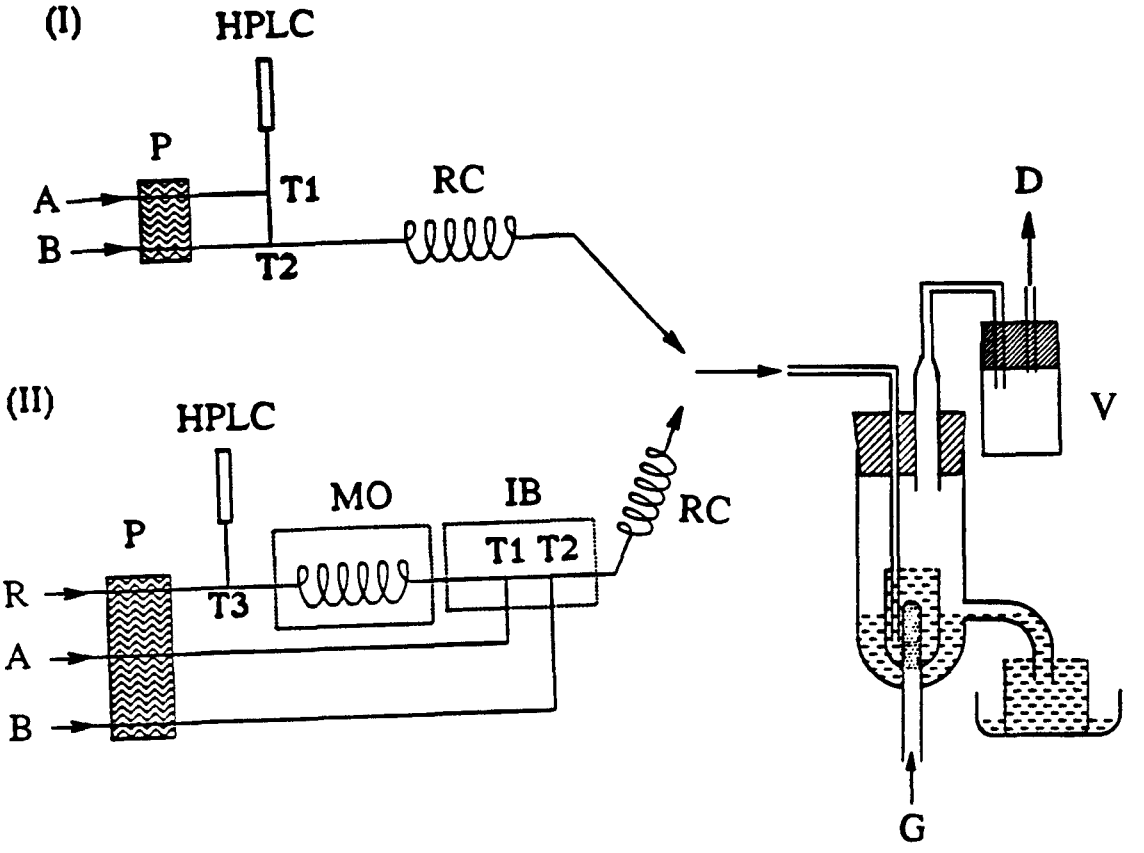


Figure 5.1. Schematics of HPLC/HGAAS system (I) and HPLC/MD/HGAAS system (II)

A -- hydrochloric acid	B -- $NaBH_4$
R -- digestion reagent	P -- peristaltic pump
MO -- microwave oven	RC -- reaction coil
IB -- ice water cooling bath	G -- carrier gas(nitrogen)
V -- glass vial (10 ml)	D -- to detector (AAS)

T1, T2, and T3 -- Teflon T-joints (1/16")

The HGAAS system for HPLC detection is schematically shown in Figure 5.1. In Scheme (I) of the Figure effluent from the HPLC directly meets at two Teflon T-joints T1 and T2 (1/16" joints, Mandel), with continuous flows of hydrochloric acid (A) and sodium borohydride (B) introduced by using a peristaltic pump (P) (Gilson Minipuls 2). Upon mixing the HPLC effluent, acid and sodium borohydride solutions, hydride generation takes place. A complete reaction is achieved by using a 1-m reaction coil (RC) (1mm i.d. PTFE coil) and the combined gas/liquid separator apparatus as described previously (Figure 2.2). Hydride generated from the reaction is carried by a continuous flow of nitrogen carrier gas (G) to the atomic absorption spectrometer (D). Because the reaction solution in the gas/liquid separator is hot, particularly when the microwave oven decomposition is used on-line as in Scheme (II) of Figure 5.1, and some foaming is produced due to the use of sodium heptanesulfonate as HPLC eluent, some aerosols can be carried into the hydride transport line and cause signal fluctuation. A small glass vial (V) (inner volume 10 ml) is installed between the hydride generator and the AAS to trap the aerosols.

In Scheme (II) of Figure 5.1, the HPLC effluent undergoes microwave assisted decomposition before hydride generation takes place. As demonstrated previously in section 4.3.2, a solution containing 0.1M potassium persulfate and 0.3M sodium hydroxide efficiently decomposes organoarsenicals to arsenate with the aid of microwave energy, and this mixture reagent was used in the present study. The HPLC effluent and the decomposition reagent (R) meet at a T-joint (T3) (1/16", Mandel, Canada). This solution mixture flows through a PTFE decomposition coil (5m x 0.8mm i.d.) located in a continuously operating microwave oven (MO) (500W, 2450MHz, Sharp Electronics, Japan), where the decomposition takes place. The hot solution from the microwave oven then meets the continuous flows of acid (A) and borohydride (B). An ice water cooling bath (IB) is used following the microwave decomposition in order to reduce dispersion and to reduce the amount of aerosol produced. After the hydride generation reaction and

gas/liquid separation, arsines are introduced to the flame heated quartz tube for atomic absorption measurement. The time that an analyte spends in the microwave decomposition hydride generation system is approximately 30 seconds. This was determined by by-passing the HPLC column and measuring the time between the injection of an arsenate standard and the appearance of the signal maximum.

A VG PlasmaQuad 2 Turbo Plus inductively coupled plasma mass spectrometer (ICPMS) (VG Elemental, Fisons Instrument) equipped with a SX300 quadrupole mass analyzer, a standard ICP torch (Fassel configuration) and either a Meinhard concentric nebulizer or a de Galan V-groove nebulizer was used. A mini-chiller (Coolflow CFT-25, Neslab) was used to cool the spray chamber to approximately 4 ^{o}C. The sampling position and ion lens voltages were optimized with respect to signal-to-noise ratio at m/z 75 by introducing a solution containing 30 ng ml^{-1} of arsenite in 1% nitric acid. The quadrupole mass analyzer was operated in the single ion monitoring mode. The instrumental operating conditions are listed in Table 5.1. A PTFE tubing (20cm x 0.4mm i.d.) with appropriate fittings was used to connect the outlet of the HPLC analytical column directly to the inlet of the ICP nebulizer. Signals at m/z 75 were monitored by using a multichannel analyzer and data were automatically transferred to and stored in the VG data system. Once a chromatographic run was complete, a chromatogram was plotted on a Epson FX-850 printer.

A UV/visible spectrophotometer (Waters Lambda-Max Model 481 LC Spectrophotometer) was used as the HPLC detector for creatinine analysis.

5.2.2. Standards and Reagents

All HPLC eluents, carbonate (BDH) buffer, phosphate (BDH) buffer, and a number of sulfonic acids ranging from methanesulfonic acid to octanesulfonate (Aldrich) and tetraethylammonium hydroxide (Eastman Kodak) were prepared in distilled water and filtered through a 0.45 μm membrane. The pH of these eluents was adjusted by using

sodium hydroxide and nitric acid. An appropriate amount of methanol (HPLC grade, Fishier) is added prior to the pH adjustment and the filtration.

5.2.3. Procedures

HPLC/MD/HGAAS and HPLC/ICPMS:

For both anion exchange and ion pair chromatography, the columns were equilibrated with the appropriate eluent flowing at 1 ml min^{-1} for at least two hours before any sample injection was made. Urine samples were centrifuged to remove any suspended particulates before they were subjected to HPLC analysis. Arsenic compounds in the urine samples were identified by matching the retention times of the chromatographic peaks of the urine samples with those of standards spiked into the sample. Detailed experimental conditions are listed in Table 5.1.

Table 5.1. Summary of Experimental Conditions

HGAAS

	No Digestion	With Microwave Digestion
HCl Conc. (Flow Rate):	2 M (3.4 ml min^{-1})	3 M (3.4 ml min^{-1})
$NaBH_4$ Conc. (Flow rate):	0.65 M (3.4 ml min^{-1})	0.65 M (3.4 ml min^{-1})
Carrier Gas Flow Rate:	160 ml min^{-1}	160 ml min^{-1}
Digestion Reagents:	none	0.1 M $K_2S_2O_8$ & 0.3 M NaOH (4 ml min^{-1})
Digestion Coil:	none	5 m x 0.5 mm i.d.

ICPMS

Forward r.f. power:	1.35 kw
Reflected power:	< 10 w
Outer gas (Cooling) flow rate:	13.8 L min^{-1}
Intermediate gas (Auxiliary) flow rate:	0.70 L min^{-1}

Nebulizer gas flow rate:	0.96 L min^{-1}
Spray chamber:	water cooled to approximately 4 ^{o}C
Sampling cone orifice diameter:	1.0 mm
Skimmer cone orifice diameter:	0.70 mm
Single ion monitoring mode:	m/z 75

HPLC

Column	Mobile Phase	Flow Rate (ml min^{-1})
(i) PolySphere SAW (BDH) anion exchange 12 cm x 4.6 mm i.d.	(a) 50 mM phosphate buffer (b) 50 mM carbonate buffer pH 7.5, 9.0, 10.3, 10.9	1.0
(ii) Bondclone 10 C18 (Phenomenex) 30 cm x 3.9 mm i.d. or µBondapak 10 C18 (Waters, Millipore) 30 cm x 3.9 mm i.d.	(a) 10 mM Heptanesulfonate & 0.1% methanol, pH 3.5 (b) 10 mM propanesulfonate & 0.1% methanol, pH 3.5 (c) 10 mM methanesulfonic acid & 0.1 or 2% methanol, pH 3.5 (d) 10 mM heptanesulfonate & 4 mM $(CH_3)_4NOH$, pH 3.5 (e) 10 mM butanesulphonate & 4 mM malonic acid, pH 3.5 (f) 10 mM heptanesulphonate, pH 3.5 for the initial 2 min followed by 10 mM mathanesulfonic acid & 4 mM malonic acid, pH 3.0	1.0
(iii) Inertsil ODS-2 (GL Sciences, Japan) 25 cm x 4.6 mm i.d.	10 mM $(C_2H_5)_4NOH$ & 4 mM malonic acid, 0.1% methanol pH 6.8	0.8

HG/GC/AAS:

Urine samples were also analyzed for inorganic arsenic, MMAA, and DMAA concentrations by using HG/GC/AAS, as described previously in Section 3.2.4.2.

Determination of creatinine in urine samples:

Creatinine in urine samples was determined by using HPLC with UV absorption spectrophotometric detection, essentially as described by Achari *et al.* (252). Urine samples were diluted by 50 times with deionized water and a 5-10 µl aliquot was injected onto a C18 column (Bondclone 10 C18, 3.9 x 300 mm, Phenomenex, California). The eluent was 50 mM sodium acetate (pH 6.5) in 98:2 (by volume) water: acetonitrile. The flow rate was 1.0 ml min^{-1}. A spectrophotometer (Waters Lambda-Max Model 481) set at 254 nm wavelength was used as the HPLC detector. Chromatograms were recorded by using a Hewlett Packard 3390A integrator and creatinine peak area was measured for quantitation.

Figure 5.2 shows chromatograms obtained from creatinine standards, a 50-fold diluted urine sample, and the diluted urine sample spiked with creatinine standards. The linear response with the increase of creatinine concentration in both the standards and the urine sample indicates that there is no interference from the urine matrix and the method is suitable for the determination of creatinine in urine.

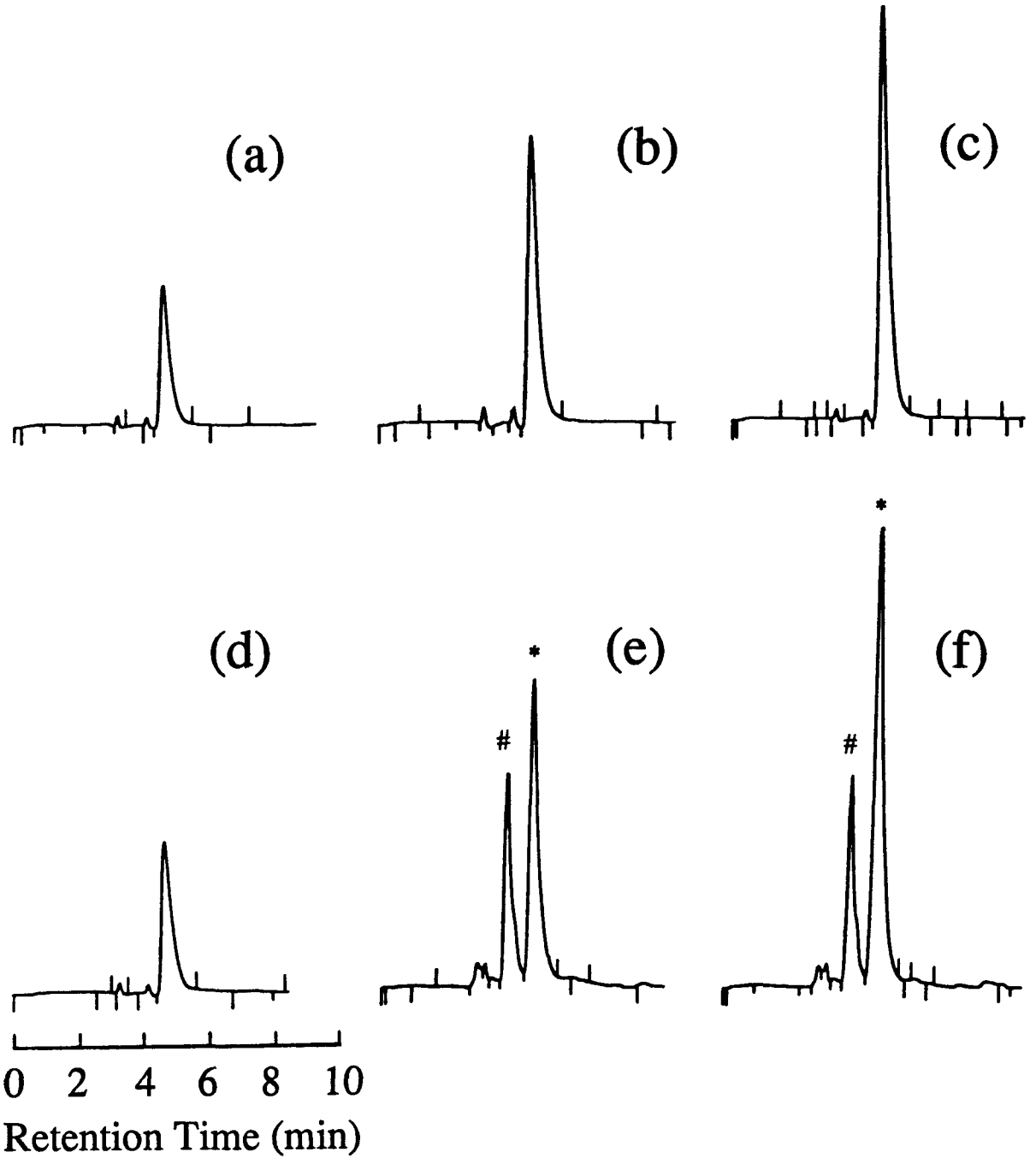


Figure 5.2. Chromatograms obtained from creatinine standards and a diluted urine sample. (5 µl injected)
(a), (b), and (c) - 10, 20, and 30 µg ml^{-1} of creatinine standard, respectively
(d) - 10 µg ml^{-1} of creatinine standard
(e) - a urine sample diluted (50 times) with deionized water
(f) - 10 µg ml^{-1} of creatinine spiked into the diluted urine sample as in (e).
The peaks marked with * and # in (e) and (f) are due to creatinine and other components in the urine sample, respectively.

5.3. RESULTS AND DISCUSSION

5.3.1. Anion exchange chromatography

Phosphate (141, 145, 150, 168, 249, 250) and carbonate (144, 148, 187, 244) buffers have been used commonly as mobile phases to separate anionic arsenic compounds on an anion exchange column. Therefore, we chose these systems to begin our study on arsenic speciation and to evaluate the capability of the on-line microwave decomposition/hydride generation atomic absorption spectrometric system (MD/HGAAS) as a HPLC detector. Figure 5.3 shows chromatograms obtained by using an anion exchange column and 50 mM phosphate (pH 7.5) buffer as mobile phase. The chromatogram in Figure 5.3a was obtained by using the set up shown in Scheme (I) of Figure 5.1, where the HPLC effluent does not undergo microwave decomposition. Although five arsenicals are present in the injected standard, only four of these are detected and appear in the chromatogram; arsenobetaine does not form a hydride without prior decomposition to a hydride forming species and therefore is not detected by HGAAS. When the on-line microwave decomposition system is used immediately before the hydride generation step (Scheme (II) of Figure 5.1), arsenobetaine is decomposed to arsenate, a hydride forming species, and all the five arsenic compounds are detected (Figure 5.3b). The response of the system to arsenobetaine alone is shown in Figure 5.3c.

At pH 7.5, $As(OH)_3$ remains undissociated as its pKa_1 value is 9.3 (28); and arsenobetaine is probably present as a neutral zwitterion. Therefore, neither $As(OH)_3$ nor arsenobetaine is retained on the anion exchange column and both co-elute in the void time (volume) of the system. This experiment was repeated by using an ICPMS as the HPLC detector, and similar results to those shown in Figure 5.3b were obtained. Thus arsenobetaine and arsenite can not be separated from each other on the anion exchange column by using a pH 7.5 buffer as mobile phase. This problem has also been encountered by other workers (145, 187, 249); yet it is often ignored and arsenobetaine is

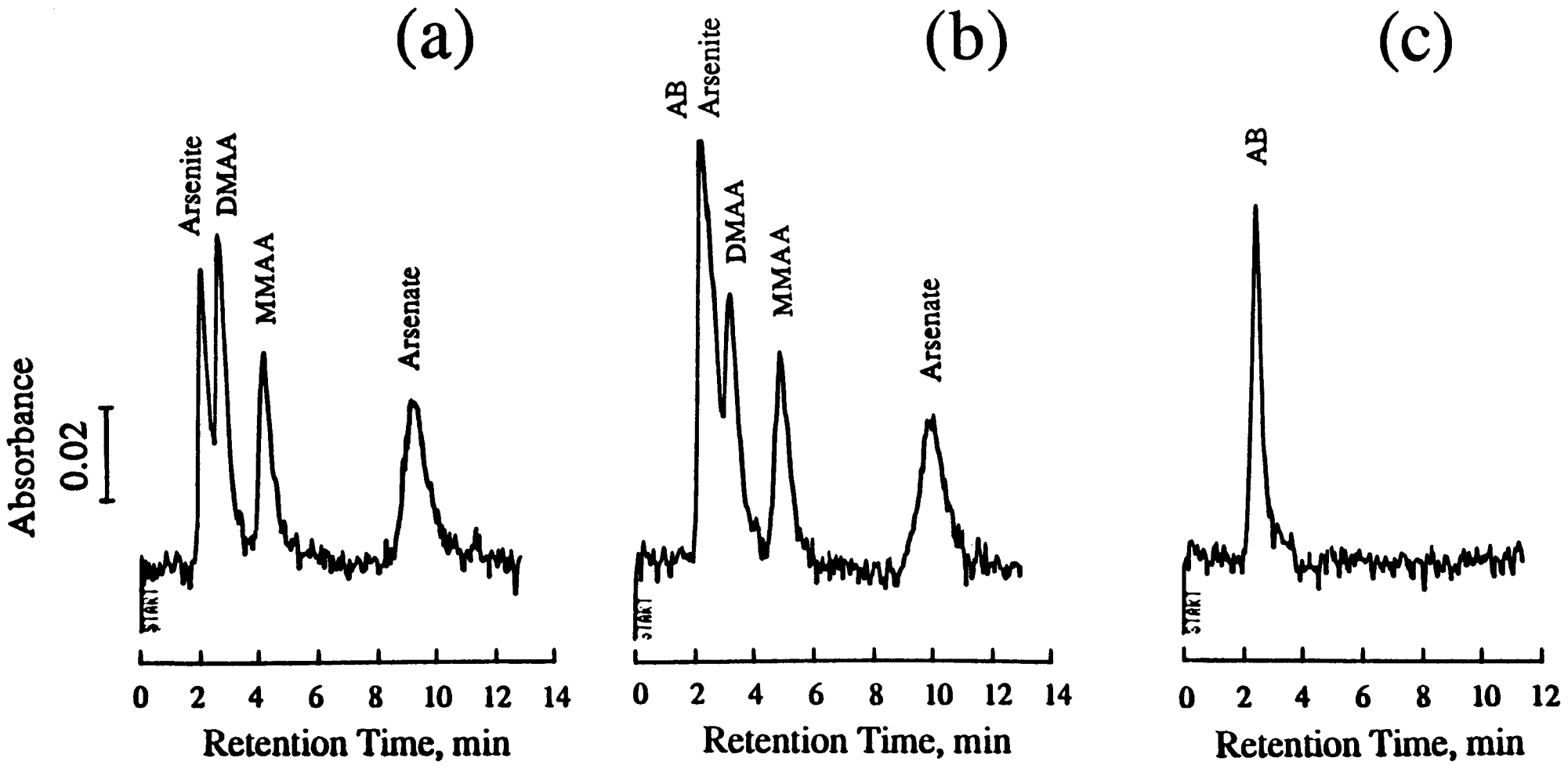


Figure 5.3. Chromatograms of arsenic compounds obtained by using an anion exchange column with 50 mM phosphate buffer (pH 7.5) as mobile phase and HGAAS detection

(a) - 2 ng of 5 arsenic species; no digestion; AB not detected

(b) - 2 ng of 5 arsenic species; with microwave assisted digestion

(c) - 3 ng of AB; with microwave assisted digestion.

excluded from many speciation studies even though it is environmentally and biologically a very important arsenic compound. It is certainly the major arsenic compound in many kinds of seafood (5). The detection system developed here, however, allows an easy solution to the problem, and the concentration of each can be measured by difference, by making two chromatographic runs: one with on-line microwave assisted decomposition of the HPLC effluent prior to HGAAS detection (Scheme II) and the other without digestion (Scheme I). Thus, selective determination can be achieved by using the new system developed here. Specific application of this is demonstrated below where the speciation of arsenic compounds in urine samples is described.

Figure 5.4 shows chromatograms obtained from urine samples collected 2 hr before (a and d), and 7 hr (b and e) and 17 hr (c and f) after a volunteer ingested some crab meat. The chromatograms a, b, and c were obtained without using microwave assisted digestion; whereas d, e, and f were obtained after on-line microwave assisted digestion. As is shown in Figure 5.4, only a trace amount (less than 20 ng ml^{-1}) of DMAA is detected by using HGAAS without microwave decomposition. When the same samples were subjected to chromatography and MD/HGASS determination, arsenobetaine is clearly detected in urine samples collected 7 hr (Figure 5.4e) and 17 hr (Figure 5.4f) after the ingestion of crab meat. The first peaks in Figures 5.4e and 5.4f account for approximately 120 and 37 ng ml^{-1}, respectively, of arsenic as arsenobetaine since arsenite is not detectable in the samples (Figure 5.4a-5.4c). If there were detectable arsenite, the difference of the first peak in peak height (or peak area) between the second run (with microwave decomposition) and the first run (without microwave decomposition) would account for arsenobetaine. By using the detection system developed here for HPLC, all five arsenic compounds can be easily speciated with two chromatographic runs of approximately 12 minutes per run.

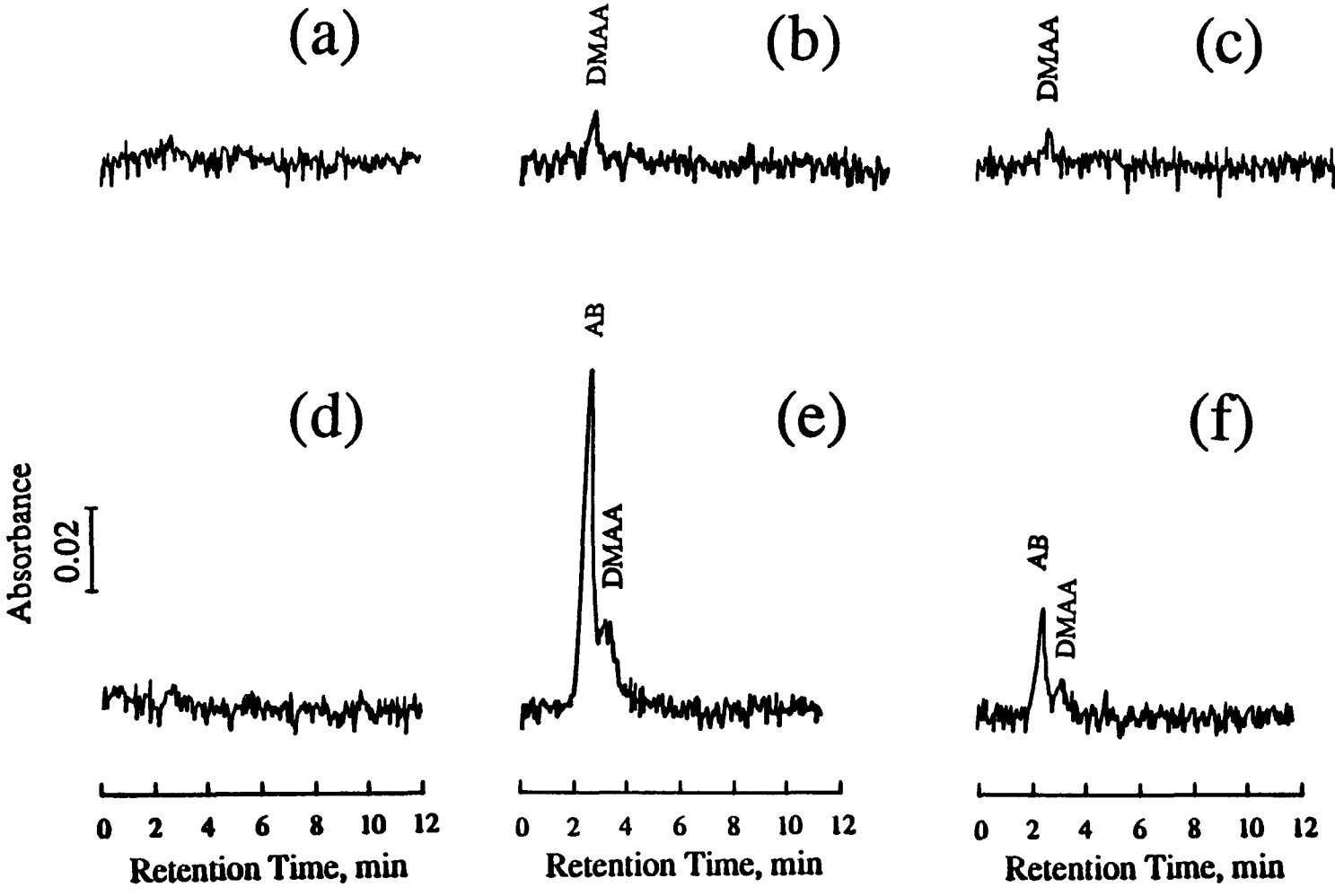


Figure 5.4. Chromatograms obtained from urine samples collected 2 hr before the ingestion of crab meat (a and d), and 7 hr (b and e) and 17 hr (c and f) after the ingestion of crab meat

(a), (b), and (c) - no digestion

(d), (e), and (f) - with microwave assisted digestion

The chromatograms in Figure 5.4 also illustrate the time course of human urinary excretion of arsenobetaine after the ingestion of crab. Maximum arsenobetaine concentration in urine, 120 ng ml^{-1}, is reached approximately 7 hr following the ingestion of crab meat (Figure 5.4e). After seventeen hours, the arsenobetaine in the urine has decreased to 37 ng ml^{-1} (Figure 5.4f). Analysis of urine samples collected beyond 40 hr showed that the arsenobetaine concentration was reduced to below the detection limit of the method (10 ng ml^{-1}). Little variation is seen in the DMAA concentration, and there are no other arsenic compounds detected.

The detection limits, defined as three times the standard deviation of the blank, are approximately 10 ng ml^{-1} (or 200 pg for a 20µl sample injection) for AB, DMAA, and arsenite, 15 ng ml^{-1} (or 300 pg) for MMAA, and 20 ng ml^{-1} (or 400 pg) for arsenate by using the HPLC/MD/HGAAS system.

5.3.2. Effect of mobile phase pH on anion exchange chromatography

Separation of arsenic compounds using anion exchange chromatography is usually performed with a mobile phase at around neutral pH. Although it is known that the pH of the mobile phase is very important for the separation, very little work has been done to study systematically the effect of pH. This is partly because, in most of the studies, silica based anion exchange columns were used, limiting the mobile phase to a narrow pH range near neutral. However, with the use of polymer based column, mobile phases in the pH range 1-14 can be used without column deterioration. Figure 5.5 shows chromatograms from four arsenicals when 50 mM phosphate buffers at pH 9.0 and 10.3 are used as mobile phase. When the pH is increased from 7.5 (Figure 5.3a) to 9.0 (Figure 5.5a), arsenite and DMAA co-elute. This is consistent with the results of Sheppard *et al.* (187), who concluded that pH 7.5 is preferred for the separation of these two species on a silica-based anion exchange column. However, our results show that when the mobile phase pH is further increased to 10.3 (Figure 5.5b) chromatographic peaks from DMAA

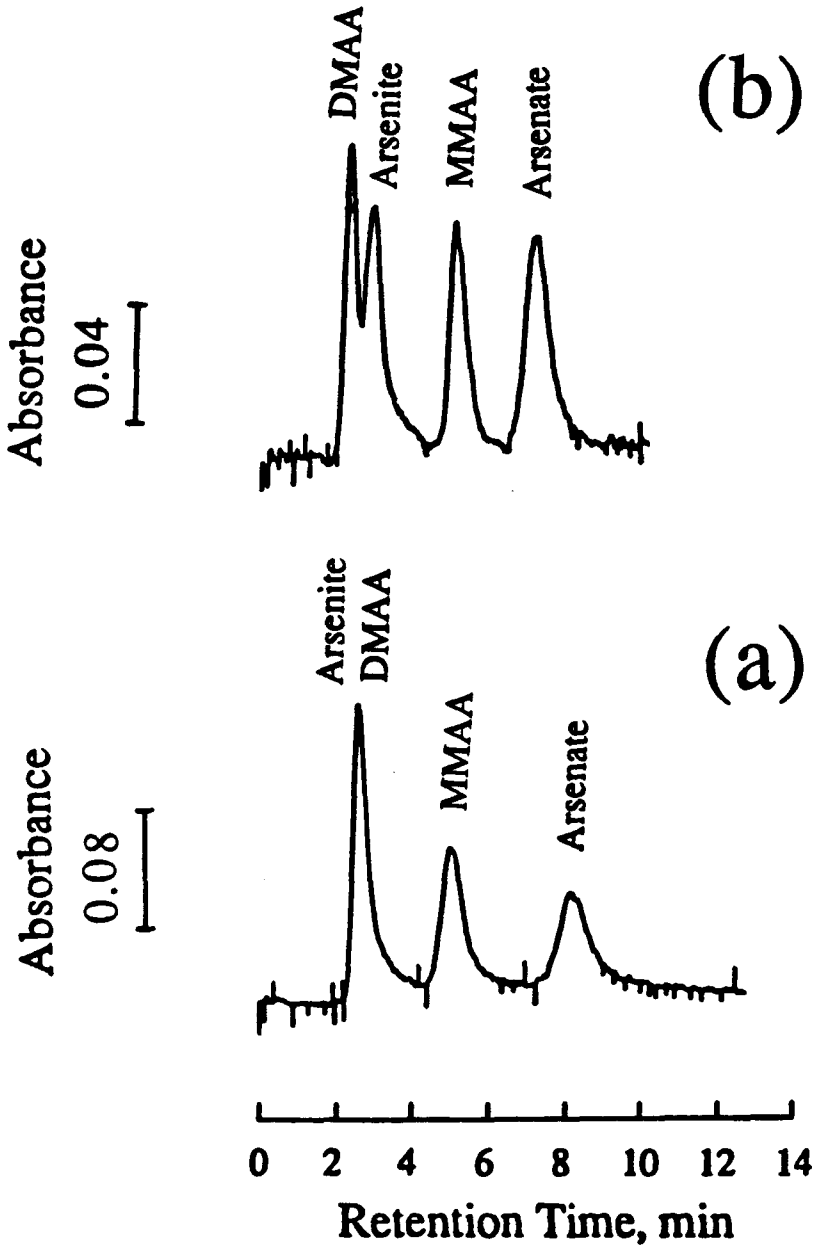


Figure 5.5. Chromatograms of four arsenicals obtained by using a polymer based anion exchange column and 50 mM phosphate buffer, at two pH's, as eluent. No microwave decomposition was used.
(a) - pH 9.0; 8 ng of 4 arsenicals
(b) - pH 10.3; 4 ng of 4 arsenicals

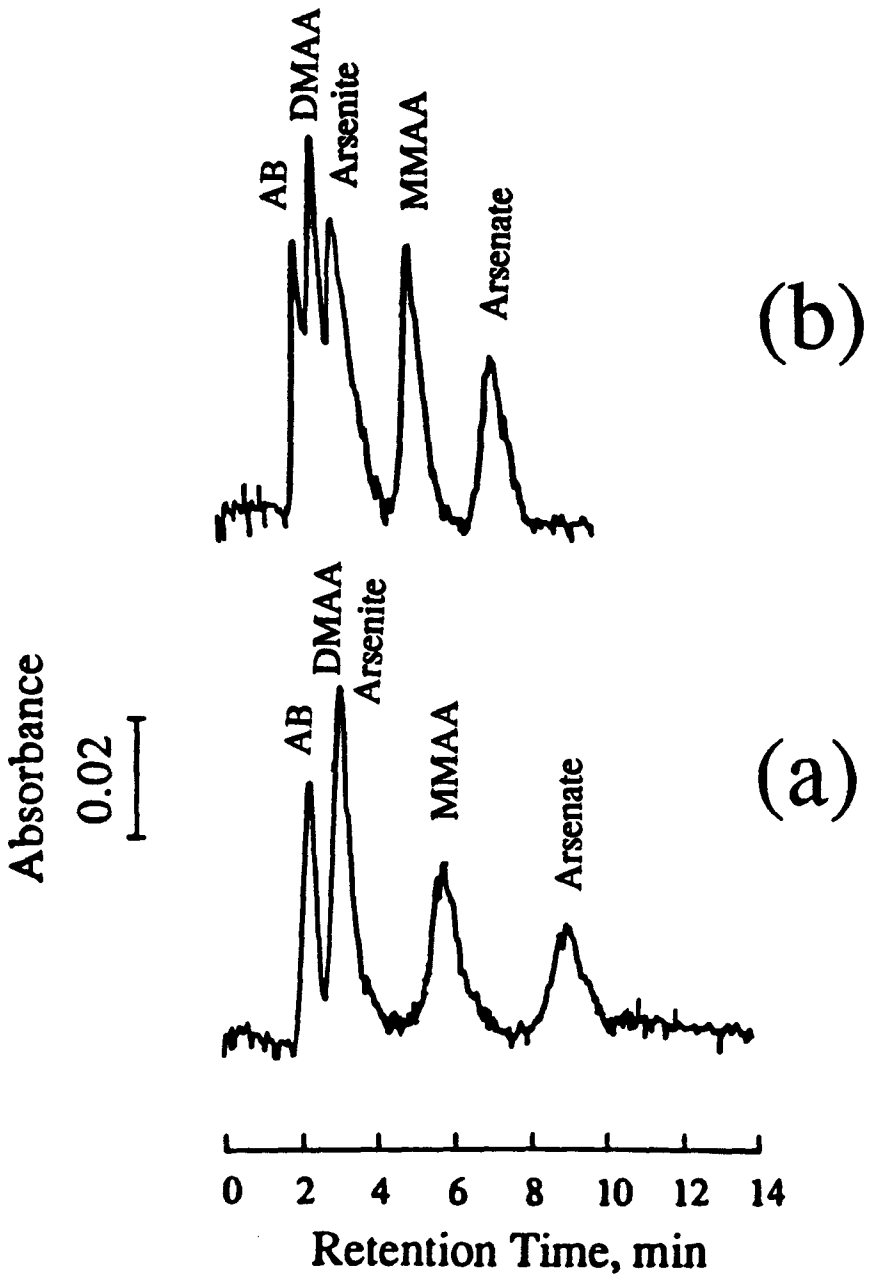


Figure 5.6. Chromatograms of five arsenicals (4 ng each) obtained by using a polymer based anion exchange column and 50 mM phosphate buffer, at two pH's, as eluent. On-line microwave decomposition was used.
(a) - pH 9.0, and (b) - pH 10.3

and arsenite are separated again, and a longer retention is observed for arsenite. The increased retention of arsenite has an advantage in that this species is now separated from arsenobetaine. This is shown in Figure 5.6, where five arsenic species are determined using HPLC/MD/HGAAS. Arsenite and DMAA co-elute at pH 9.0 (Figure 5.6a), whereas arsenite and arsenobetaine co-elute at pH 7.5 (Figure 5.3b). When the pH is increased to 10.3 (Figure 5.6b) a stronger retention of arsenite results in arsenobetaine, arsenite and DMAA being partially separated from one another and being well separated from MMAA and arsenate.

As noted above, the pK_{a1} value of H_3AsO_3 is 9.3 (28). At pH 7.5, very little H_3AsO_3 is dissociated so the species is not retained on the column and is eluted in the void volume. Arsenobetaine, a zwitterion ($(CH_3)_3As^+CH_2COO^-$), is also co-eluted in the void volume. As the pH is increased, more H_3AsO_3 is dissociated to give the anion $H_2AsO_3^-$, which is retained more strongly on the anion exchange column than the undissociated species. Arsenobetaine apparently remains a zwitterion within the pH range studied and is eluted in the void volume. Thus arsenite and arsenobetaine are separated from each other.

Further increase of pH to 10.9 resulted in the partial overlap of peaks from arsenite and MMAA. In the pH region below 7.5 no improvement was observed in the separation of the five arsenic compounds and arsenate retained strongly on the column resulting in a prolonged elution time. Therefore, for optimum separation the pH of the mobile phase should be 10.3.

Similar results were obtained when 50 mM sodium bicarbonate buffer was used as mobile phase: no difference was found in the chromatographic separation when using either phosphate or carbonate buffers. As an example, Figure 5.7 shows a chromatogram obtained by using the same anion exchange column as described above and 50 mM sodium bicarbonate buffer (pH 10.2) as the mobile phase. As expected, AB, AC

$((CH_3)_3As^+CH_2CH_2OH)$, and $(CH_3)_4As^+$ do not retain on the anion exchange column and co-elute in the void volume.

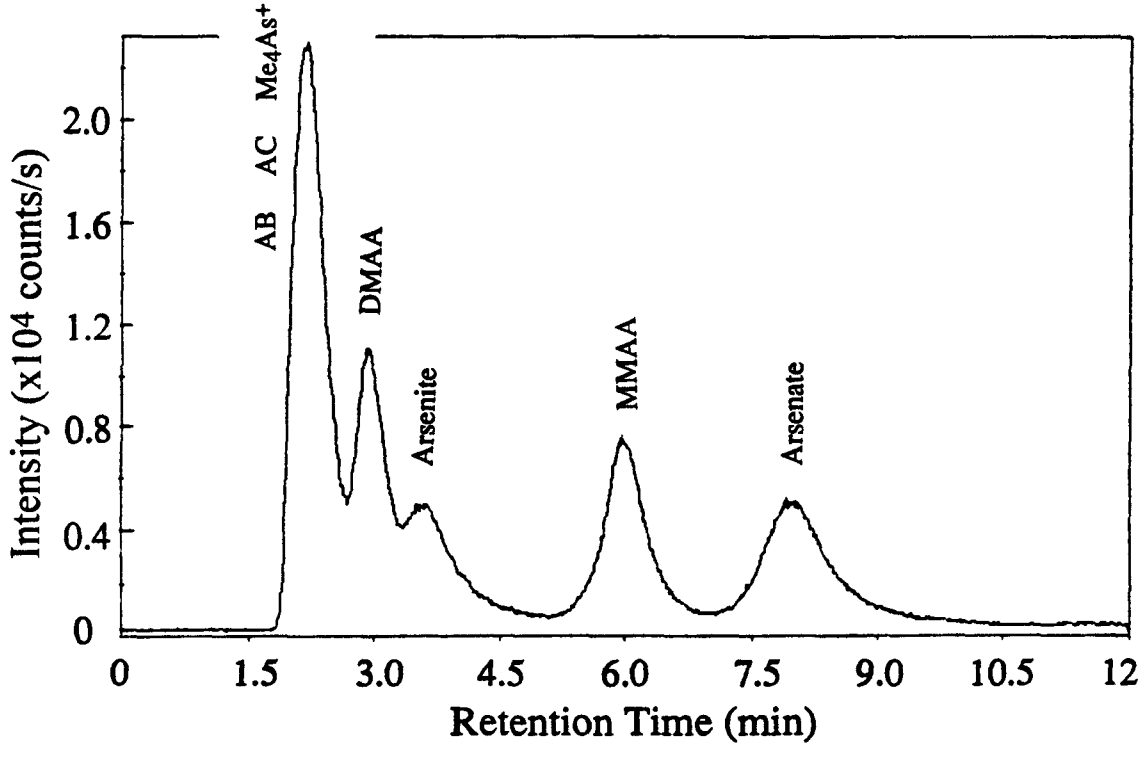


Figure 5.7. Separation of seven arsenicals (10 ng each) on an anion exchange column with 50 mM $NaHCO_3$ buffer (pH 10.2) as eluent and using ICPMS detection.

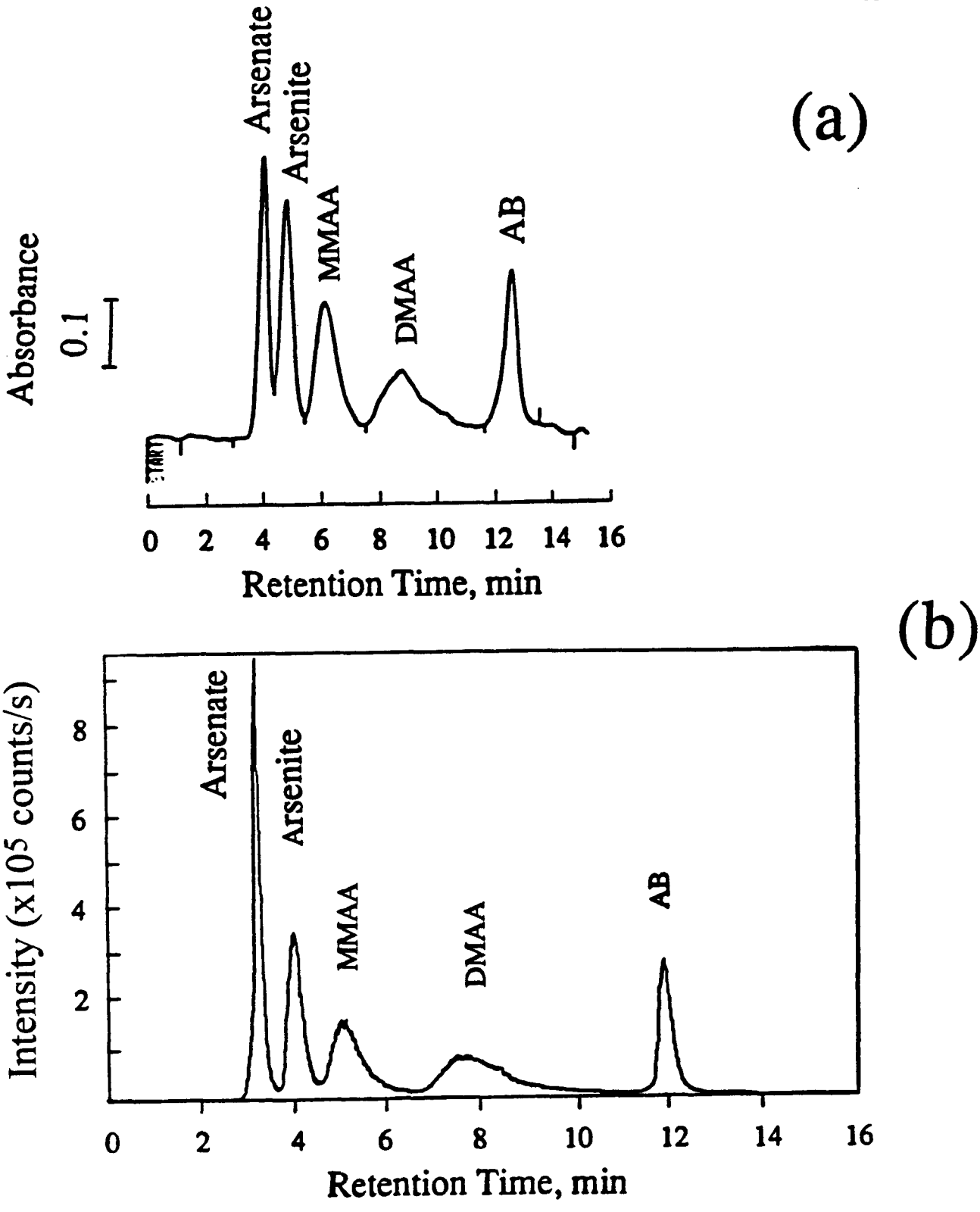


Figure 5.8. Chromatograms of five arsenicals (20 ng each) obtained on a reversed phase C18 column with 10 mM heptanesulfonate (pH 3.5), an ion pair reagent, as eluent.

(a) - with MD/HGAAS detection

(b) - with ICPMS detection

5.3.3. Ion pair chromatography

The anion exchange chromatography system described above is simple, but it is convenient only for studies involving arsenite, arsenate, MMAA and DMAA. Although the new detection system developed can be used to speciate arsenobetaine as well, it requires two chromatographic runs. Consequently, ion pair chromatography was studied in order to improve the separation of arsenic compounds. In this type of chromatography, a solution containing an ion pairing reagent (counter ion) is used as mobile phase. This causes a change in the solute-solvent interactions, resulting in a change in retention behavior compared to a similar system which does not contain the counter ions (254).

An anion pair chromatographic system that uses heptanesulfonate as an ion pairing reagent is studied and the separation conditions, including the concentration, pH, and flow rate of the mobile phase, are optimized. Figure 5.8 shows chromatograms of five arsenic compounds that are well separated under the optimum conditions on a reversed phase C18 column and are detected by using MD/HGAAS (Figure 5.8a) and ICPMS (Figure 5.8b). Similar resolution in the two chromatograms indicates that the dispersion in the detection systems is comparable. This is probably because the average time that an analyte spends in the microwave assisted digestion hydride generation unit is only 30 seconds.

AC and Me_4As^+ are also important arsenic compounds present in biological systems (49, 52, 55, 58, 74-76), however, they do not elute from the column, even after 40 min, under the conditions used to obtain the separation shown in Figure 5.8. Consequently, further studies were carried out in an effort to speciate these compounds.

It has been suggested that the addition of a small amount of second ion pair reagent that has the same charge as the analytes of interest (positive charge in this case) into the mobile phase reduces the retention of the analytes. As a result of the repulsion between the ions of the same charge and the competition between the introduced ions and the analyte ions for the site of oppositely charged pairing ion, a shorter retention is

expected. Thus to reduce the retention of AB, AC and Me_4As^+ which are positively charged, 5 mM tetramethylammonium hydroxide ($(CH_3)_4NOH$) was added into the 10 mM heptanesulfonate mobile phase. As shown in Figure 5.9, AB, AC and Me_4As^+ are now all eluted faster with the longest retention time of approximately 10 min for Me_4As^+. A good separation of AB, AC and Me_4As^+ is obtained within the 10-min chromatographic run. However, AB and DMAA now co-elute as a result of the faster elution of AB.

Another approach that was studied to reduce the retention of AB, AC and Me_4As^+ is to decrease the hydrophobicity of the ion pair reagent (counter ion) by using a sulfonic acid that has a shorter hydrocarbon chain. A number of sulfonic acids of hydrocarbon chain length ranging from one carbon (methanesulfonic acid) to eight carbons (sodium octanesulfonate), were each used as the HPLC eluents and the retention of the arsenicals was studied. It was found that as the length of hydrocarbon chain attached to the sulfonic acid group decreases the retention times for AB, AC, and Me_4As^+ on the reversed phase C18 column also decrease. For example, when propanesulfonate instead of heptanesulfonate is used as an ion pair reagent (Figure 5.10), faster elution of the compounds studied is observed. The Me_4As^+ ion elutes at approximately 20 min when propanesulfonate is used as the ion pair reagent in the mobile phase, it did not elute from the column after 40 min when heptanesulfonate was used. It has been shown that the ion pair reagent in an aqueous based mobile phase interacts with the reversed phase (253-256). Sulfonic acid is ionized under the present pH condition (3.5) and acts as an anion exchanger to interact with the solutes at the polar end while it interacts with the reversed phase C18 at its hydrophobic end. The decreased interaction between the sulfonic acid and the stationary phase of the C18 column, by reducing the hydrophobicity of the sulfonic acid from heptanesulfonate to propanesulfonate, results in the shorter retention of the solutes.

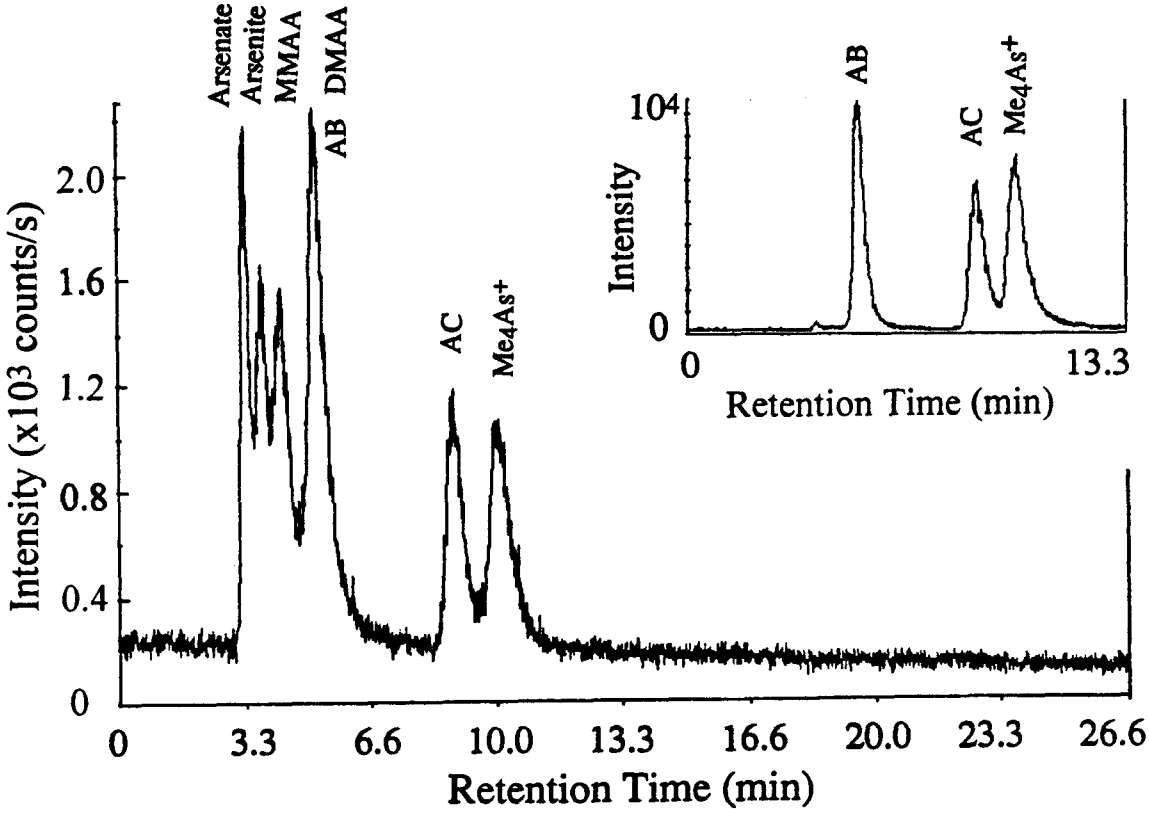


Figure 5.9. Chromatogram of seven arsenicals (2 ng each) obtained on a reversed phase C18 column with 10 mM heptanesulfonate and 4 mM $(CH_3)_4NOH$ (pH 3.5) as eluent and using ICPMS detection.
The insert is a chromatogram of 20 ng of AB, AC, and Me_4As^+, obtained under the same conditions.

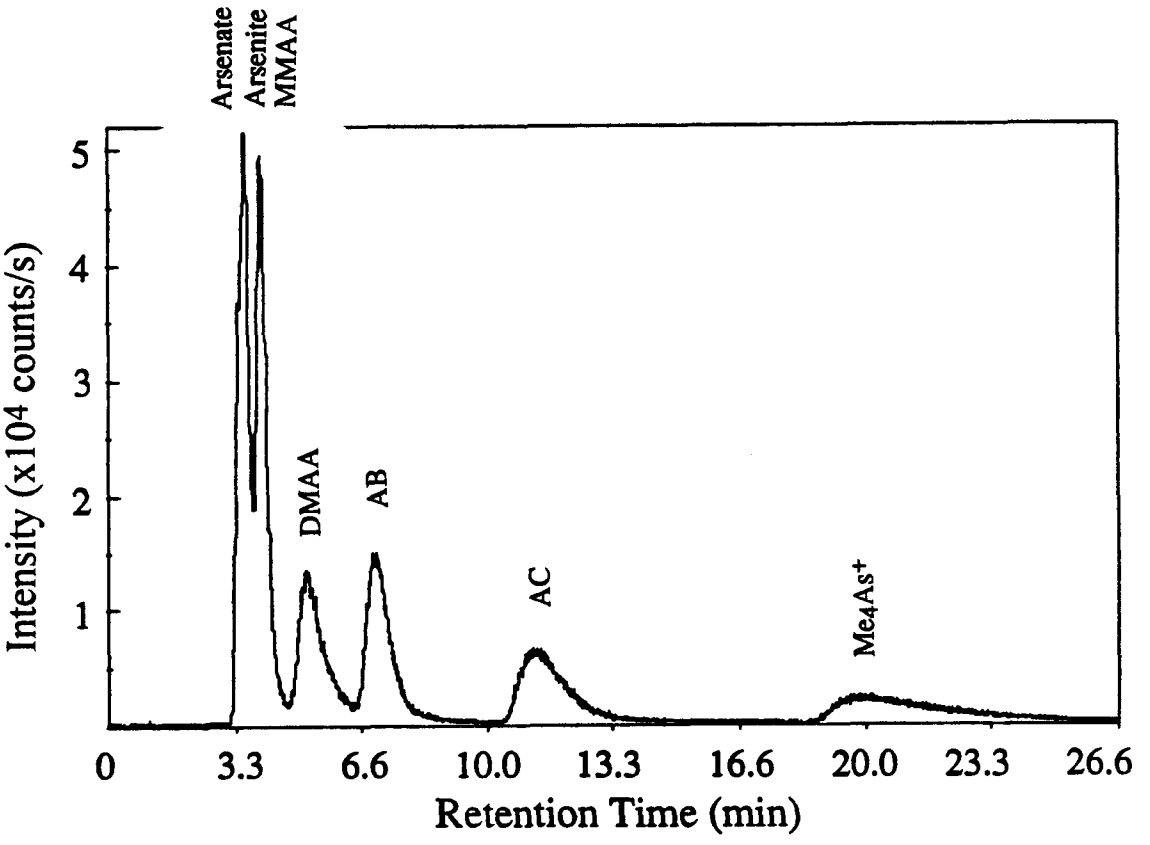


Figure 5.10. Chromatogram of seven arsenicals (20 ng each) obtained on a reversed phase C18 column with 10 mM propanesulfonate and 4 mM malonic acid (pH 3.5) as eluent and using ICPMS detection.

The use of an ion pair reagent that has an even shorter hydrocarbon chain further reduces the retention time of the strongly retained analytes. As demonstrated in Figure 5.11, the retention times for AC and Me_4As^+ in particular are further reduced to approximately 8.5 and 12.5 min, respectively, when methanesulfonic acid is used as the ion pair reagent.

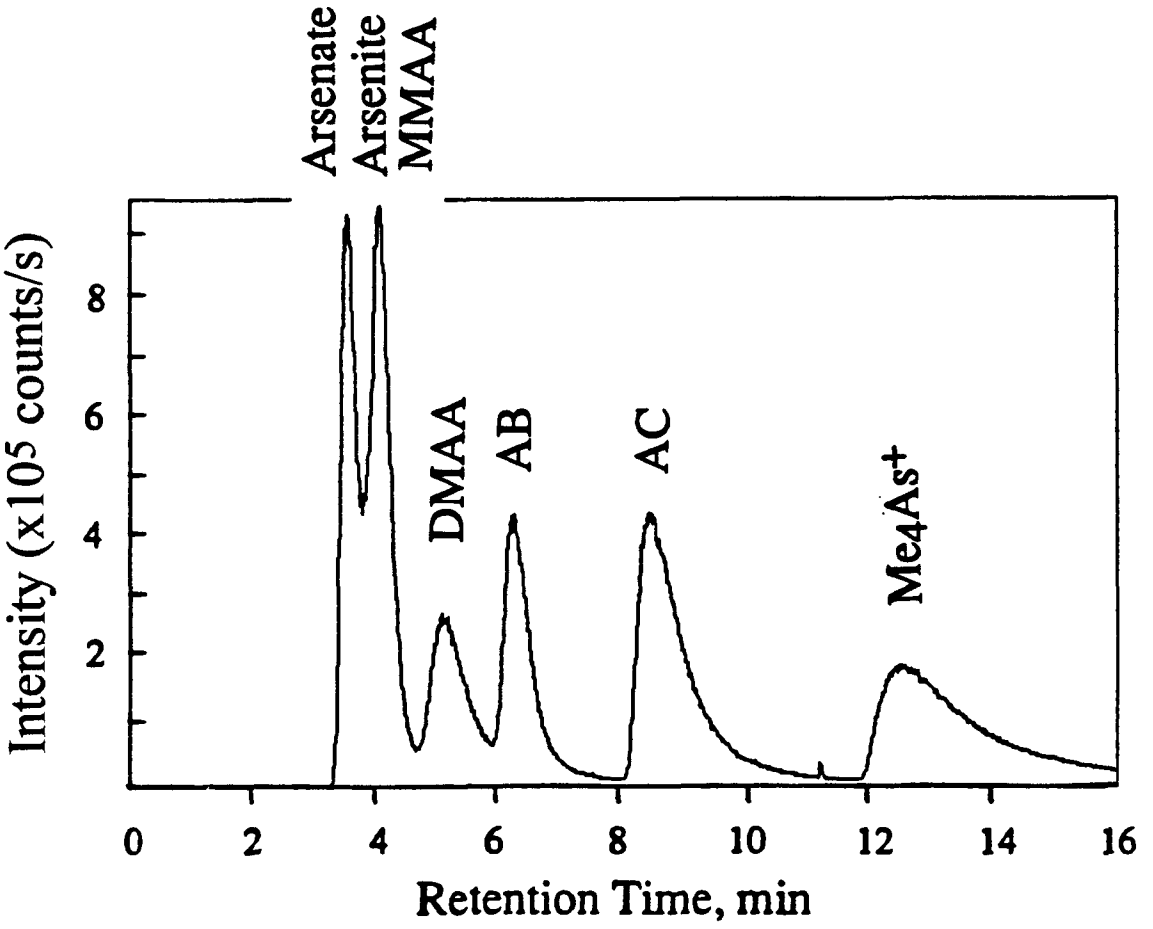


Figure 5.11. Chromatogram of seven arsenicals (20 ng of arsenate, arsenite, MMAA, DMAA, and AB; 40 ng of AC and Me_4As^+) obtained on a reversed phase C18 column with 10 mM methanesulfonic acid and 4 mM malonic acid (pH 3.5) as eluent and using ICPMS detection.

The addition of an organic solvent into the aqueous mobile phase is also known to reduce the retention of analytes in an ion pair chromatography system (254). As demonstrated in Figure 5.12, the retention times of AB, AC and Me_4As^+ are reduced to 5.7, 7.6, and 10.5 min, respectively, when 2% methanol is added to the mobile phase compared with the retention times of 6.3, 8.5, and 12.5 min for these same arsenicals when only 0.1% methanol is present in the mobile phase (Figure 5.11). This is because the introduction of organic solvent into the mobile phase reduces the hydrophobic interaction between the C18 stationary phase and the ion pair reagent to which analyte ions are paired. The use of a higher concentration of organic solvent is not advisable because the sample cone of the ICP-MS interface may become blocked because of deposition of carbon residue as a result of incomplete combustion of organic compounds (1, 145, 257). The introduction of small amount of air into the ICP nebulizer gas can reduce this problem (145, 257). However a shorter life time of the instrument sample cone is observed (1, 257) as a consequence of air introduction.

Although AB, AC, $Me_4As^+$and DMAA are well separated from each other (Figure 5.10-5.12) by using propanesulfonate or methansulfonic acid as eluents, arsenate, arsenite, and MMAA peaks partially overlap. In a routine analysis, two chromatographic runs of the same sample, one under the conditions shown in Figure 5.8 and the other under the conditions indicated in Figure 5.11 could be performed to speciate all these seven arsenicals. Alternatively a simple step gradient operation involving heptanesulfonate as an initial mobile phase (Figure 5.8) followed by methanesulfonic acid as the mobile phase (Figure 5.11) can be used. Figure 5.13 show an example obtained from a such step gradient chromatographic operation. A complete separation of the seven arsenicals is achieved on the single column in one run.

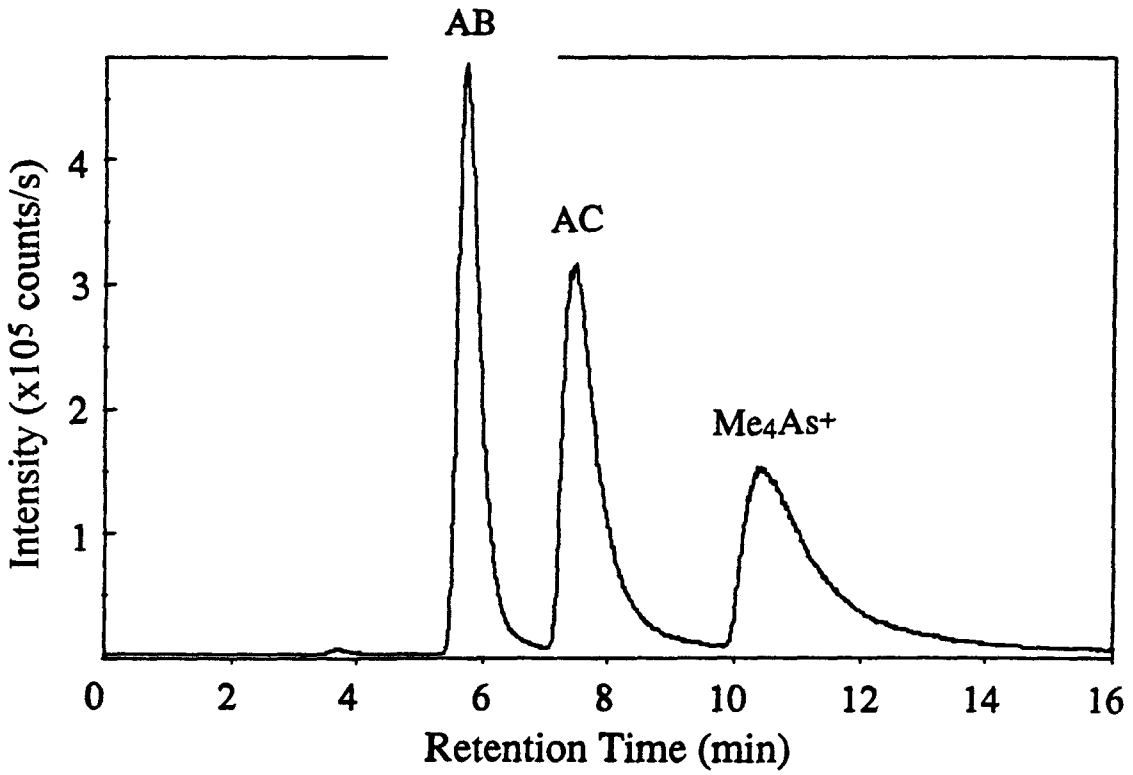


Figure 5.12. Chromatogram of three arsenicals (20 ng each) obtained on a reversed phase C18 column with 10 mM methanesulfonic acid and 2% methanol (pH 3.5) as eluent and using ICPMS detection.

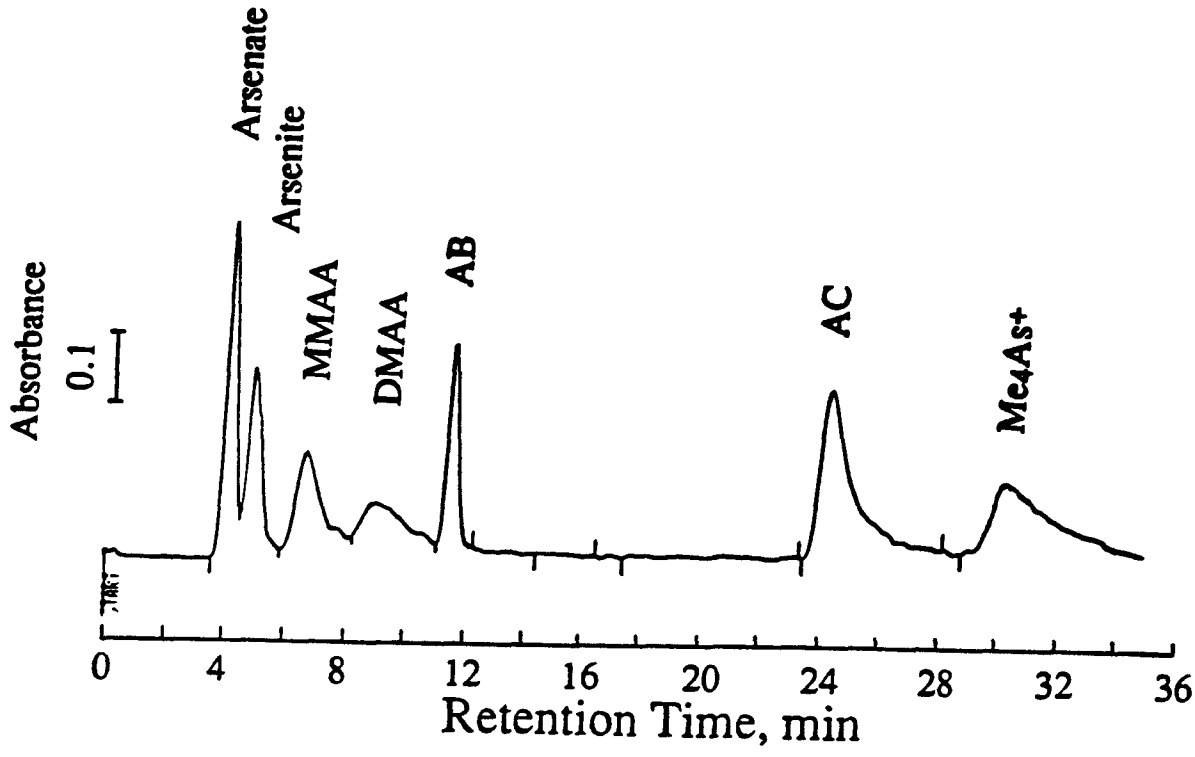


Figure 5.13. Chromatogram of seven arsenicals (20 ng of arsenate, arsenite, MMAA, DMAA, and AB; 40 ng of AC and Me_4As^+) obtained by using a step gradient elution and MD/HGAAS detection
Initial eluent: 10 mM heptanesulfonate, pH 3.5
2 min after, switch to: 10 mM methanesulfonic acid and 4 mM malonic acid (pH 3.0); flow rate: 1.0 ml min^{-1}.

The ion pair chromatographic system discussed above is based on the pairing between sulfonate anions and the analyte cations. This has been shown to be very useful to separate cations such as AB, AC and Me_4As^+. Some other arsenicals, arsenosugars in particular, usually exist as anions, and their separation may be best carried out by using a cation pairing chromatographic system. Only few studies, mainly by a Japanese group (6, 61, 149), have dealt with the separation of arsenosugars and they have reported (6, 61, 149) that ion pair chromatography using tetramethylammonium hydroxide and malonic acid as the mobile phase is useful. We have adopted this approach in our studies. Figure 5.14 shows a chromatogram obtained from a standard reference material (1566a Oyster

tissue, National Institute of Standard and Technology). Shibata *et al.* (61) have rigorously identified the presence of arsenobetaine and two arsenosugars (X and XI as shown in Table 1.1 on page 2) as the major arsenic species in this standard reference material. The chromatogram shown in Figure 5.14 is similar to that reported by Shibata *et al.*(61). A sample from the same batch was submitted to Dr. Shibata for analysis and the arsenic species identified (258) are in good agreement with our results shown here. Thus in the absence of pure sample of X and XI, the NIST 1566a standard reference material can be used as a standard for chromatography identification purposes.

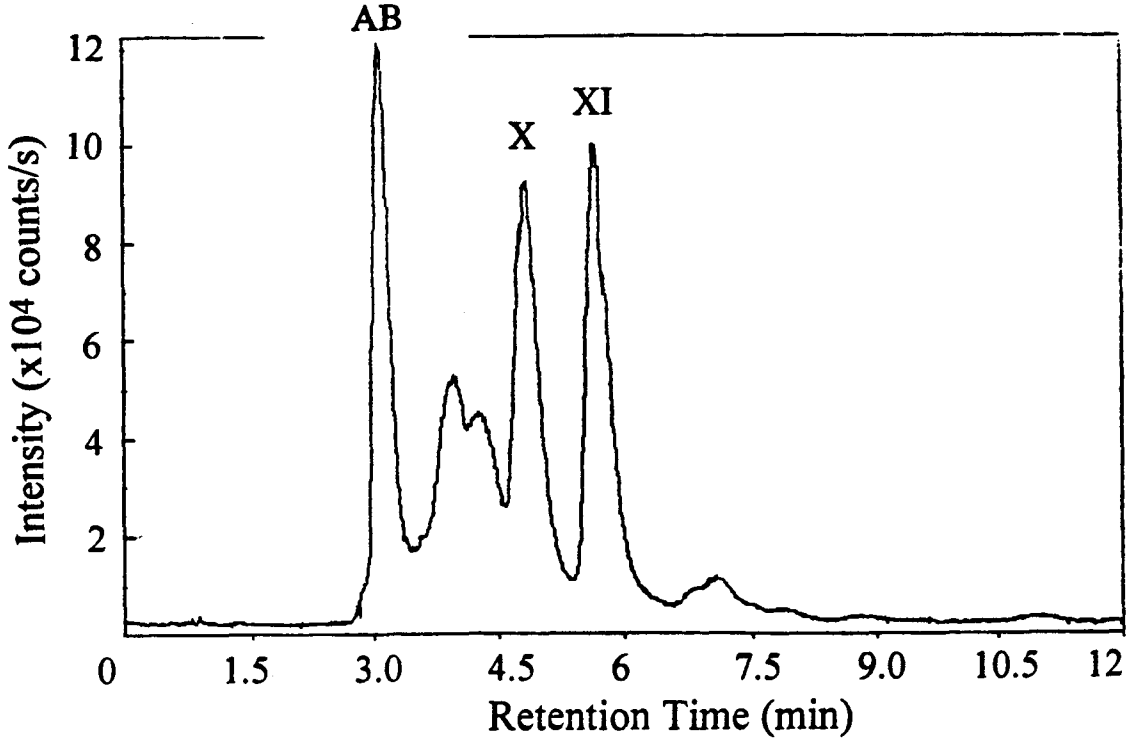


Figure 5.14. Chromatogram of a standard reference material (NIST 1566a Oyster Tissue) extract obtained on a reversed phase column (Inertsil ODS-2) with 10 mM $(C_2H_5)_4NOH$ and 4 mM maloinc acid (pH 6.8) as eluent and using ICPMS detection.

5.3.4. Speciation of arsenic compounds in human urine

Urine samples collected from a volunteer who ingested some shrimps were studied for arsenic speciation. Ion pair liquid chromatography with 10 mM butanesulfonate and 4 mM malonic acid as eluent was used with ICPMS detection. HG/GC/AAS was also used to determine concentration of inorganic arsenic, and DMAA. MMAA is not detected in the urine samples from either measurement. The detection limit for MMAA using HG/GC/AAS is 1 ng (or 0.3 ng ml^{-1}). The concentrations of arsenic species, AB, DMAA, and inorganic arsenic (mainly arsenate) in the urine samples, normalized against their creatinine concentration, are summarized in Figure 5.15. Creatinine is known to be excreted constantly from an individual human body (259, 260). Thus the relative concentration of arsenic over creatinine in the urine is a measure in which the uncertainty due to the volume change is taken into consideration. Therefore, the normalized results in Figure 5.15 roughly illustrate the rate of excretion of the arsenic compounds. It is clear that arsenobetaine is rapidly excreted from the human body following the ingestion of shrimps. It is also clear that the ingestion of arsenobetaine (through the consumption of shrimps) does not increase inorganic arsenic, MMAA and DMAA concentration in urine as would be expected for a species that is simply excreted without metabolic change.

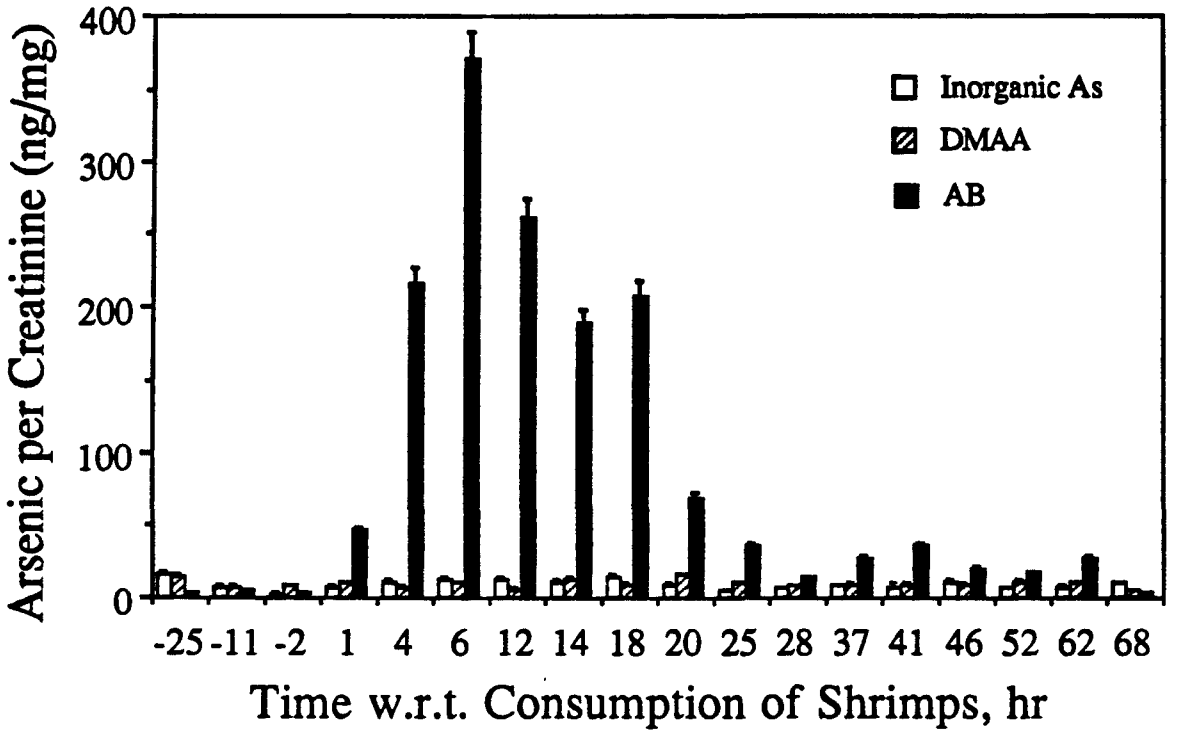


Figure 5.15. Speciation of arsenicals in urine samples collected encompassing the consumption of shrimps
(□) - Inorganic As; (▨) - DMAA; (■) - AB

Chapter 6
SPECIATION OF ARSENIC COMPOUNDS IN THE MARINE ENVIRONMENT

6.1. INTRODUCTION

Arsenic occurs in seawater mainly as inorganic arsenate, at levels of approximately 2 mg L^{-1}, whereas in marine animals the levels can be much higher, up to 100 μg kg^{-1} (wet weight), as arsenobetaine (5, 7, 9, 12). Arsenobetaine was first isolated and unequivocally characterized (46, 47) in 1977 from the tail muscle of the western rock lobster, *Panulirus cygnus*. Since then this compound has been found to be present as the most abundant arsenical in almost all the marine animals so far investigated (5, 7, 12) Several reports have indicated that marine animals do not make arsenobetaine directly from arsenate present in ambient seawater, but rather accumulate it through the food chain (33, 63-66). It has been suggested that arsenosugars, the major arsenicals found in marine algae (6, 15, 32, 36), are involved in the production of arsenobetaine at some stage in the food chain (5, 7, 9, 12, 67-69). However, the origin of arsenobetaine is still not clear. Detailed studies on arsenic speciation in a variety of marine algae and animal samples are necessary in order to gain a better understanding of the cycling of arsenic in the marine environment.

Sample handling, storage, and preparation procedures are crucial steps in obtaining meaningful analytical results for environmental analysis (261, 262). The most important requirement is to maintain the integrity of a sample which should be representative of the specific sampling site under study. When chemical speciation is concerned, this requirement includes preserving the total concentration of the analytes as well as the individual chemical species. A freezing procedure has been commonly used to preserve biological samples (263). However, little is known about the effect on arsenic speciation of the freezing/defrost process and long-term storage. In order to gain

information on this aspect of sample preservation, arsenic speciation in fresh and defrosted samples has been carried out.

6.2. EXPERIMENTAL

6.2.1. Reagents

Standard solutions of arsenic compounds used in this work have been listed in Table 1.1 and were prepared in deionized water. The HPLC eluents, solutions of (i) 10 mM tetraethylammonium hydroxide and 4.5 mM malonic acid (pH 6.8) and (ii) 10 mM sodium heptanesulfonate and 4 mM tetramethylammonium hydroxide (pH 3.5), were prepared in distilled deionized water and filtered through a 0.45 μm membrane filter. The pH of all HPLC eluents was adjusted by using diluted nitric acid and sodium hydroxide. Methanol (0.1%) (HPLC grade, Fisher) was added to both eluents before the pH adjustment and the filtration.

6.2.2. Samples

The commercial seaweed products Nori, powdered "kelp", and Dulse were purchased from a local food store (Vancouver, Canada), fresh crabs and shrimps were purchased from a local fish market. Other marine animal samples such as mussels, clams, and oysters were collected from the British Columbia coast (Canada) on a cruise in May-June, 1991. The samples were frozen (-20 ^{o}C) immediately after collection and stored at this temperature. The frozen samples were each placed in a beaker and allowed to defrost at room temperature. The defrosted samples and the "defrost juice" collected in the beaker were used for further analysis. Nori, "kelp" or Dulse (0.5-1 g dry weight), or marine animal samples (5-10 g wet weight) were extracted by using a procedure similar to that described by Shibata and Morita (61). Each sample, defrosted if necessary, was weighed into a test tube (15 ml). To each tube was added 5-10 ml of a methanol/water

mixture (1:1, V/V). The tube was sonicated for 10 min and after centrifugation, the extract was removed and placed in a round-bottom flask. The extraction process with the aid of sonication was repeated a further four times for each sample. The extracts were combined in the flask, evaporated to dryness, and the residue dissolved in 10 ml of deionized water. The sample was analyzed by using both FIA/HGAAS and HPLC/ICPMS.

6.2.3. FIA/HGAAS

An aliquot (1 or 2 ml) of the sample extract was diluted with an equal amount of deionized water. An appropriate amount of cysteine was added to make its concentration in the sample approximately 1-2%. The sample was analyzed for total hydride-forming arsenic species by using FIA/HGAAS as described in Section 3.2.4.1. Another aliquot (2 ml) of the sample extract was placed in a 125-ml Erlenmyer flask to which was added 20 ml of deionized water, approximately 2 g of potassium persulfate and 1.5 g of sodium hydroxide. The sample was digested by using the batch type microwave assisted decomposition procedure as described in Section 4.2.3.4. After the microwave decomposition, the sample was diluted with deionized water to 50 ml and analyzed for total arsenic concentration by using the same FIA/HGAAS method. The "defrost juice" from the previously frozen samples was analyzed in the same manner.

6.2.4. HPLC/ICPMS

Two chromatographic systems were used, one primarily designed for the separation of arsenosugars and the other designed for arsenobetaine. An ODS-2 column (GL Sciences, Japan) with eluent (i): 10 mM tetraethylammonium hydroxide and 4.5 mM malonic acid (pH 6.8), at flow rate of 0.7 or 0.8 ml min^{-1} was used for the separation of arsenosugar derivatives and metabolites resulting from arsenosugar ingestion. Another C18 column (Phenomenex, California) was used when running eluent

(ii): 10 mM sodium heptanesulfonate and 4 mM tetramethylammonium hydroxide (pH 3.5), at a flow rate of 1 ml min^{-1} for the separation of arsenobetaine. The HPLC column was equilibrated with the appropriate eluent for at least 2 hr before any sample injection was made. All samples were centrifuged and filtered through a 0.45 μm membrane filter prior to injecting 5-10 μl of the sample onto the HPLC column for chromatographic analysis. Arsenic compounds in the samples were identified by matching the retention times of the chromatographic peaks of the sample with those of standards, sometimes spiked into the sample. It should be pointed out that the chromatograms shown in Figures 6.1 and 6.2 were obtained under slightly different flow rate (0.7 ml min^{-1}) and void volume conditions from those shown in Figures 6.3-6.11 (0.8 ml min^{-1} and a larger void volume), although the same eluent and column were used for these analyses.

6.3. RESULTS AND DISCUSSION

6.3.1. Arsenic speciation in seaweed and shrimp samples

Four seaweed samples and three shrimp samples, each in duplicate, were extracted as described in the Experimental Section. The arsenic concentration in each sample extract was initially determined by using FIA/HGAAS. Measurements were made both before and after the microwave assisted digestion of the sample extract and the results are summarized in Table 6.1. As discussed previously, direct FIA/HGAAS analysis of the sample without a prior digestion gives arsenic concentrations corresponding to the hydride-forming species, whereas the total arsenic concentration is obtained by analyzing the same sample after it has undergone the microwave assisted digestion. The results in Table 6.1 show that less than 10% of the arsenic is present as hydride-forming species in the seaweed product Nori and two "kelp" samples; whereas in the seaweed product Dulse 24% of the total arsenic compounds respond to HGAAS before the digestion. In shrimp samples less than 1% of the total arsenic concentration is

detected by using HGAAS in the absence of digestion. The total arsenic concentrations are determined following microwave assisted digestion of the samples, and the results are in general agreement with those previously reported (5, 6) for the same category of marine samples.

Table 6.1. Arsenic concentration in seaweed and shrimp samples determined by using HGAAS with and without microwave assisted digestion.

Samples	Arsenic concentration ($\mu g\ g^{-1}$)	
	Without digestion	Microwave assisted digestion
Nori *	0.68 ± 0.04	21.0 ± 0.8
Powdered "kelp" *	1.9 ± 0.1	19.6 ± 0.7
Whole "kelp" *	1.7 ± 0.1	23 ± 1
Dulse *	1.5 ± 0.1	6.2 ± 0.3
Small Shrimp (unfrozen whole)	0.02	1.4 ± 0.1
Tiger prawn (unfrozen whole)	0.02	1.8 ± 0.1
Tiger prawn tail (frozen)	-	0.19 ± 0.02

*: marked samples in dry weight, other samples in wet weight.

-: not detected.

Further speciation of arsenic compounds in these sample extracts was studied by using HPLC/ICPMS and some chromatograms are shown in Figures 6.1-6.4. Arsenosugar X is shown to be the major arsenic species present in the Nori sample (Figure 6.1). Three major arsenic species were contained in the "kelp" sample (Figure 6.2), two of which are identified as arsenosugars X and XI (structures shown in Table 1.1

on page 2) on the basis of a match of their retention times with those of standards. The other major arsenic compound which appears at a longer retention time (approximately 7.5 min) is believed to be arsenosugar XIV (structure also shown in Table 1.1) on the basis of the occurrence and relative retention of arsenic species previously reported for some related macroalgae (32, 36, 37, 39, 40, 73, 149). Standards containing XIV are not available to us. Four arsenic compounds are identified in Dulse as DMAA, arsenate, and the arsenosugar derivatives X and XI (Figure 6.3). The proportions of DMAA and arsenate are higher in Dulse (Figure 6.3) than those in Nori (Figure 6.1) and "kelp" (Figure 6.2). This is consistent with the results shown in Table 6.1, where a higher percentage of hydride-forming arsenic species was obtained from undigested samples of Dulse by using HGAAS, when compared to those obtained from Nori and "kelp".

Arsenobetaine is the dominant arsenic species present in shrimp as shown in Figure 6.4 in agreement with the result (Table 6.1) that 99% of the total arsenic in shrimp is present as "hidden" arsenic. Arsenobetaine is an arsenic species which is not reducible by sodium borohydride and which requires vigorous digestion to convert it to a form amenable to analysis by using hydride generation.

It has been claimed by some that arsenocholine is present in shrimps (53-55), but others (56, 57) could not confirm its presence. We have not found evidence for the presence of arsenocholine in shrimps in the present study, although looking for this compound was not an objective.

The difference in the arsenic concentration between unfrozen whole tiger prawns and frozen tiger prawn tails shown in Table 6.1 was intriguing, and the source of this difference clearly deserved further investigation. Two main possibilities for the discrepancy were considered (i) a difference in concentration between the tail and the rest of the prawn and (ii) a difference resulting from the freezing/defrost process.

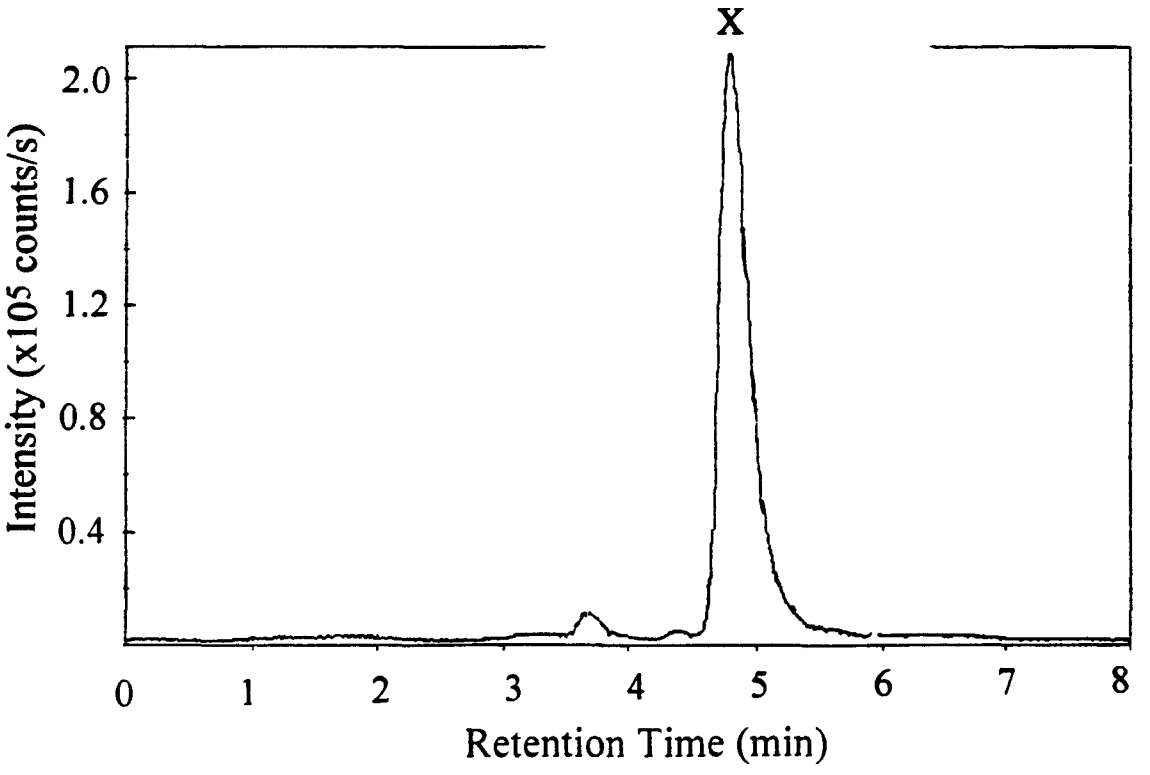


Figure 6.1. A HPLC/ICPMS trace of an extract from seaweed product Nori.
HPLC column: Inertsil ODS-2 reversed phase (4.6 x 250 mm, 10 μm)
Eluent: 10 mM tetraethylammonium hydroxide and 4.5 mM malonic acid (pH: 6.8);
Flow rate: 0.7 ml min^{-1}

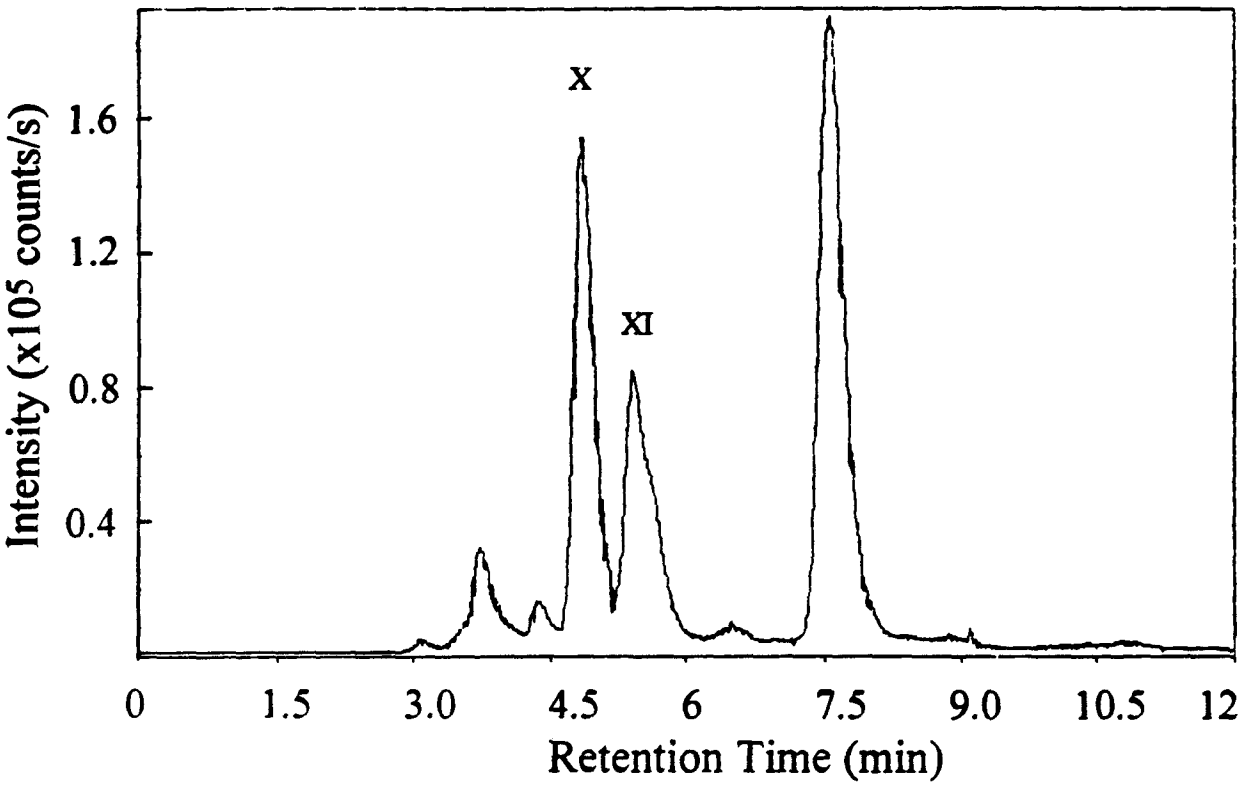


Figure 6.2. A HPLC/ICPMS trace of an extract from powdered seaweed product "kelp". Same conditions as in Figure 6.1

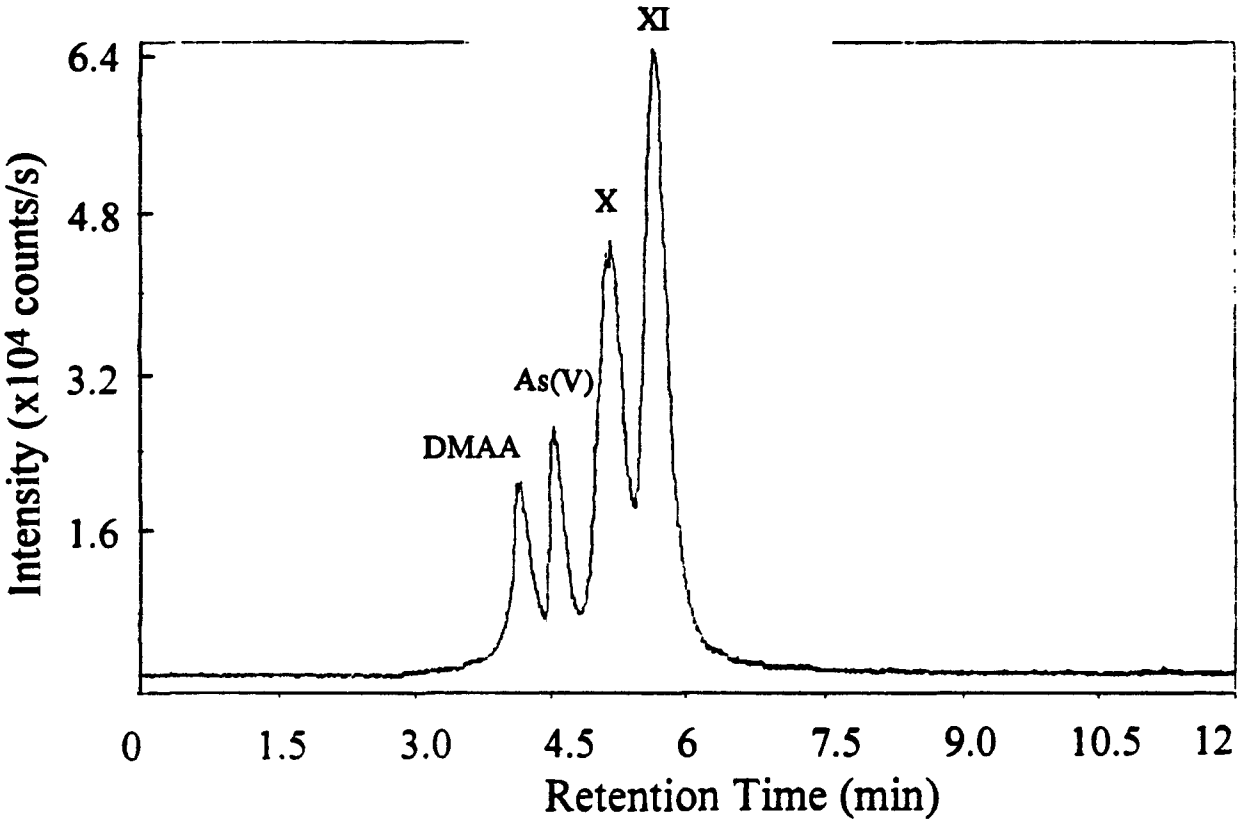


Figure 6.3. A HPLC/ICPMS trace of an extract from powdered Dulse.
HPLC column: Inertsil ODS-2 reversed phase (4.6 x 250 mm, 10 μm)
Eluent: 10 mM tetraethylammonium hydroxide and 4.5 mM malonic acid (pH: 6.8);
Flow rate: 0.8 ml min^{-1}

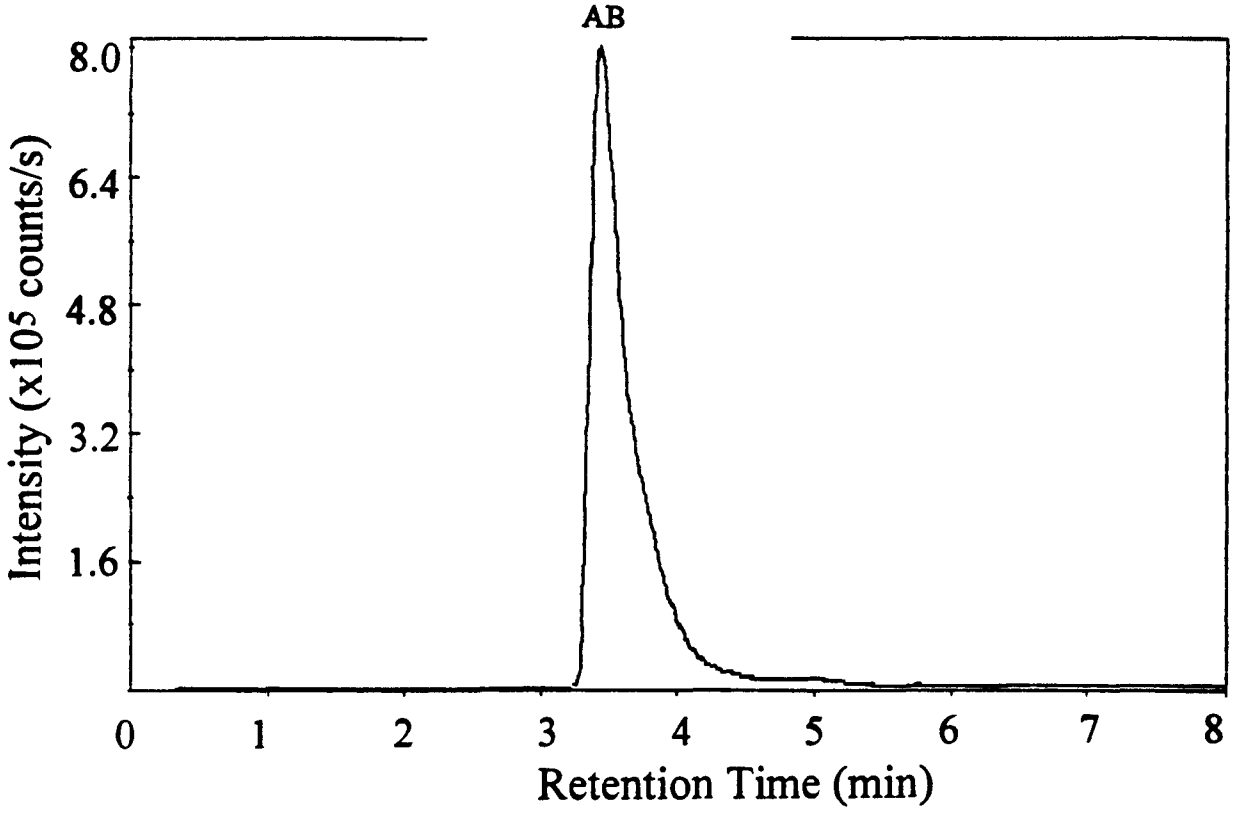


Figure 6.4. A HPLC/ICPMS trace of an extract from shrimp.
Same conditions as in Figure 6.3

To examine the first possibility, the concentration of arsenic in the heads and tails of tiger prawns was determined separately. Both live and frozen prawns were purchased from a local seafood market. Each prawn was dissected to give a head and a tail. Groups containing heads only and tails only were separately extracted and analyzed. The results as shown in Table 6.2 indicate very little arsenic concentration difference between the heads and tails of the prawns. The slightly higher concentration found in the tails probably results from the higher portion of muscle (Higher arsenic concentrations are generally found in the soft tissue of marine animals). A significant difference in arsenic concentration was obtained, however, between the fresh (live) prawns and the frozen ones giving strongly to the second possibility that a portion of the arsenic compounds might have leached out along with the body fluid on defrosting. The prawns had been frozen and stored for some time although the length of time is unknown in the present case. This possibility was studied further and the results are given in the following section.

Table 6.2. Comparison of total arsenic concentration in prawn heads and tails

Samples		Arsenic concentration (μg As/g wet weight sample)
Live (fresh) tiger prawn	Heads	7.0 ± 0.8
	Tails	8.7 ± 0.6
Frozen tiger prawn*	Heads	2.2 ± 0.5
	Tails	2.3 ± 0.1

* Defrosted prior to analysis.

6.3.2. Speciation of arsenic in sample extracts and defrost "juice" from previously frozen marine animal samples

Fresh shrimps were purchased, one sample was analyzed for arsenic and the other was immediately frozen in a household freezer for one day. The frozen shrimps were then allowed to defrost at room temperature and the defrost "juice" was collected for analysis. Approximately 6% of the total arsenic present in the fresh shrimps was found in the defrost "juice" of the frozen shrimps (Table 6.3).

Similarly, a number of other previously frozen marine animal samples were analyzed for arsenic and the results are summarized in Table 6.3. These samples had been frozen for approximately two years before this investigation. The results illustrate that significant amounts of arsenic are present in the defrost "juice", ranging from 3 to 48% of the total arsenic.

The arsenic concentrations reported in Table 6.3 are the results of three replicate measurements of duplicate sample extracts. When microwave assisted digestion was involved, duplicate or triplicate aliquots of each sample extract or defrost "juice" were subjected to the digestion prior to HGAAS analysis. The relatively high standard deviation observed is probably due to the heterogeneity of the sample as has been suggested by others (61). Nevertheless, it is clear that appropriate care should be taken in sampling and storage processes when the concentration of arsenic in field samples is to be determined. If a freezing procedure is involved, the possible loss of arsenic on defrosting should be taken into consideration.

Table 6.3. Comparison of arsenic concentration in sample extracts and in defrost "juice"

Sample	Amount	As found (μg)		As concentration (μg g^{-1})		%
		No digestion	Microwave digestion	No digestion	Microwave digestion	Defrost/ Total
Prawns (from local fish market)						
Tissue			549		8.3 ± 0.4	
"Juice"			35			6%
California Mussel (*Mytilus californianus*)						
Tissue	105.8 g	5.46	93.4	0.05 ± 0.01	0.9 ± 0.1	
"Juice"	45 ml	0.73	23.1			20%
Oyster *(Crassostrea Gigas)*sample 1						
Tissue	18.6 g	1.5	11.7	0.08 ± 0.01	0.63 ± 0.07	
"Juice"	21 ml	0.34	8.5			42%
Oyster *(Crassostrea Gigas)*sample 2						
Tissue	25.5 g	2.1	16.6	0.08 ± 0.01	0.65 ± 0.05	
"Juice"	21	0.36	6.9			29%
Clam, Soft-Shell (*Mya arenaria*)						
Tissue	76.6 g	5.9	32.2	0.08 ± 0.01	0.42 ± 0.03	
"Juice"	77 ml	2.1	29.4			48%
File Yoldia (*Yoldia limatula*)						
Tissue	26.2 g	2.9	100.9	0.11 ± 0.03	3.8 ± 0.5	
"Juice"	4.4 ml	0.16	34.8			26%
Triton (*Fusitriton*)#						
Tissue	109.3 g	8.2	2644	0.08 ± 0.01	24.2 ± 2.8	
"Juice"	7.1 ml	0.10	80.4			3%

*: Prawns were frozen for one day, all other samples were frozen for approximately two years.

#: The *Fusitriton* sample had been previously dissected and soft tissue was kept frozen. Other samples were dissected to give soft tissue after they were defrosted.

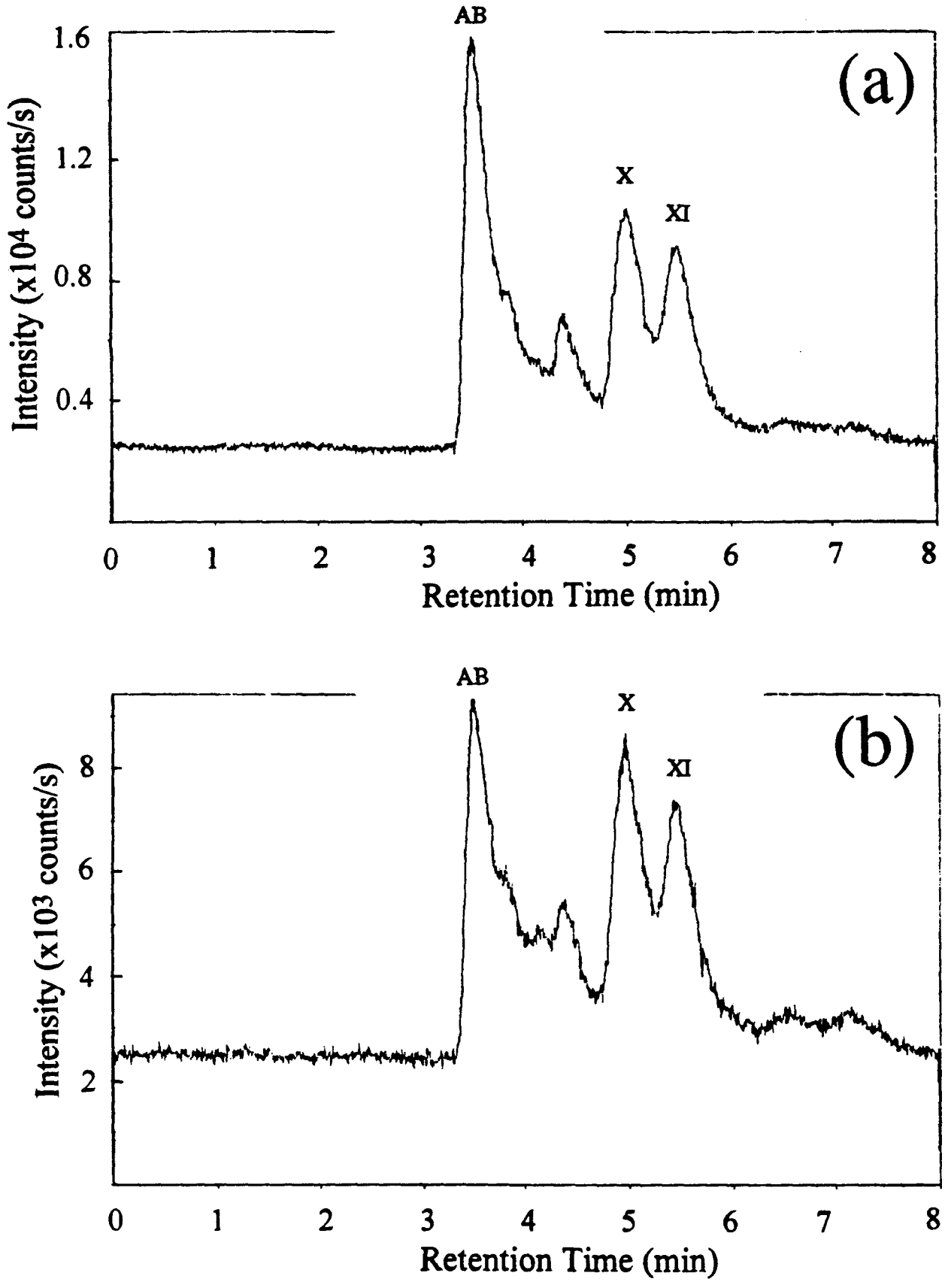


Figure 6.5. HPLC/ICPMS traces from an extract (a) and defrost "juice" (b) of an oyster sample. Same conditions as in Figure 6.3

HPLC/ICPMS studies reveal that the arsenic species found in the defrost "juice" are identical with those present in the original sample. As an example, Figure 6.5 shows two chromatograms obtained from an oyster extract (a) and oyster defrost "juice" (b). In both cases arsenobetaine, arsenosugars X and XI are found to be the major arsenic species and arsenate and DMAA are minor components. It seems that there is little discrimination against specific arsenic species as a result of the defrosting process probably because most of these arsenic compounds are water soluble and are not membrane bound. Thus they can be readily released after the cells are ruptured by freezing.

Figures 6.6-6.10 show chromatograms obtained from extracts of a number of bivalves. These results reveal that arsenobetaine is the major arsenic compound in the soft shell clam (*Mya arenaria*) (Figure 6.6), in a small deep water clam (*Yoldia*) (Figure 6.7), and a gastropod (*Fusitriton*) sample (Figure 6.8). A small peak corresponding to arsenosugar X is also detected in these samples (Figures 6.6-6.8). It is particularly interesting to note the presence of the arsenosugar X and the relatively high concentration of arsenic in the deep water clam (*Yoldia*). Very little is known about arsenic speciation in deep water bivalves, most attention has been given to their intertidal counterparts. Two arsenosugars X and XI along with arsenobetaine are found as the major arsenic species in the California mussel (*Mytilus californianus*) (Figure 6.9) and intertidial Pacific oyster (*Crassostrea gigas)* (Figure 6.10). The proportion of arsenosugars in these samples is quite high. These results, in agreement with those in a recent report (61), confirm that bivalves contain not only arsenobetaine but also arsenosugars as the major water-soluble arsenic compounds.

A number of previous studies (49-52) on the speciation of arsenic compounds in bivalves did not report the presence of the arsenosugar derivatives. This discrepancy may result from difference in the samples but more likely is a result of the different analytical techniques that were used.

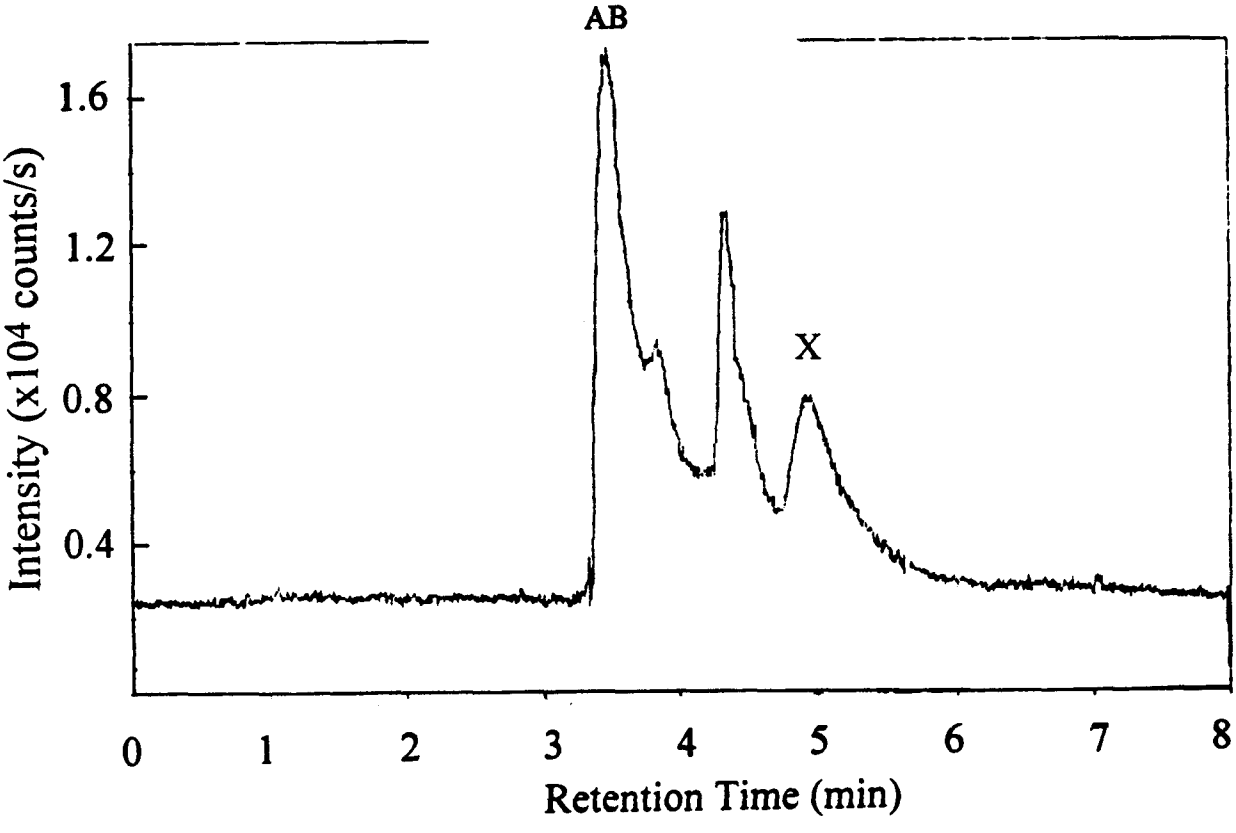


Figure 6.6. A HPLC/ICPMS trace of an extract from soft shell clam (*Mya arenaria*). Same conditions as in Figure 6.3

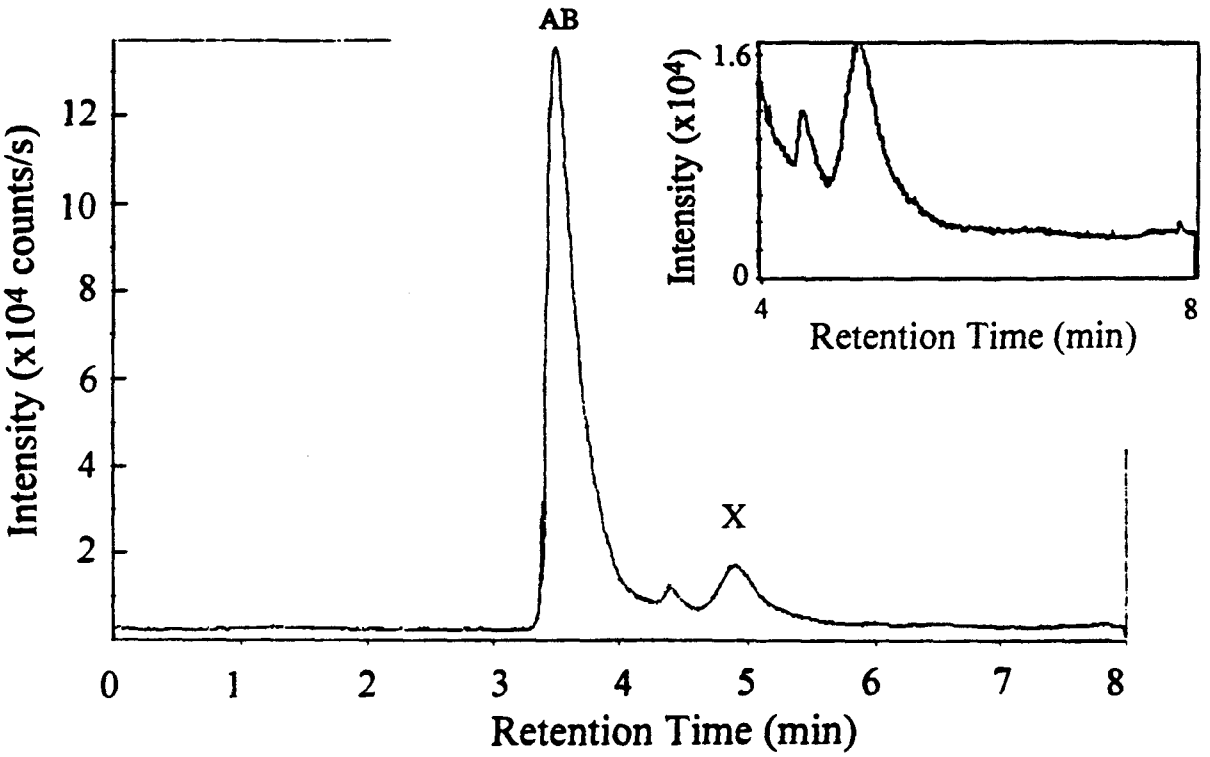


Figure 6.7. A HPLC/ICPMS trace of an extract from *Yoldia*..
Same conditions as in Figure 6.3

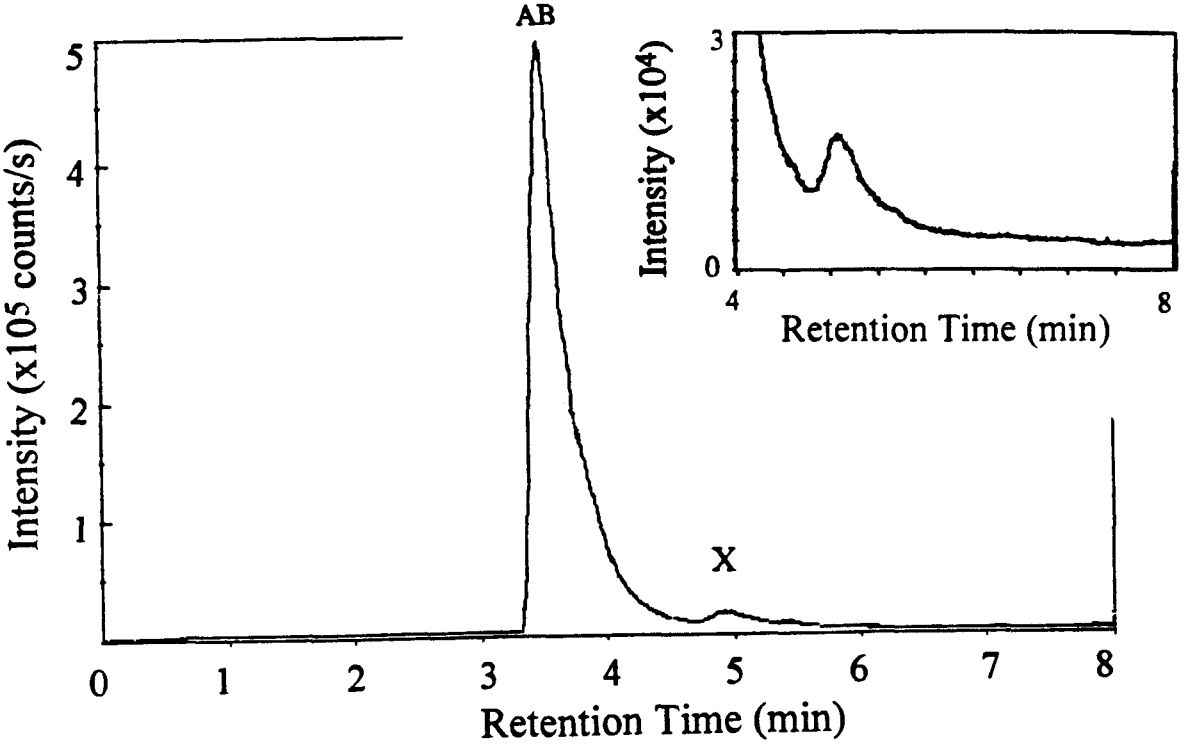


Figure 6.8. A HPLC/ICPMS trace of an extract from *Fusitriton*. Same conditions as in Figure 6.3

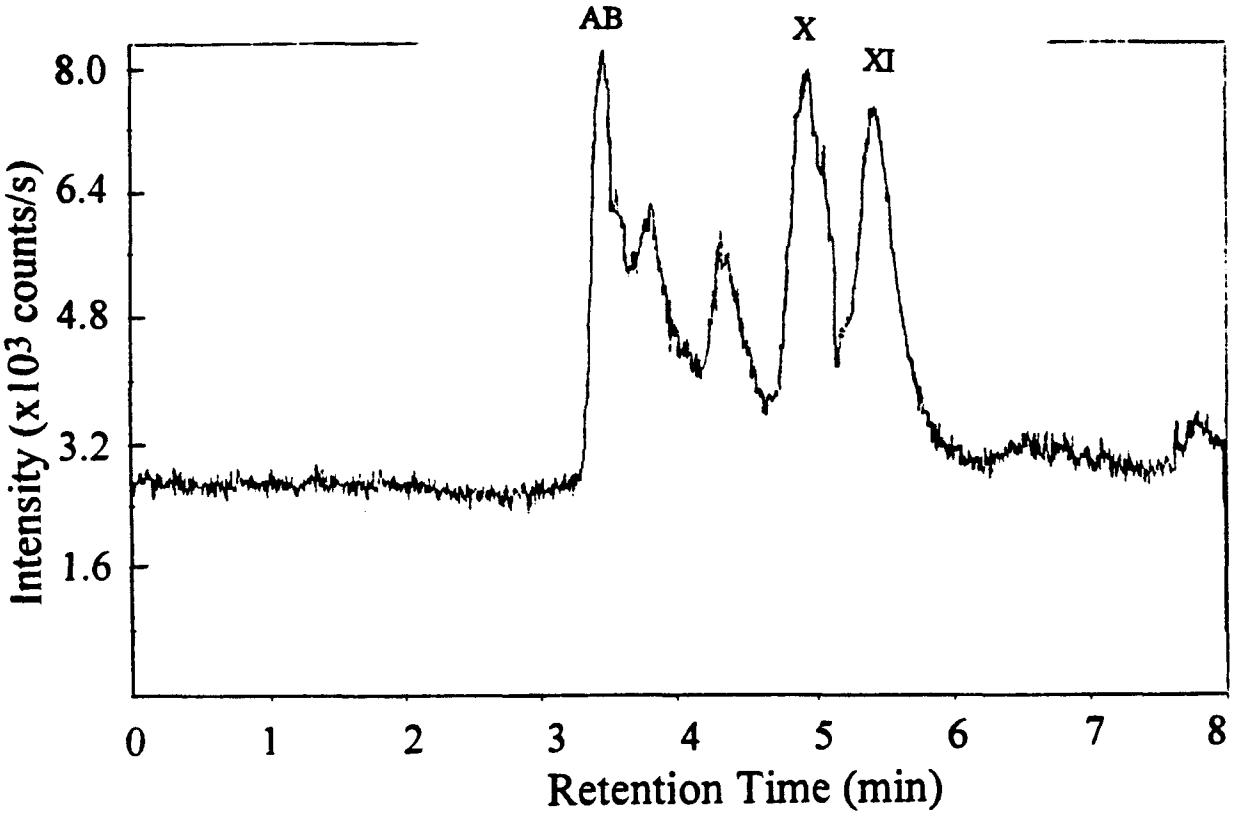


Figure 6.9. A HPLC/ICPMS trace of an extract from the California mussel (*Mytilus califonianus*).
Same conditions as in Figure 6.3

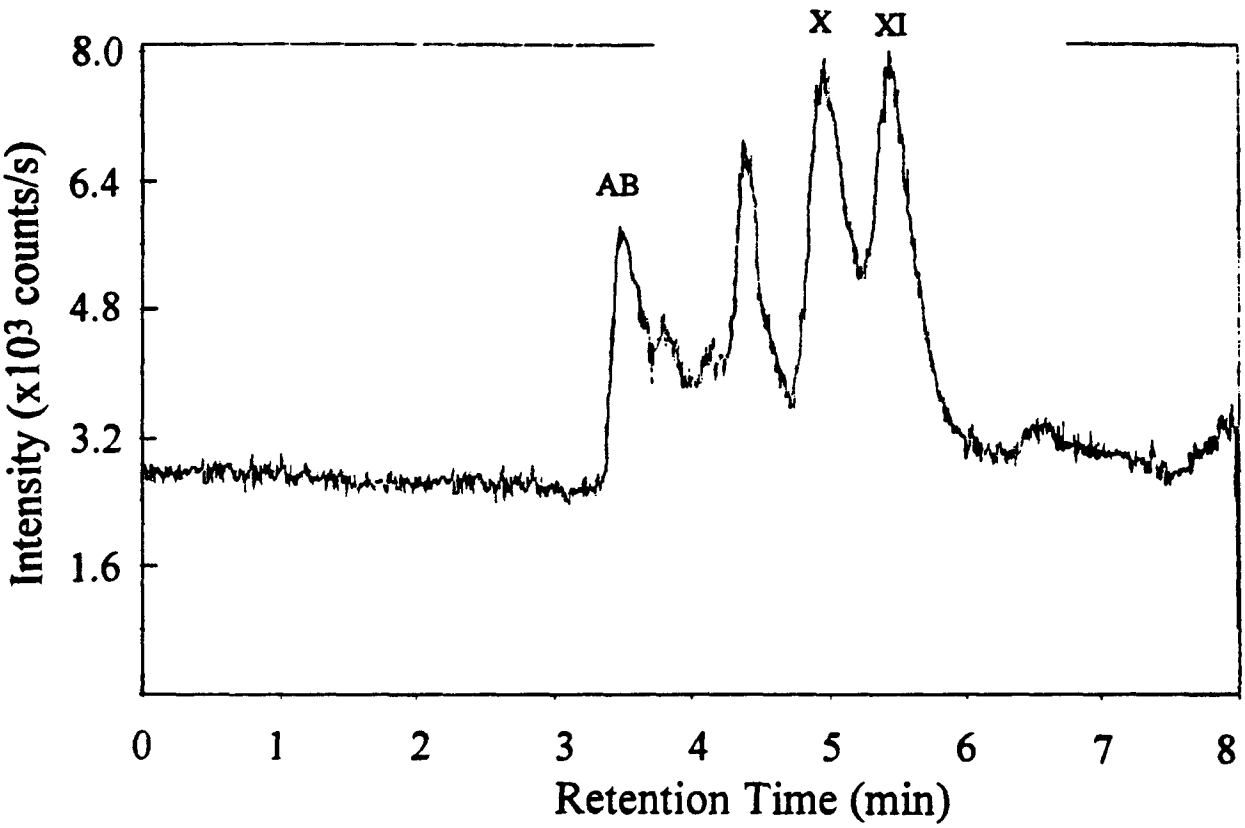


Figure 6.10. A HPLC/ICPMS trace of an extract from oyster (*Crassostrea gigas*). Same conditions as in Figure 6.3

The samples studied in this work were all collected during a cruise in May-June, 1991 along the British Columbia coast, Canada. There is the possibility that arsenic speciation in bivalves varies temporally (seasonal variation) and/or spatially (regional variation). There is no information available, however, on the seasonal and regional variations of arsenic speciation in seaweed.

Our preliminary results on the speciation of arsenic in two brand names of Nori showed that only arsenosugar X was present in one sample whereas two arsenosugars X and XI were found at approximately 2:1 ratio in another Nori sample. Both samples were extracted with a mixture of methanol/water (50/50) at nearly neutral pH, and no decomposition of these arsenicals is expected. The difference in arsenic speciation between the two batches of Nori may be due to: (i) the two products named Nori might have been cultivated and processed from different seaweed species or (ii) the raw seaweed material for Nori preparation might have been harvested at different seasons and/or locations. A recent report by Shibata *et al.* (15) supports the first contention. They (15) noticed that the proportion of the two arsenosugars X and XI was different in two types of red algae *Porphyra*, which are both used for Nori preparation.

As mentioned above, the analytical techniques used by others (49-52) during previous speciation studies of marine animals may not have been suitable for isolating arsenosugars. The extensive sample clean-up and column separation process usually employed strong anion exchange and cation exchange column separation procedures. A primary objective of most of these studies was to separate and identify arsenobetaine and as a result of the lengthy and potent separation procedure, arsenosugars could have been changed or lost before they reached the final clean up steps. Edmonds and Francesconi (37) have commented that arsenosugars are quite labile and can be decomposed to DMAA at extreme pH conditions. Such conditions are commonly used for eluting arsenic compounds from either a strong cation exchange column or a strong anion exchange column. In the present study, however, aliquots of a sample extract obtained under mild

conditions, were injected directly onto the HPLC column for separation. The arsenic species that eluted were continuously monitored on-line by using ICPMS. Only the minimum sample treatment (extraction) was involved, minimizing the possibility of changing the integrity of arsenic species present in the original sample.

Arsenobetaine has been identified as the major or sole water-soluble arsenic species in many marine animals (5, 7). On the other hand, all the macroalgae so far investigated have been reported to contain arsenosugars and no detectable arsenobetaine. Bivalves, which belong to the phylum Mollusca, seem to be different from other marine animals with regard to their arsenic speciation. While some members of the Mollusca, such as squid, cuttlefish (140) and octopus (264) have been reported to contain arsenobetaine as the dominant or sole arsenic species, the giant clam, *Tridacna maxima,* was found to have arsenosugars X and XI present in its kidney (62). This result was treated as an exception and the authors (62) attributed the source of the arsenosugar derivatives to symbiotic algae. However, the present results show that mussels, oysters and clams also contain arsenosugars as major arsenic species. Shibata and Morita (61) also found that a number of bivalves contain arsenosugar derivatives in addition to arsenobetaine. These results reveal that further studies of bivalves may provide answers to the question: at what stage in the food chain are arsenosugars transformed into arsenobetaine? The source and possible physiological implications of arsenosugars in these samples also remain to be studied.

The tetramethylarsonium ion (Me_4As^+) has been found (49-52) in some bivalves such as clams and sea anemone. In the present study, Me_4As^+ co-elutes with arsenobetaine under the given chromatographic conditions, and thus no identification is made for the presence or absence of this arsenic species.

Chapter 7
HUMAN URINARY ARSENIC EXCRETION FOLLOWING ONE TIME INGESTION OF ARSENOSUGARS AND ARSENOBETAINE

7.1. INTRODUCTION

Chemical information on the transformation and metabolism of arsenic compounds is necessary in order to understand the toxicological effect of these compounds and the cycling of arsenic in the environment. While the metabolism of arsenite, arsenate, MMAA, DMAA, and arsenobetaine has been studied extensively (13, 80, 84-89, 93-97) in both animal and human subjects, very little is known about the metabolism of arsenosugars (265, 266), the major arsenic compounds present in marine algae - "vegetable of the sea". For example, Nori, a foodstuff processed from red algae (*Porphyra tenera* and other *Porphyra* species), contains 17-28 $\mu g\ g^{-1}$ of arsenic (in dry sample), almost all of which is present as arsenosugars (15). Average daily consumption of seaweed by Japanese has been reported to be 20-30 g with a high of 120 g (wet weight) (267). Although it has been suggested that arsenosugars have no detectable cytotoxicity and mutagenecity (15), the metabolites of arsenosugars resulting from the ingestion of seaweed are not known. Such information is vital in order to evaluate any toxicological implications and health risks accompanying seaweed consumption because the toxicity of arsenic is chemical species dependent (11-16). Since urinary excretion is known to be the major pathway for the elimination of arsenic from the body, it was decided to study the speciation of arsenic compounds in the urine of human subjects who had ingested arsenosugar-containing seaweed. In this chapter, urinary arsenic excretion patterns and information on chemical speciation of urinary arsenic following human consumption of seaweed are reported.

7.2. EXPERIMENTAL

Instrumentation, reagents, procedures, and marine samples dealt with in this chapter are similar to those described in Chapter 6. The same procedures for the determination of creatinine in urine samples as described in section 5.2.3 were used.

Urine samples were obtained from the 9 volunteers listed in Table 7.1. All volunteers refrained from eating any seafood for at least 72 hr prior to commencing the seafood ingestion experiment. Each volunteer was instructed to collect 2-3 urine samples during the 12 hr period prior to the consumption of seafood. These samples were used to determine the background level of arsenic in the urine of the volunteers resulting from a regular diet that excluded seafood.

Ingestion of Nori:

Each volunteer refrained from eating any seafood for 3 days and then consumed approximately 9.5 g (dry weight) of the seaweed product Nori (4 sheets) in one meal. The time of this meal is referred to as time zero. Following the one time consumption of Nori, urine samples (usually middle stream) were collected at approximately 3-5 hr intervals for the next 4 days or longer. No other seafood was eaten during the experiment.

Ingestion of powdered kelp:

In another experiment, volunteers 1 and 2 ingested "kelp" and urine samples were collected as described above for Nori. Similar protocols to those above were followed except that kelp instead of Nori was ingested.

Ingestion of crab and shrimps:

In another set of experiment, volunteers 3 and 4 were asked to ingest steam-cooked crab meat, and volunteers 1, 2, 5, and 6 were chosen to ingest shrimps. As in the Nori experiment, mid-stream urine samples from volunteers 3-6 were collected for 3

days. Volunteers 1 and 2, however, were asked to eat a known amount of stir-fried shrimps including the juice from the cooking and to collect all urine samples for 3 days following the consumption of shrimps. The volume of each urine sample was measured. This procedure enabled the total amounts of all arsenic species excreted by the volunteers after the consumption of shrimps to be determined.

All urine samples were stored at 4 ^{o}C and were analyzed within 48 hr. No preservative was added.

Table 7.1. Information on 9 volunteers who participated in the study of seafood ingestion

Volunteer	Sex	Age	Occupation
1	M	32	Graduate Student
2	F	30	Graduate Student
3	M	59	Retired Farmer
4	F	53	Retired Farmer
5	F	20	Student
6	F	20	Student
7	M	60	University Professor
8	F	48	School Teacher
9	M	24	Graduate Student

Note: Volunteers 1-4 are from the same family and have a similar diet, likewise volunteers 7 and 8.

7.3. RESULTS AND DISCUSSION

7.3.1. Urinary arsenic excretion after the ingestion of "kelp"

The arsenic concentration in urine samples collected from volunteer one prior to and following the ingestion of the seaweed product "kelp" is shown in Figure 7.1. Both microwave digested and undigested urine samples were analyzed for arsenic species by using hydride generation atomic absorption spectrometry (HGAAS), and the results from both measurements of each sample are compared. As described previously (Chapter 4), only hydride-forming arsenic species are determined by using HGAAS analysis on samples that have not been subjected to microwave assisted digestion. Arsenosugars, arsenobetaine, arsenocholine, and the tetramethylarsonium ion are "hidden" and are not detected. When urine samples are decomposed in the presence of potassium persulfate and sodium hydroxide, all arsenic compounds are converted to arsenate, which is readily determined by using HGAAS. Under these conditions, the total arsenic concentration is measured. By performing the two analyses on each sample, the concentration of "hidden" arsenic species is revealed.

Figure 7.1 shows that a considerable amount of direct hydride-forming arsenic compounds is present in urine, particularly in the period of 16 to 43 hr after the ingestion of kelp. The small difference in the arsenic concentrations measured before and after the microwave assisted decomposition of the urine sample indicates that only small amounts of "hidden" arsenic compounds are present in the urine samples. The urinary excretion of arsenic following the ingestion of kelp shown in Figure 7.1 is much slower than in the excretion of arsenic following the ingestion of crab meat (Figure 4.14). The drastic difference in the urinary arsenic excretion patterns between the consumption of kelp and crab meat is believed to be due to the different arsenic compounds ingested. Arsenobetaine is the major arsenic compound present in crab (5, 6), and the ingestion of crabs resulted in a fast excretion of this arsenical, unchanged (86, 94, 47). In contrast, the

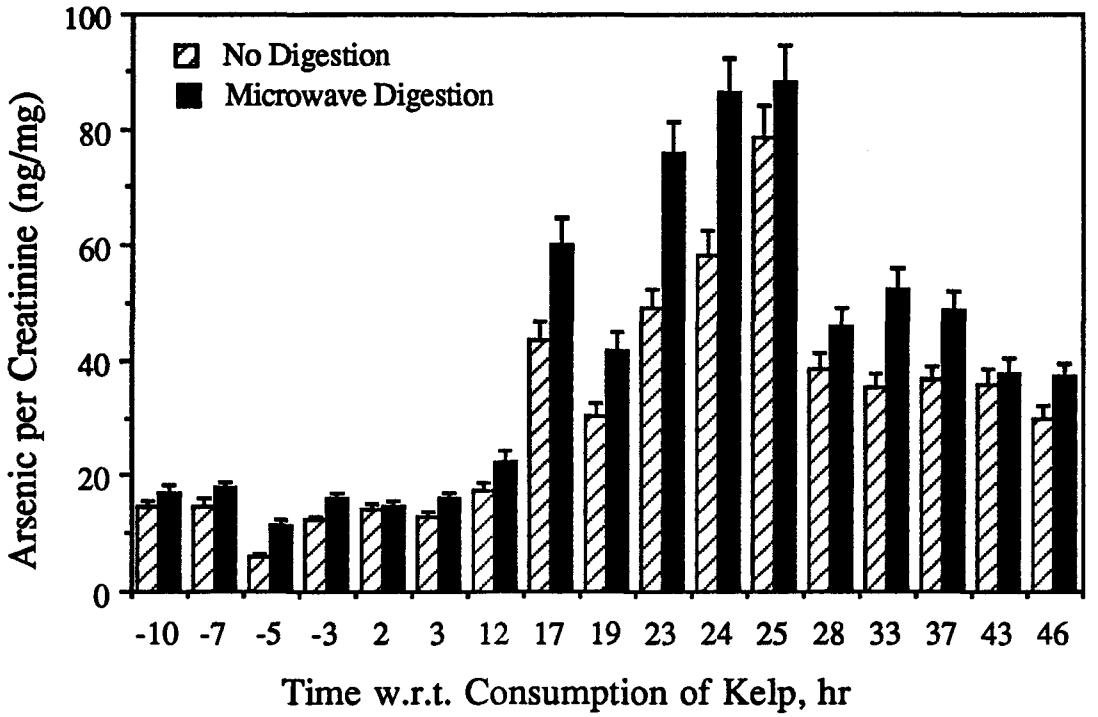
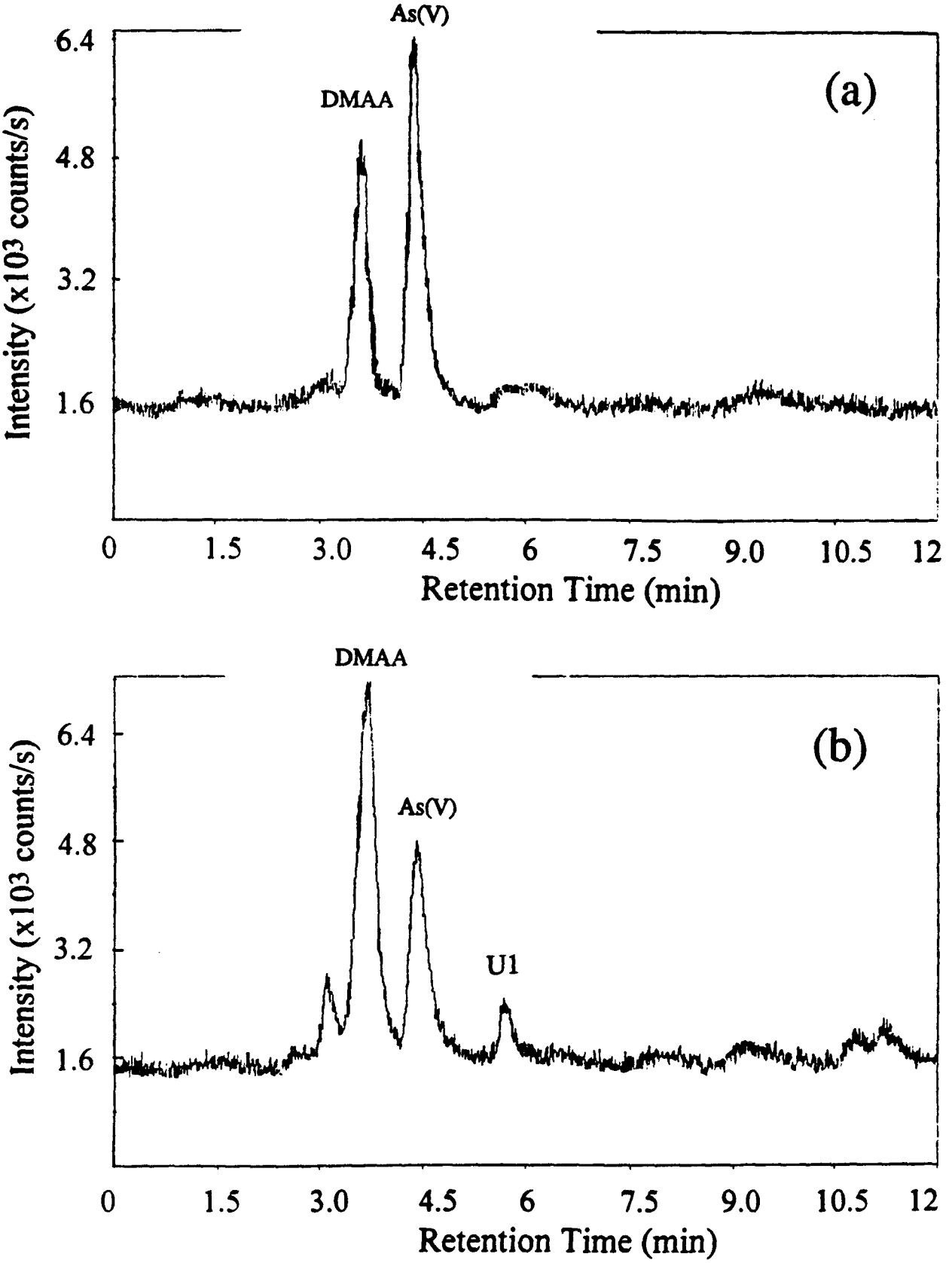


Figure 7.1. Relative concentration of arsenic in urine samples, normalized against the concentration of creatinine in the appropriate samples, collected from volunteer one prior to and following the consumption of "kelp". The determination was carried out by using FIA/HGAAS
(▨) - without digestion
(■) - with microwave assisted digestion of the sample

product "kelp", prepared from brown alga, contains arsenosugars as the major arsenic compounds (32, 36, 37). Analysis of a kelp sample extract by using HGAAS gives an arsenic concentration of 1.9 $\mu g\ g^{-1}$. When the sample is analyzed following microwave assisted digestion the arsenic content is 19.6 $\mu g\ g^{-1}$. The difference between the two measurements is attributed to the presence of arsenosugars. As indicated in Chapter 6, an analysis of the extract of the powdered kelp by using HPLC/ICPMS shows the presence of three major arsenic species, arsenosugars X, XI, and possibly XIV (Figure 6.2).

The arsenic species in urine samples were also studied by using HPLC/ICPMS. Chromatograms obtained from urine samples collected 7 hr before and 12, 23, and 46 hr after the ingestion of kelp are shown in Figure 7.2(a-d). Only arsenate and DMAA are the major arsenic species in urine samples, each at approximately 5-7 ng ml^{-1} concentration level, prior to the ingestion of kelp (Figure 7.2a). After eating kelp, a small peak U1 with the same retention time as that of arsenosugar XI (approximately 5.7 min) appears in the chromatogram of the 12 hr urine sample (Figure 7.2b). In addition to this compound, two other peaks appear (at retention times approximately 9.1 and 10.8 min) in the 23 hr urine samples (Figure 7.2c). The retention times of these two peaks do not match those of any of the standard arsenic compounds currently available to us (Table 1.1). These two peaks may indicate two new arsenic species that are metabolic products from X, XI, and XIV. These new compounds are not detected in the 46 hr urine sample (Figure 7.2d).

Analysis on a 25 hr urine sample from another volunteer (volunteer 2) shows three unknown arsenic compounds (U3, U4, and U6) (Figure 7.3). However, the arsenic compound U1, probably arsenosugar XI is not observed in the urine samples from this volunteer. The difference between the two chromatograms (Figure 7.2c and Figure 7.3) suggests that the metabolism of arsenosugars in the human body could vary from person to person. Therefore, it was decided to repeat the experiment, involving a few more volunteers.


As(V)
DMAA
(a)
6.4
4.8
3.2
1.6
Intensity (x103 counts/s)
0
1.5
3.0
4.5
6
7.5
9.0
10.5
12
Retention Time (min)
DMAA
As(V)
U1
(b)
6.4
4.8
3.2
1.6
Intensity (x103 counts/s)
0
1.5
3.0
4.5
6
7.5
9.0
10.5
12
Retention Time (min)

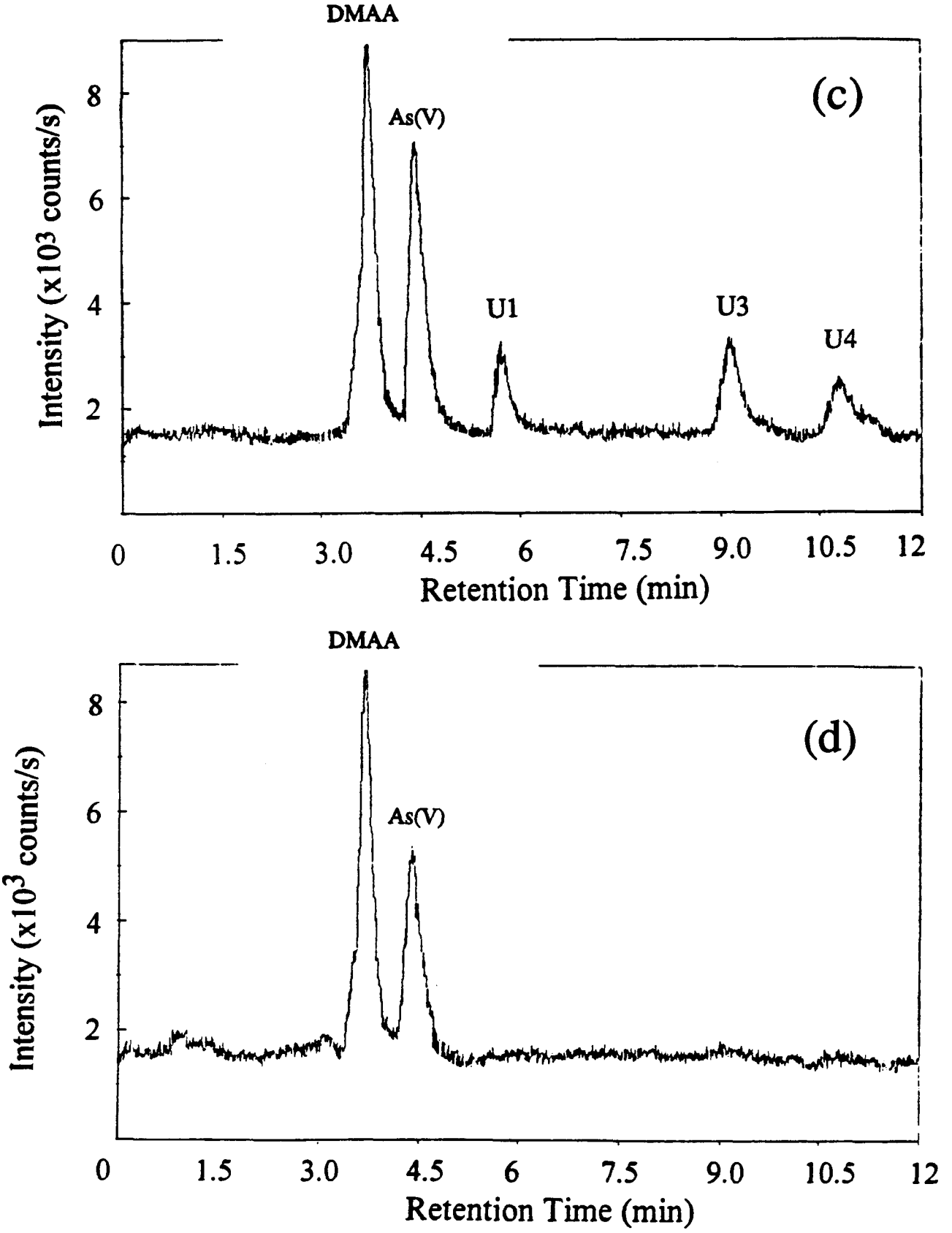


Figure 7.2. HPLC/ICPMS traces of urine samples collected 7 hr bofore (a) and 12 hr (b), 23 hr (c) and 46 hr (d) after volunteer one ingested "kelp"
HPLC column: Inertsil ODS-2 reversed phase (4.6 x 250 mm)
Eluent: 10 mM tetraethylammonium hydroxide and 4.5 mM malonic acid (pH 6.8); Flow rate: 0.8 ml min^{-1}

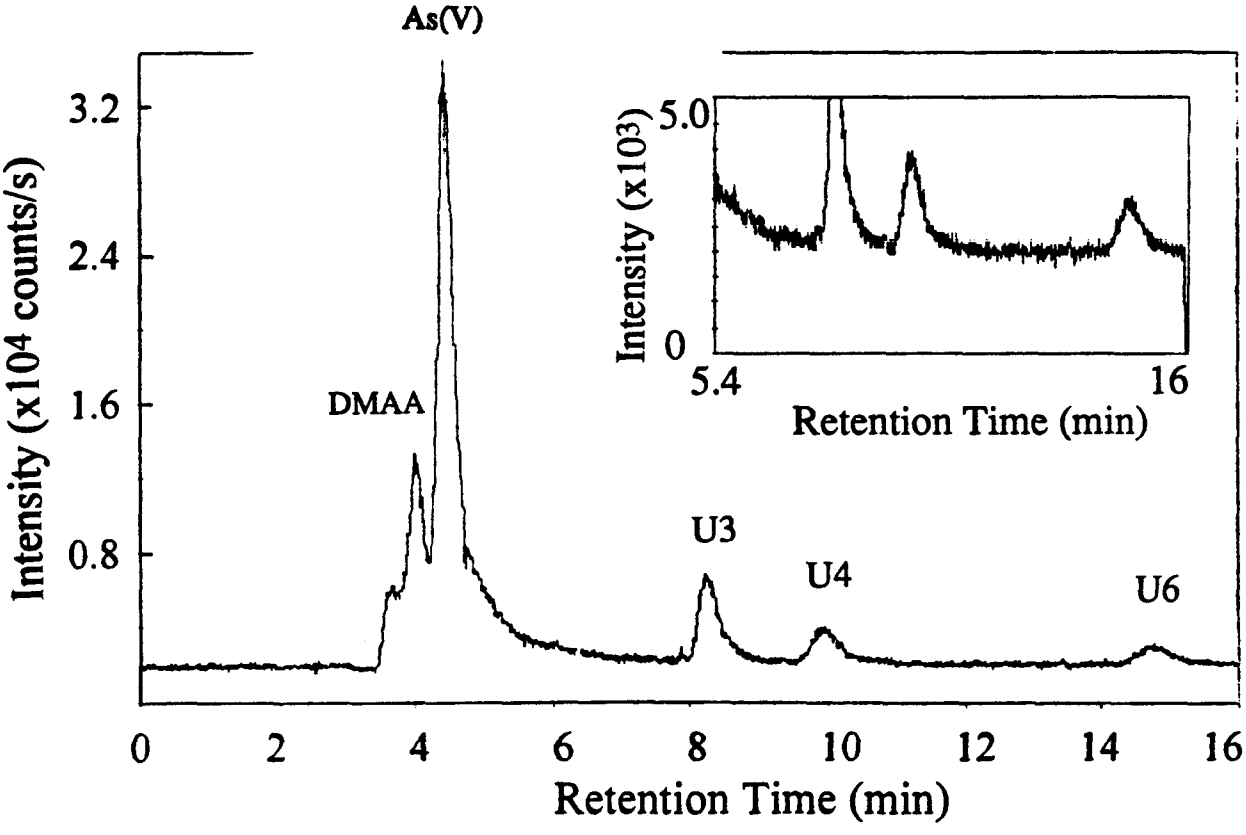


Figure 7.3. A HPLC/ICPMS trace of a urine sample collected from volunteer two 25 hr after the ingestion of "kelp"
HPLC column: Inertsil ODS-2 reversed phase (4.6 x 250 mm)
Eluent: 10 mM tetraethylammonium hydroxide and 4.5 mM malonic acid (pH 6.8); Flow rate 0.8 ml min^{-1}

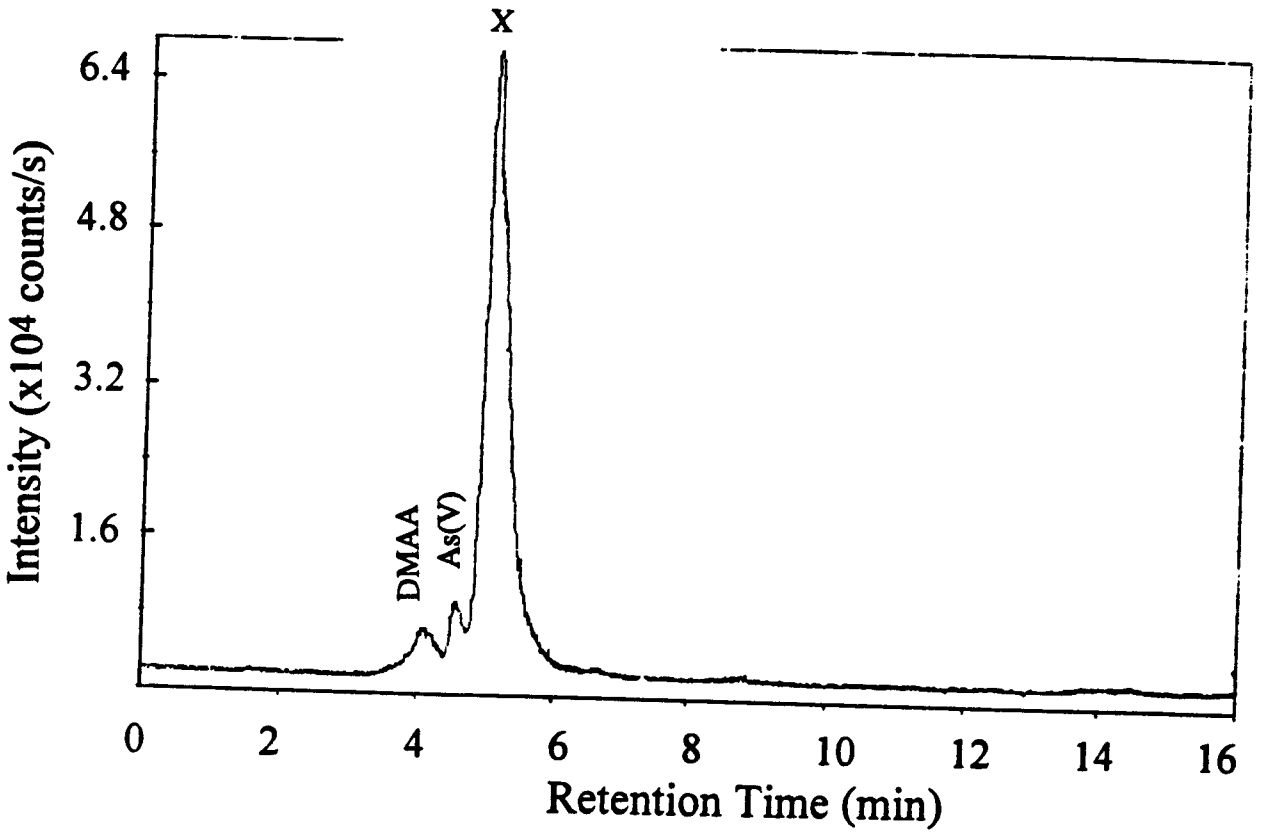


Figure 7.4. A HPLC/ICPMS trace of an extract from seaweed product Nori
Same condition as in Figure 7.3.

A small group consisting of nine volunteers was chosen so that the number of samples could be handled easily in our laboratory. For simplicity a seaweed based food product, Nori, processed from red alga was chosen because it contains a single arsenosugar, previously identified as X, as the major arsenic compound. Nori is readily available in thin sheets from local food markets. The analysis of a Nori sample extract by using HGAAS before and after microwave decomposition gives arsenic concentrations of 0.7 $\mu g\ g^{-1}$ and 21 $\mu g\ g^{-1}$, respectively. The HPLC/ICPMS trace of an extract from the same batch of Nori as used for the ingestion experiment is shown in Figure 7.4. Only one major arsenosugar, X, is present. It should be pointed out that the same analysis on another brand of Nori revealed the presence of arsenosugars X and XI. The difference in arsenic species present in Nori samples of two brand names probably arises because they were processed from different red alga species as was discussed in the previous chapter.

7.3.2. Urinary arsenic excretion after the ingestion of Nori

Each of the 9 volunteers (Table 7.1) was asked to eat approximately 9.5 g (4 sheets) of Nori (containing approximately 193 μg of arsenic as arsenosugar X), and urine samples were collected encompassing the time of ingestion. The arsenic concentration in the urine samples was determined by using HGAAS both with and without microwave decomposition of the sample, and the results are shown in Figures 7.5-7.13. It is clear that similar urinary arsenic excretion patterns are obtained from all volunteers apart from numbers 4 and 5 (Figure 7.8 and 7.9). An increase of arsenic concentration in urine is observed, and the highest concentration is found between 10 and 60 hr after eating Nori with a return to background level after approximately 80 hr. In contrast, very little change is observed in urine samples from volunteers 4 and 5 (Figures 7.8 and 7.9) even though they had ingested the same amount of Nori as the others. Only background levels of arsenic species are found in these urine samples. Both volunteers had no apparent problem in digesting the seaweed and no abnormal activities or feelings. It is interesting to note that

volunteers 1-4 are in the same family, have a very similar diet, and have similar activities. Yet their urinary arsenic excretion patterns are significantly different (Figures 7.5-7.8). These results strongly suggest that different individuals metabolize arsenosugars in different ways.

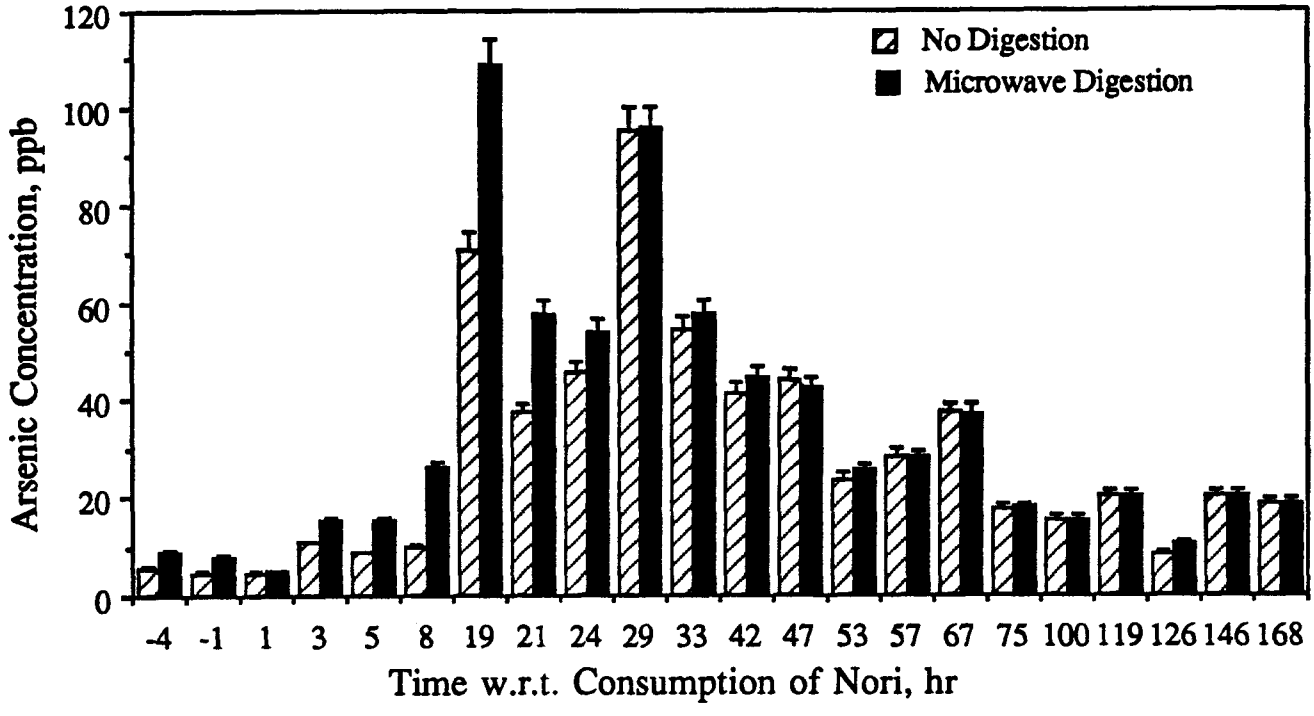


Figure 7.5. Concentration of arsenic in urine samples collected from volunteer one prior to and following the consumption of Nori, determined by using FIA/HGAAS
(▨) - without digestion
(■) - with microwave assisted digestion of the sample

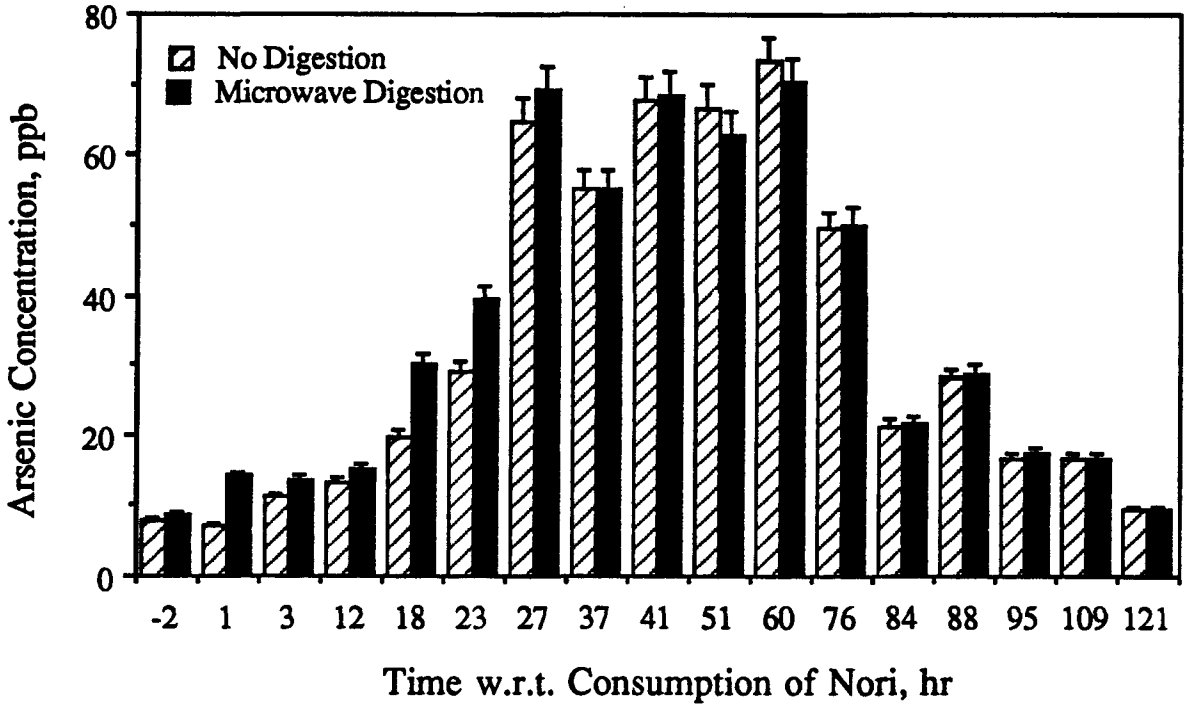


Figure 7.6. Same as in Figure 7.5 except samples from volunteer two

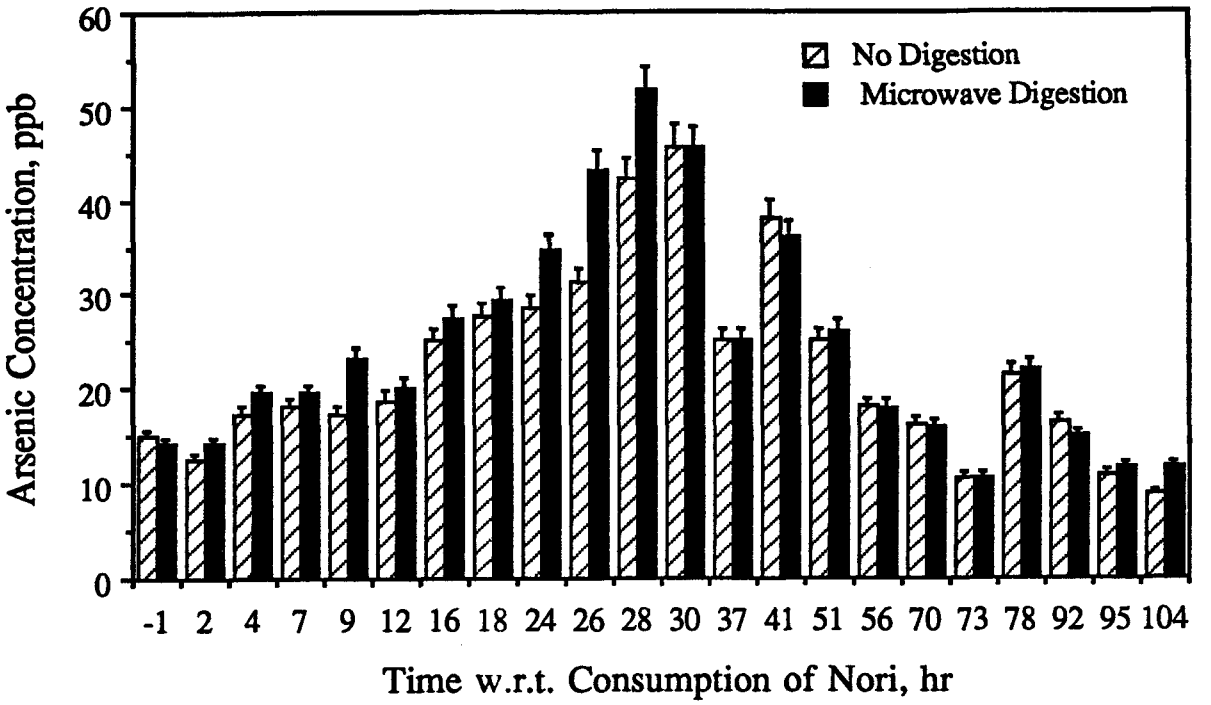


Figure 7.7. Same as in Figure 7.5 except samples from volunteer three

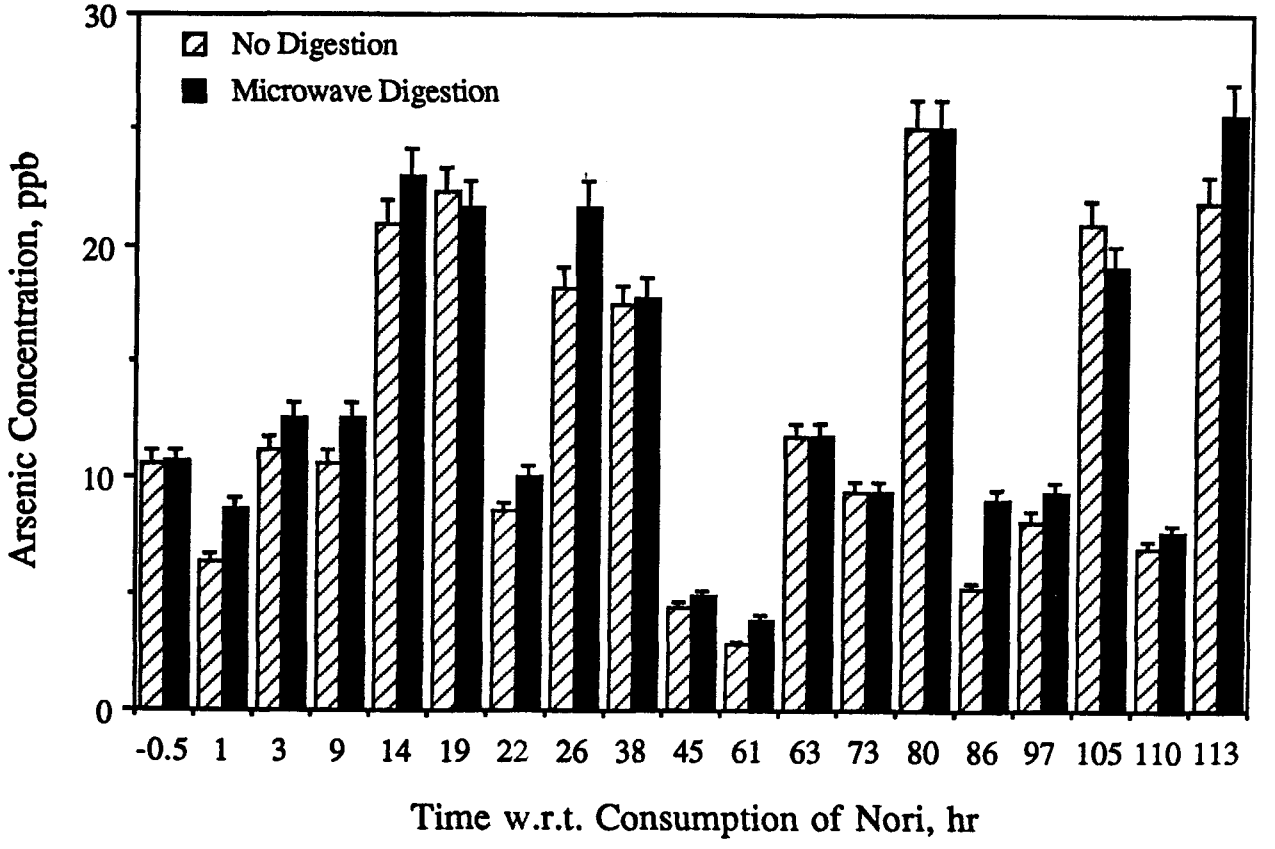


Figure 7.8. Same as in Figure 7.5 except samples from volunteer four

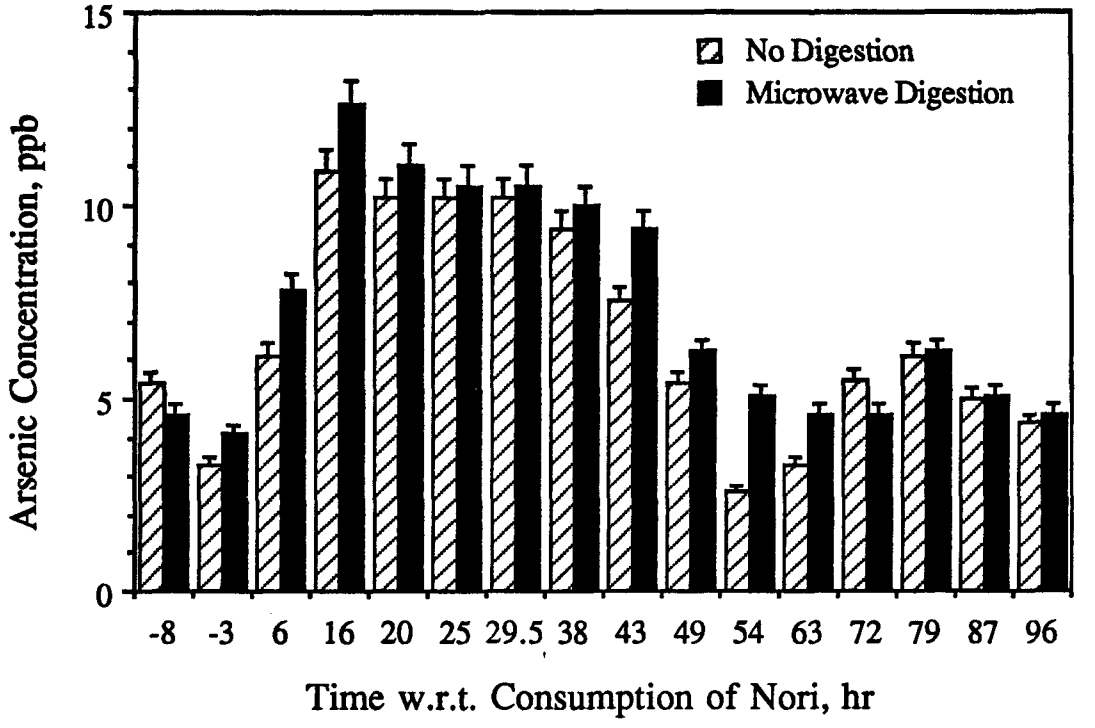


Figure 7.9. Same as in Figure 7.5 except samples from volunteer five

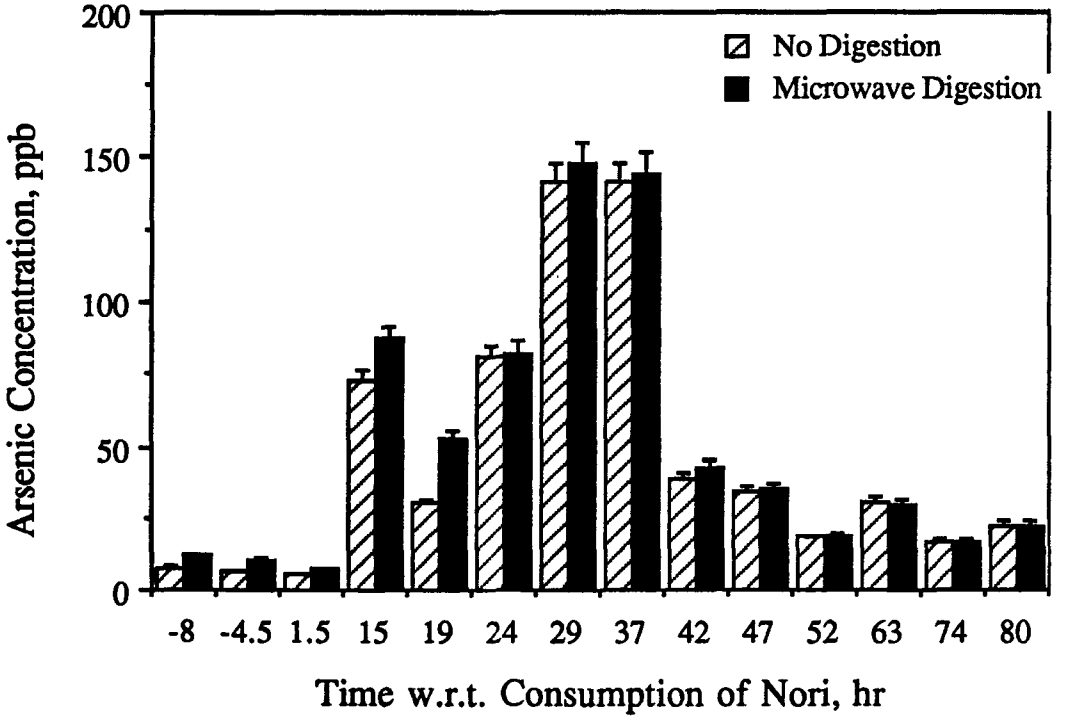


Figure 7.10. Same as in Figure 7.5 except samples from volunteer six

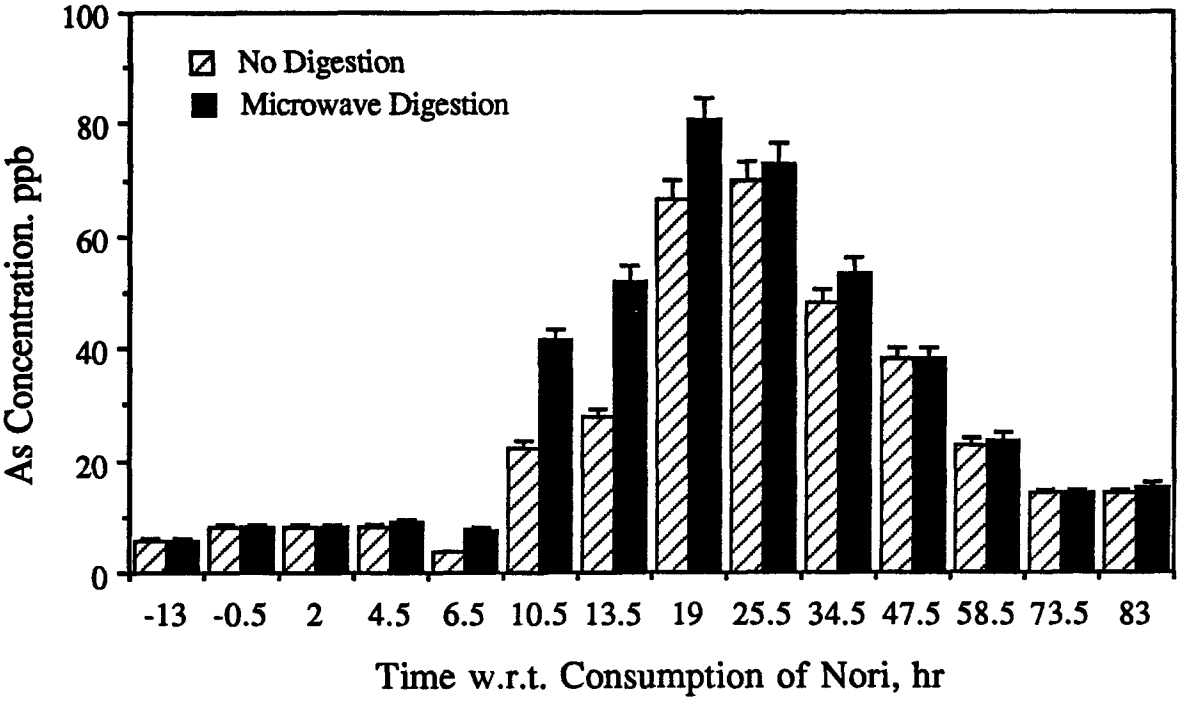


Figure 7.11. Same as in Figure 7.5 but samples from volunteer seven

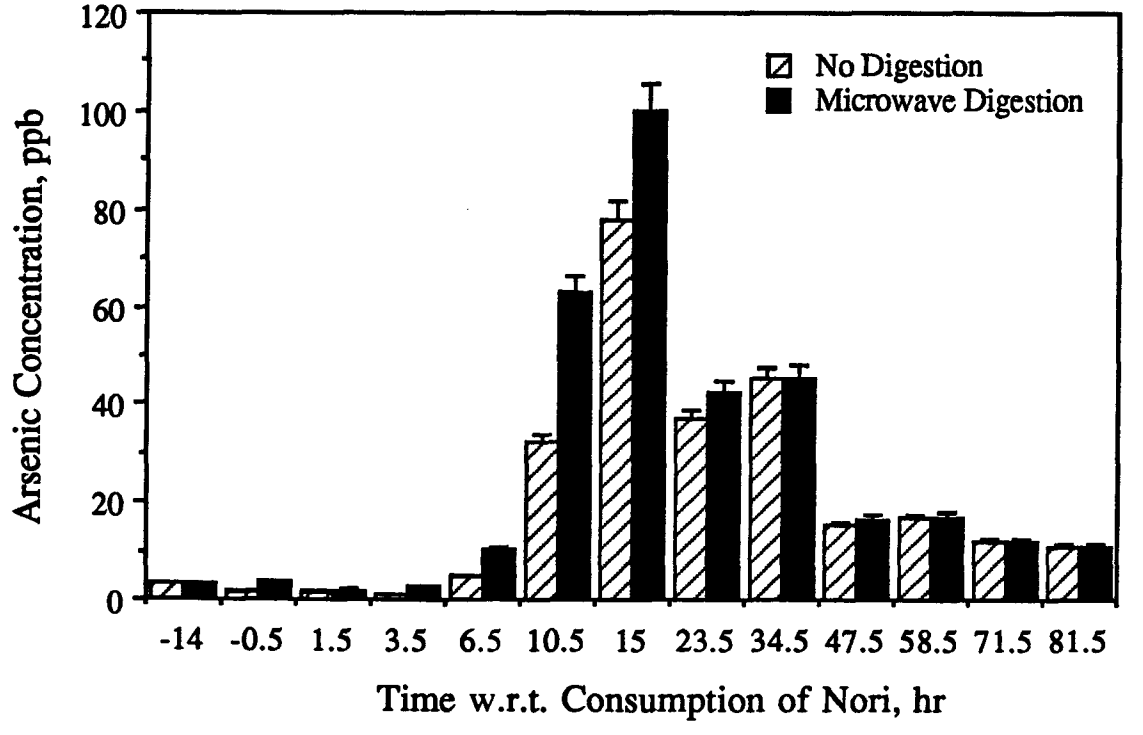


Figure 7.12. Same as in Figure 7.5 but samples from volunteer eight

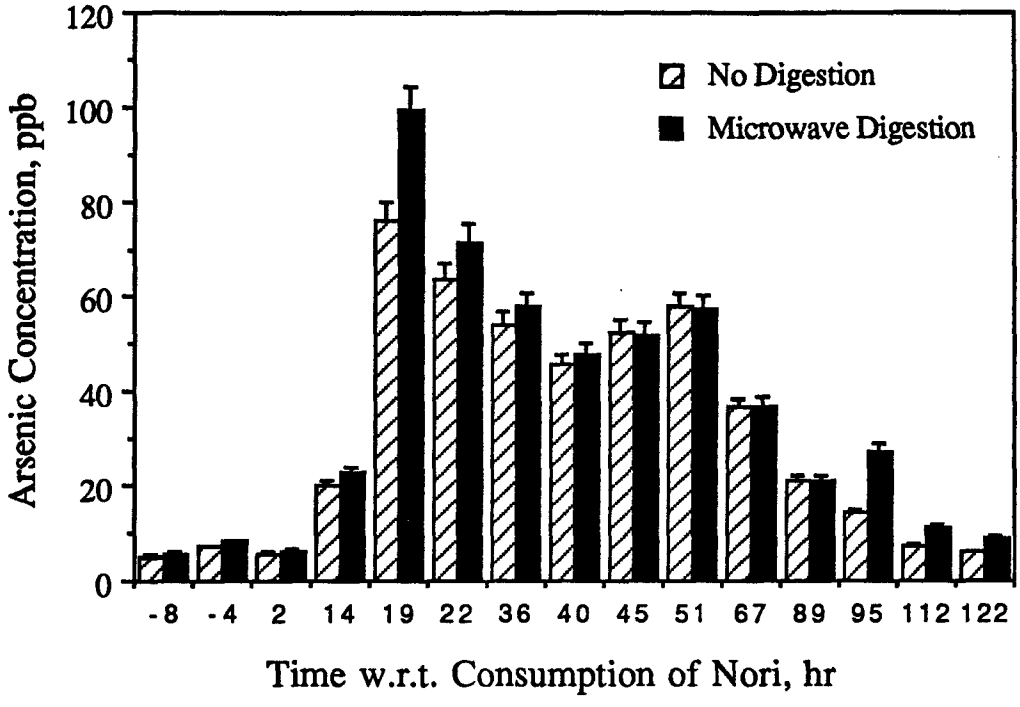


Figure 7.13. Same as in Figure 7.5 but samples from volunteer nine

To further study the excreted arsenic species, selected urine samples from each volunteer were subjected to HPLC/ICPMS analysis and the chromatograms are shown in Figure 7.14-7.23. Chromatograms obtained from urine samples collected 13 hr before (Figure 7.14a) and 13.5 hr after (Figure 7.14b) volunteer 7 ate 9.5 g of Nori are compared in Figure 7.14. It clearly shows that besides arsenate and DMAA, which are present in the background urine samples, five other arsenic compounds U1-U4 and U6, at retention

times of 5.7, 6.6, 8.2, 9.9, and 14.7 min, are now also present in the urine sample collected 13.5 hr after the ingestion of Nori. The retention time of U1 (5.7 min) is similar to that of arsenosugar XI but not X, the arsenosugar in Nori. Moreover, when another ion pair chromatographic system was used to analyze the same samples, there was no match of the retention times with the arsenosugar present in Nori (Figures 7.15, and 7.16a-16d). Therefore, the unidentified compound may be chemically modified arsenosugars. The same can be said for the four other arsenic compounds in Figure 7.14b (6.6, 8.2, 9.9 and 14.7 min) which do not match any of the eleven arsenic standards available (Table 1.1). It is confirmed that all these peaks are due to arsenic compounds and are not the result of $ArCl^+$ isobaric interference in the ICPMS measurement. A HPLC/ICPMS trace of 10 µl 3% NaCl is shown in Figure 17. Only a single peak in the void volume appears in the chromatogram probably due to $ArCl^+$. At a lower NaCl concentration, for example 0.1%, no interference peak due to $ArCl^+$ was observed.

Similarly chromatograms were obtained by analyzing urine samples from the other volunteers. The creatinine concentration in the urine samples was also determined and this was used as a reference to correct for the variation in urine volume, because creatinine has been known to be excreted at a constant rate from human body (259, 260). Concentrations of creatinine and HPLC/ICPMS peak intensities from some urine samples are summarized in Table 7.2. In addition to the appearance of various unknown metabolites U1-U6, the DMAA concentration is also significantly increased in urine samples collected after the ingestion of Nori. Figures 7.18 and 7.19 summarize detailed results on the speciation of arsenic in two sets of urine samples from volunteer 1 who ingested, in two separate experiments, "kelp" and Nori. The relative intensity shown in both Figures was obtained by normalizing the intensity of each chromatographic peak from a urine sample against the concentration of creatinine in the corresponding urine sample.

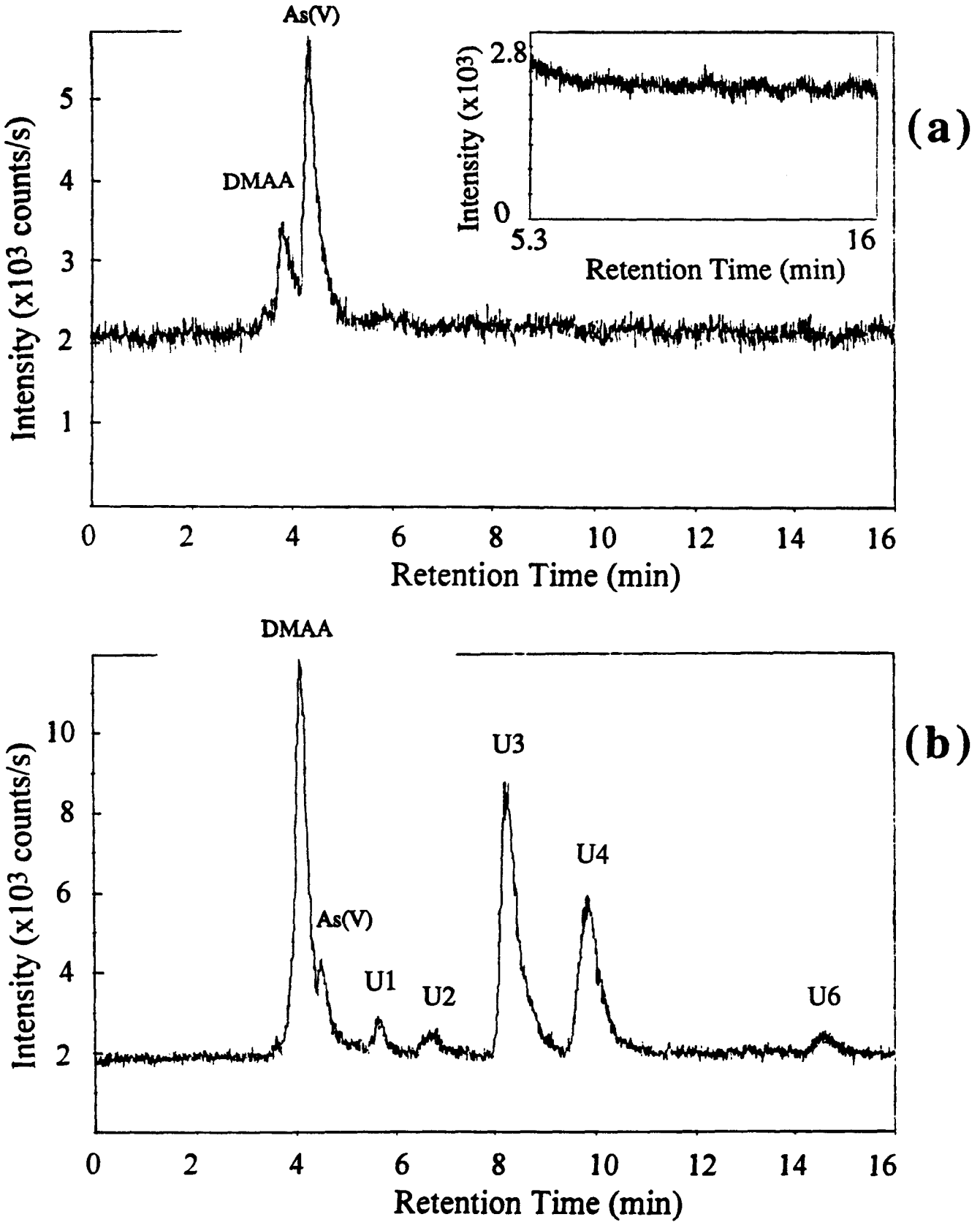


Figure 7.14. HPLC/ICPMS chromatograms of urine samples from volunteer seven 13 hr before (a) and 13.5 hr after (b) the ingestion of 9.5 g of Nori
Same conditions as in Figure 7.3.

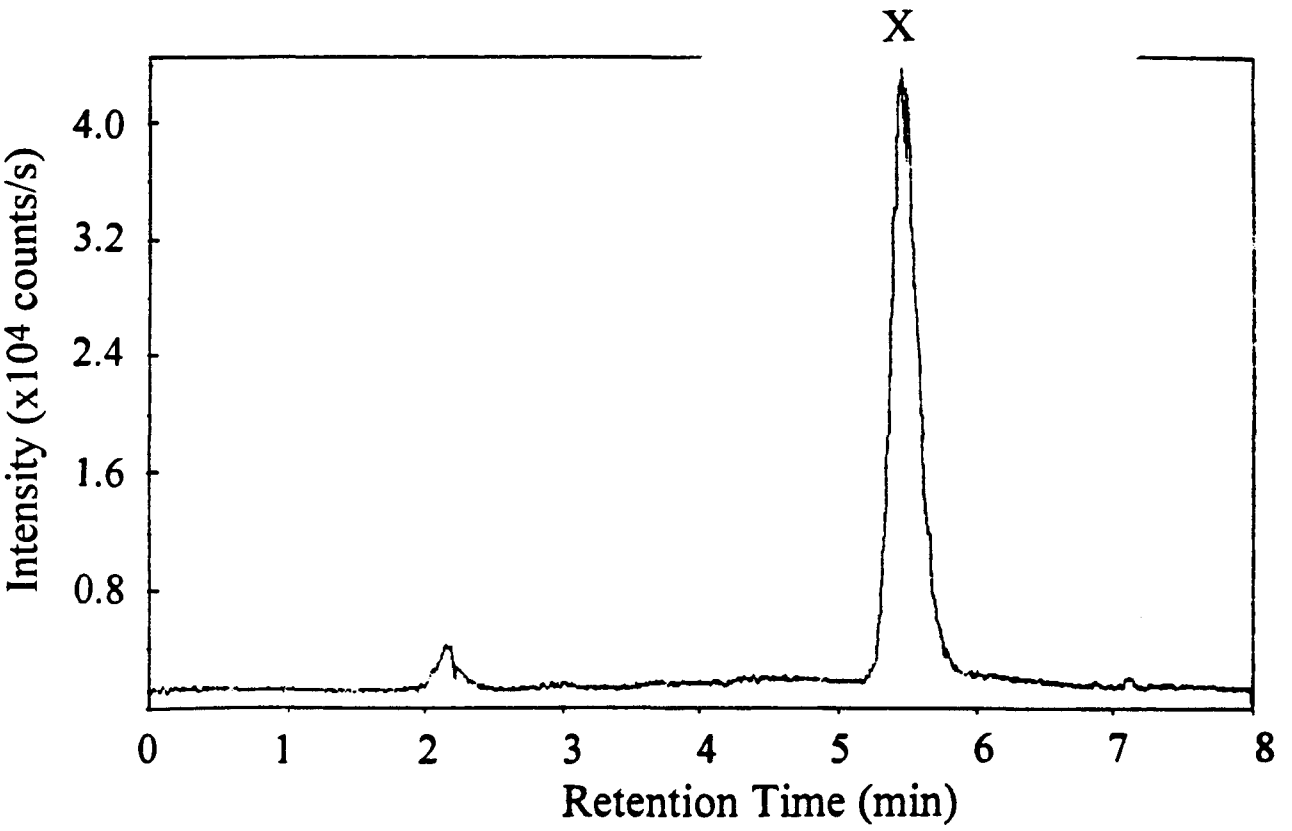
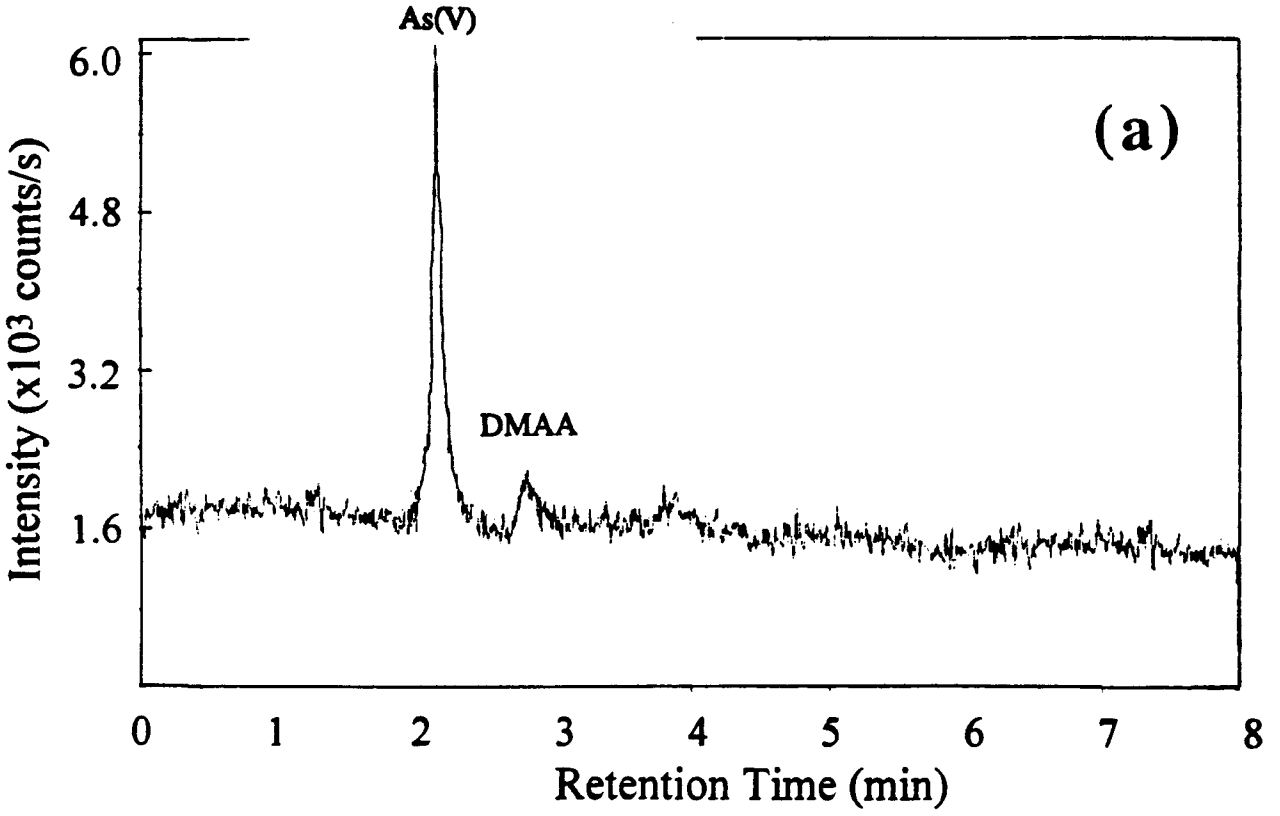
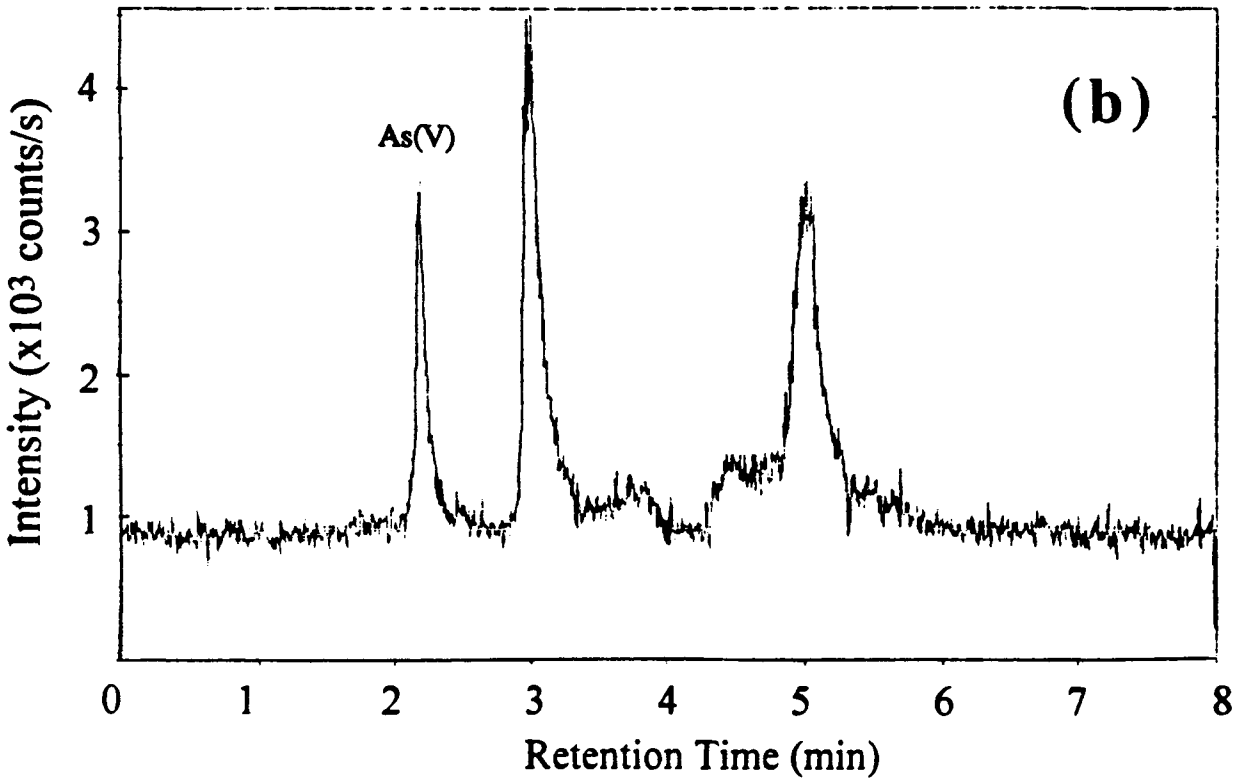


Figure 7.15. A HPLC/ICPMS trace of a Nori sample extract
HPLC column: mBondclone C18 (3.9 x 300 mm, Phenomenex)
Eluent: 10 mM sodium heptanesulfonate and 4 mM tetramethylammonium hydroxide (pH 3.5); Flow rate 1 ml min^{-1}


As(V)
DMAA
(a)
Intensity (x10³ counts/s)
6.0
4.8
3.2
1.6
0
1
2
3
4
5
6
7
8
Retention Time (min)


As(V)
(b)
Intensity (x10³ counts/s)
4
3
2
1
0
1
2
3
4
5
6
7
8
Retention Time (min)

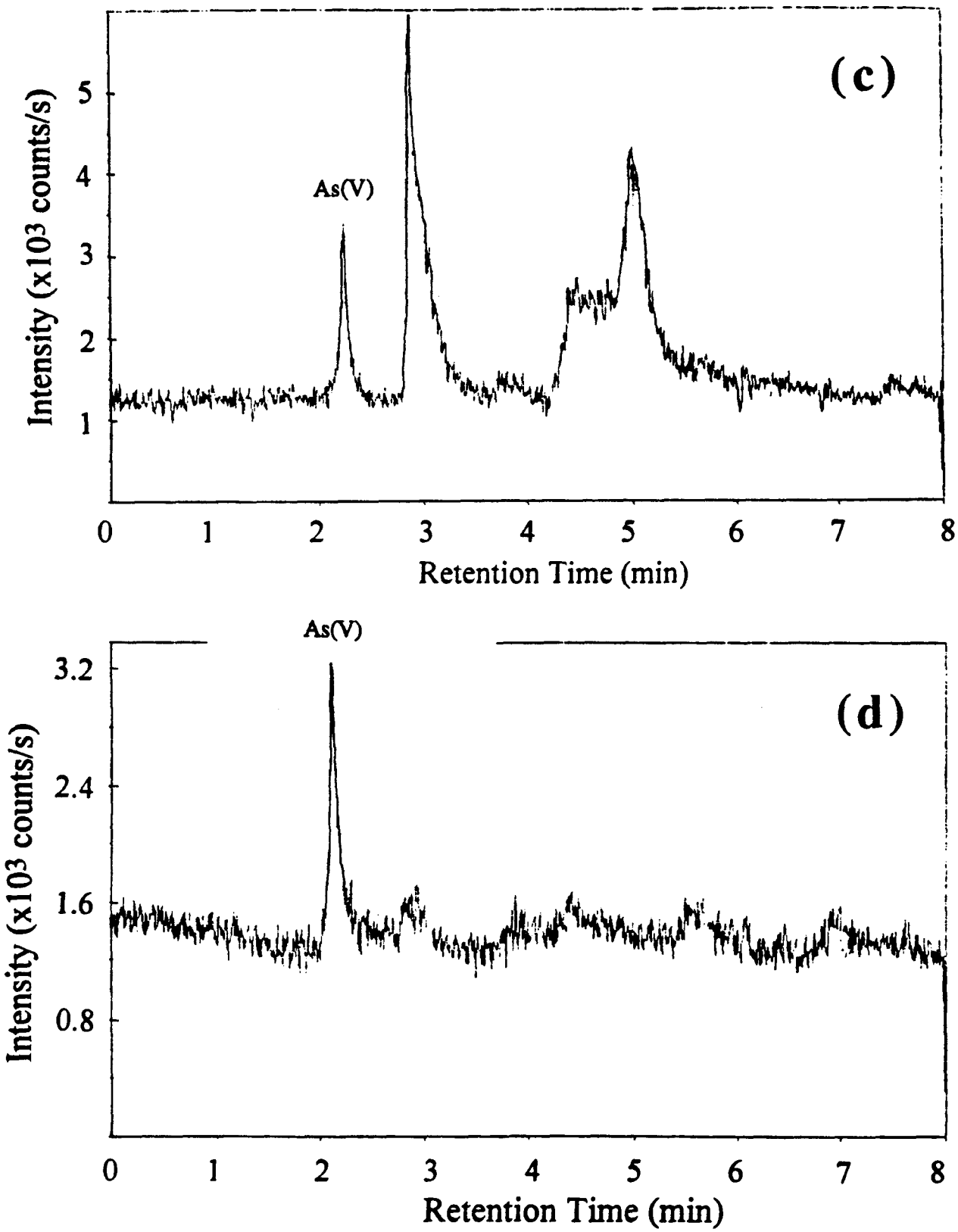


Figure 7.16. Chromatograms of urine samples from volunteer seven 13 hr before (a) and 13.5 hr (b), 19 hr (c), and 58.5 hr (d) after the ingestion of Nori
Same conditions as in Figure 7.15.

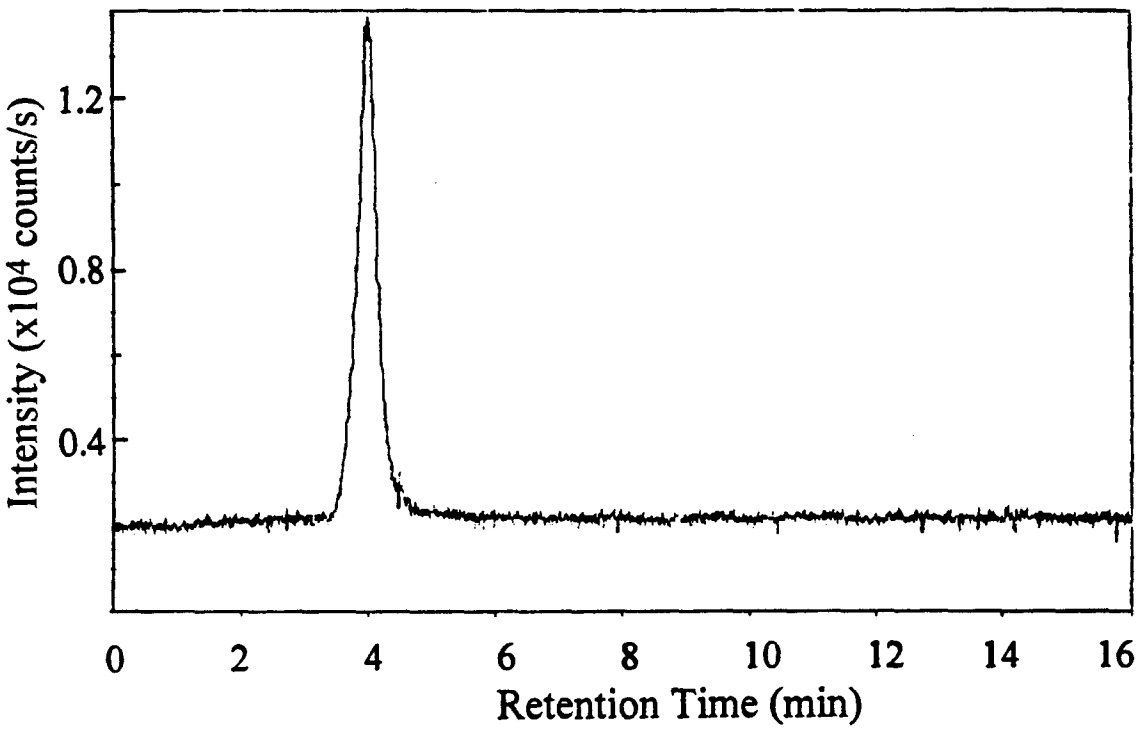


Figure 7.17. A HPLC/ICPMS trace of 10 µl 3% NaCl solution

Same conditions as in Figure 7.3.

Table 7.2. Creatinine concentration and relative HPLC/ICPMS peak intensity of arsenic compounds present in the selected urine samples

Retention Time:				5.7	6.6	8.2	9.9	13.0	14.7	Creatinine
As Compds:		DMAA	As(V)	U1	U2	U3	U4	U5	U6	Conc.
#	Time(hr)									($g\ L^{-1}$)
2	18	5	3.5	1	x	1.6	1	x	0.2	-
	27	12	8	x	x	0.4	x	x	x	1.11
3	26	12	6	x	x	2	1.6	x	0.5	0.96
4	19	8	7	1	x	x	x	x	x	-
	26	8	8	0.5	x	x	x	x	x	0.94
5	16	8	10	0.5	x	x	x	x	x	-
	29.5	7	10	x	x	x	x	x	x	2.1
6	-8	16	8	x	x	x	x	x	x	1.4
	15	43	10	0.2	0.2	2.4	1.4	x	0.8	-
	29	40	12	0.4	0.4	6.4	2	0.4	0.6	1.0
7	-13	3.5	2	x	x	x	x	x	x	0.50
	13.5	10	2	0.8	0.6	7	4	x	0.3	0.73
	19	8	u	1.5	x	9.5	3.5	x	0.5	1.6
	34.5	8	u	0.4	x	3.5	0.5	x	0.2	-
8	-14	1	3.5	x	x	x	x	x	x	1.0
	15	38	10	1.2	0.8	25	8	x	1.2	1.0
	23.5	7	7	4	x	14	2	x	0.5	-
9	19.5	34	22	0.2	0.5	12	8	0.1	0.8	1.2

x: undetected -: not determined u: unresolved from DMAA peak

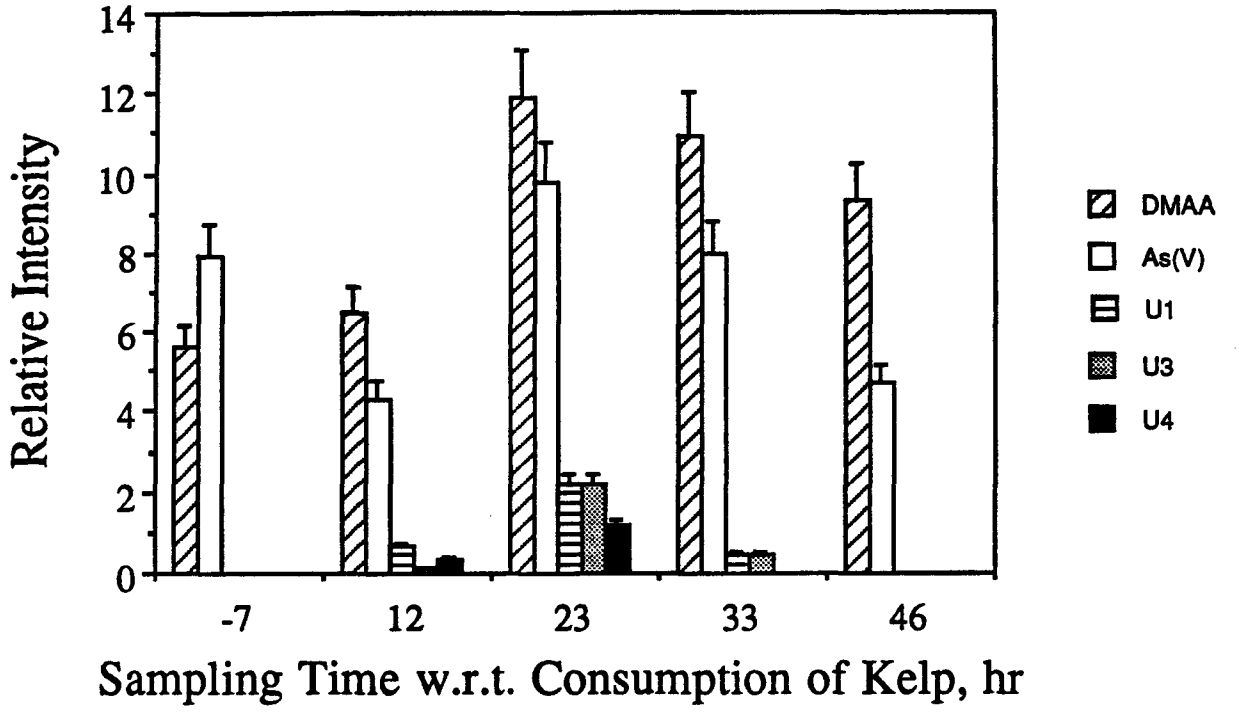


Figure 7.18. Relative intensity of chromatographic peaks of various arsenic species in urine samples from volunteer one encompassing the consumption of "kelp"

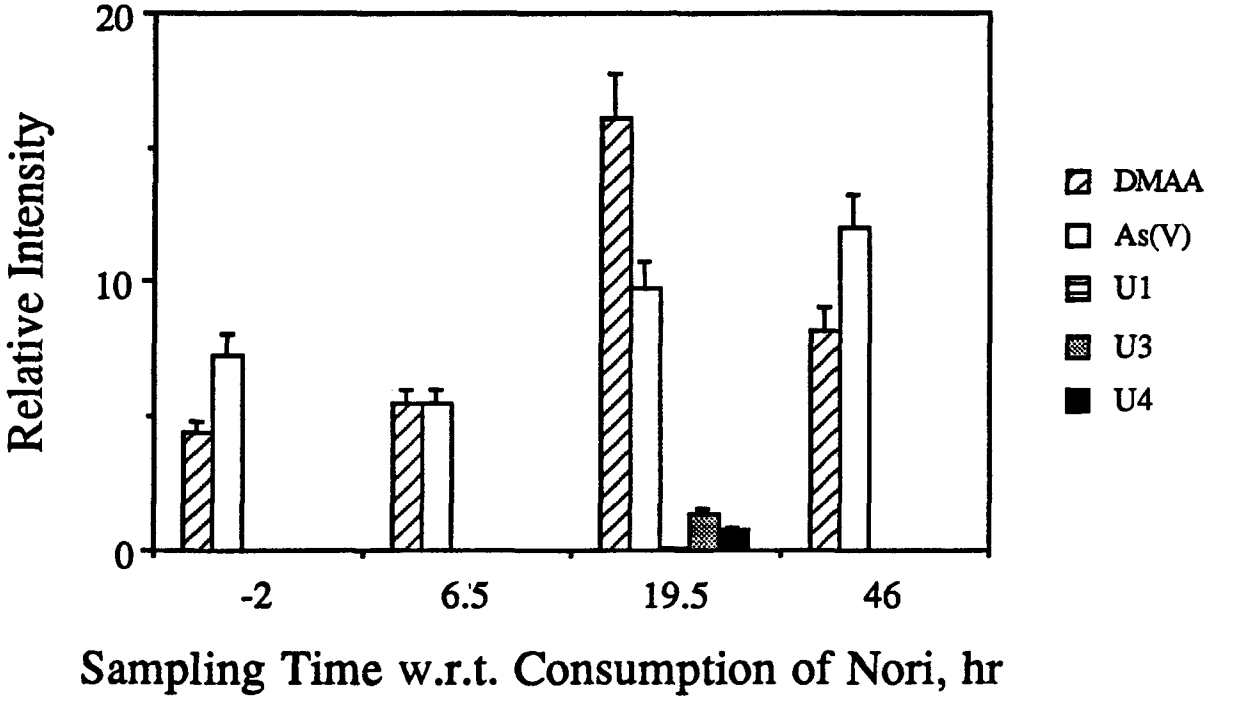


Figure 7.19. Relative intensity of chromatographic peaks of various arsenic species in urine samples from volunteer one encompassing the consumption of Nori

Figures 7.20-7.26 along with Figures 7.2 and 7.14 show chromatograms obtained by analyzing urine samples from the 9 volunteers. Up to six arsenic species (U1-U6) at retention times 5.7, 6.6, 8.2, 9.9, 13.0, and 14.7 min in addition to arsenate and DMAA are present in urine samples of these volunteers. Two members of a family, volunteers 7 and 8, each excreted 5 metabolites into their urine samples following the ingestion of Nori (Figures 7.14b and 7.25). On the other hand, volunteers 1-4 excreted 3, 4, 3, and 1 metabolite(s), respectively (Figures 7.2c and 7.20-7.22), although all these 4 volunteers are from the same family and have a similar diet. Furthermore, volunteers 5 and 6, both female and 20 years of age, also showed different metabolic behaviors towards arsenosugars. None of the arsenic metabolites were detected in the 29.5 hr urine sample of volunteer 5 (Figure 7.23), whereas 6 metabolite arsenic species were present in the 29 hr urine of volunteer 6 (Figure 7.24). These results are consistent with those obtained from the time course studies (Figures 7.5-7.13) and support the contention that the ability to metabolize arsenosugars varies from individual to individual.

7.3.3. Urinary arsenic excretion after the ingestion of crabs and shrimps

Because individuals, volunteers 4 and 5 in particular, showed different metabolic behavior toward arsenosugars, it was decided to establish if this difference applied to arsenobetaine, the main arsenical found in crabs and shrimps. Six volunteers (1-6 as shown in Table 7.1) were chosen for this study; of particular interest was a comparison between volunteers 1-3 and 4, and between 5 and 6.

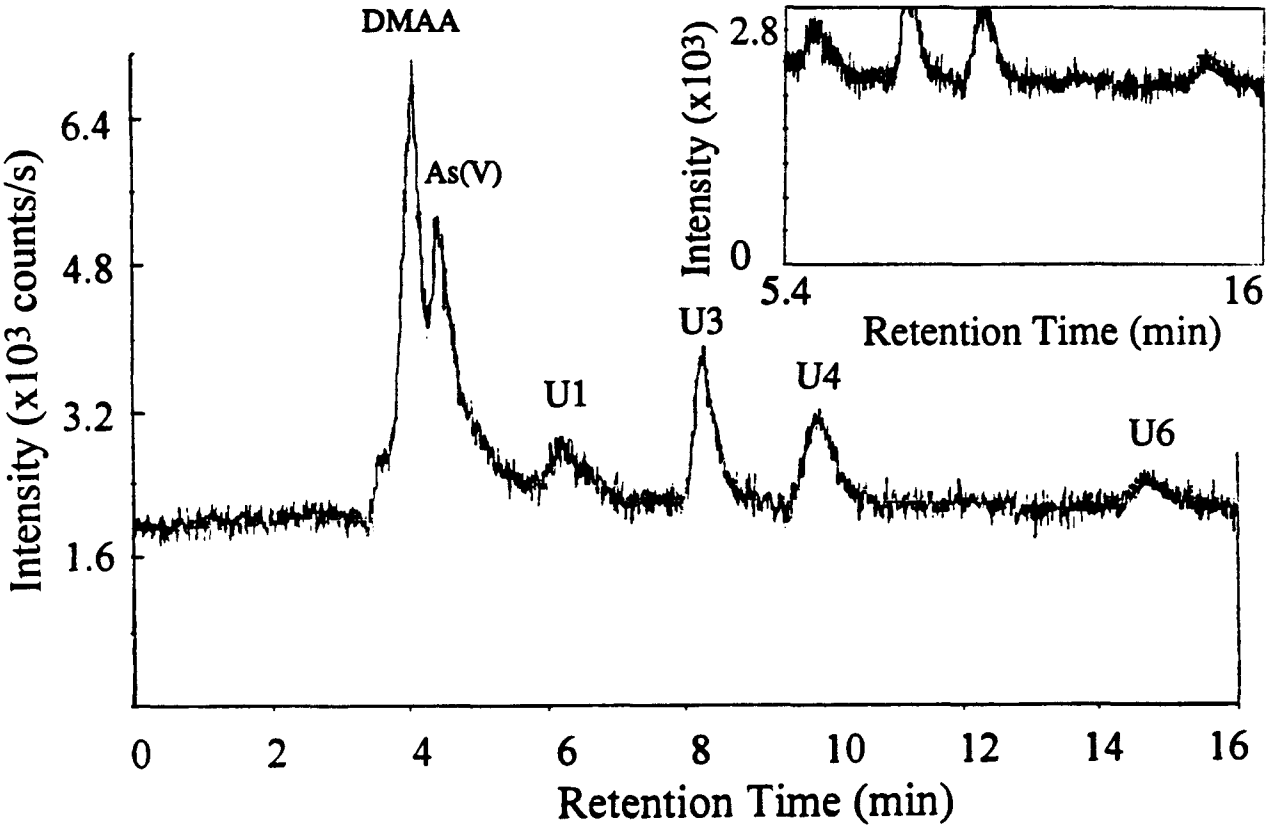


Figure 7.20. A HPLC/ICPMS trace of a urine smaple from volunteer two 18 hr after the ingestion of 9.5 g of Nori

Same conditions as in Figure 7.3.

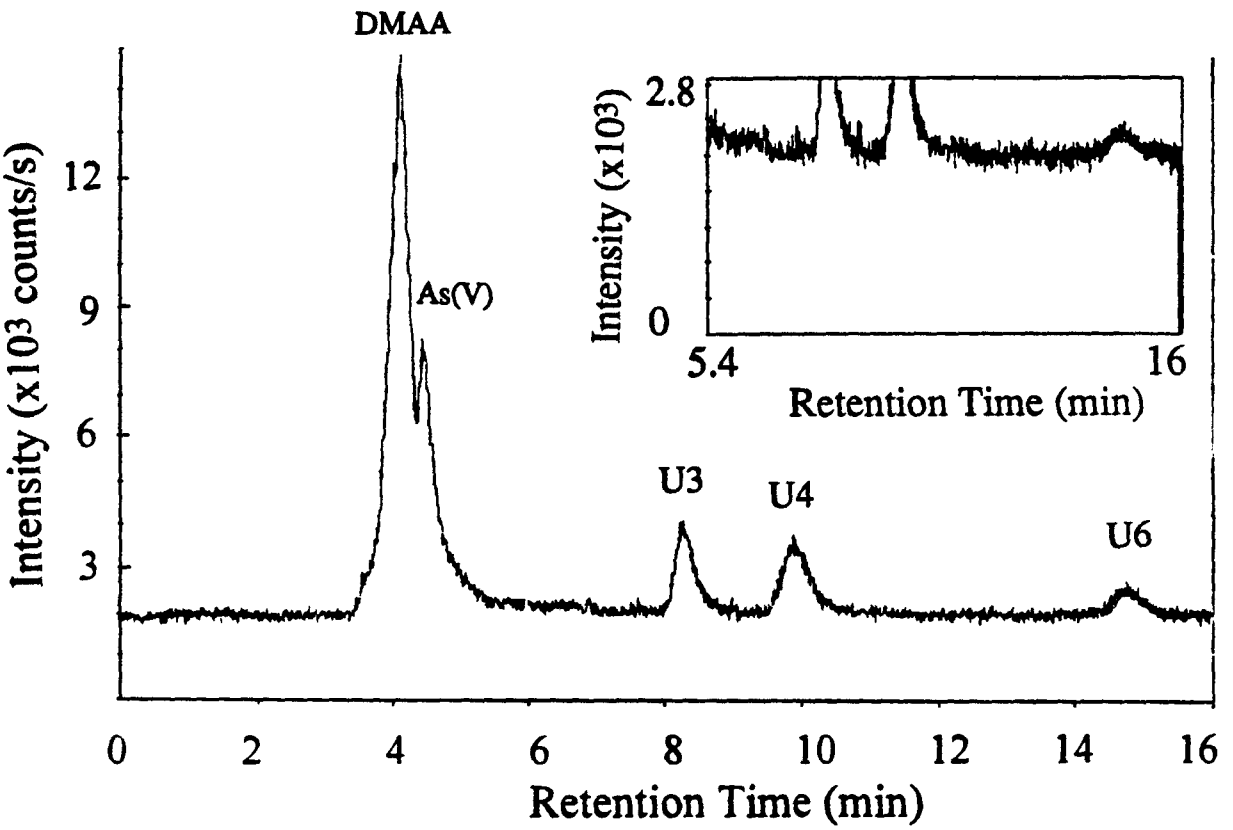


Figure 7.21. A HPLC/ICPMS trace of a urine sample from volunteer three 26 hr after the ingestion of 9.5 g of Nori. Same conditions as in Figure 7.3.

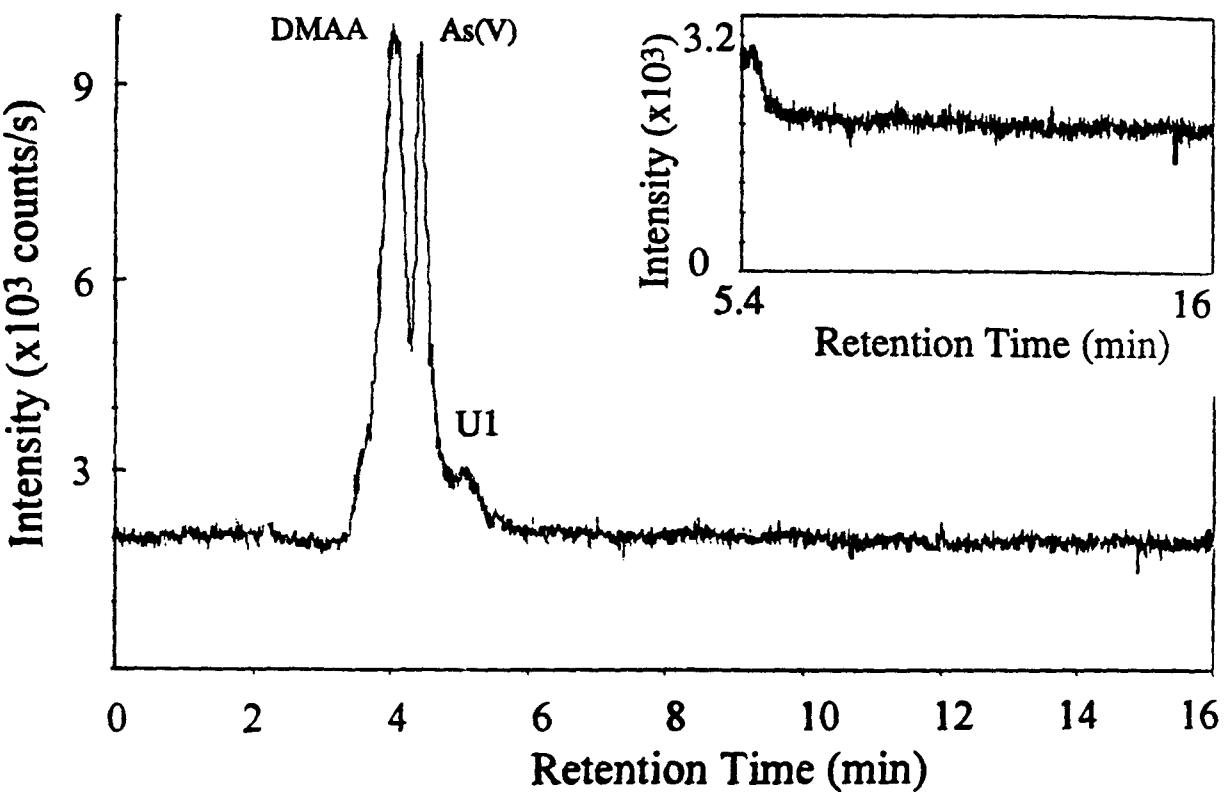


Figure 7.22. A HPLC/ICPMS trace of a urine sample from volunteer four 26 hr after the ingestion of 9.5 g of Nori. Same conditions as in Figure 7.3.

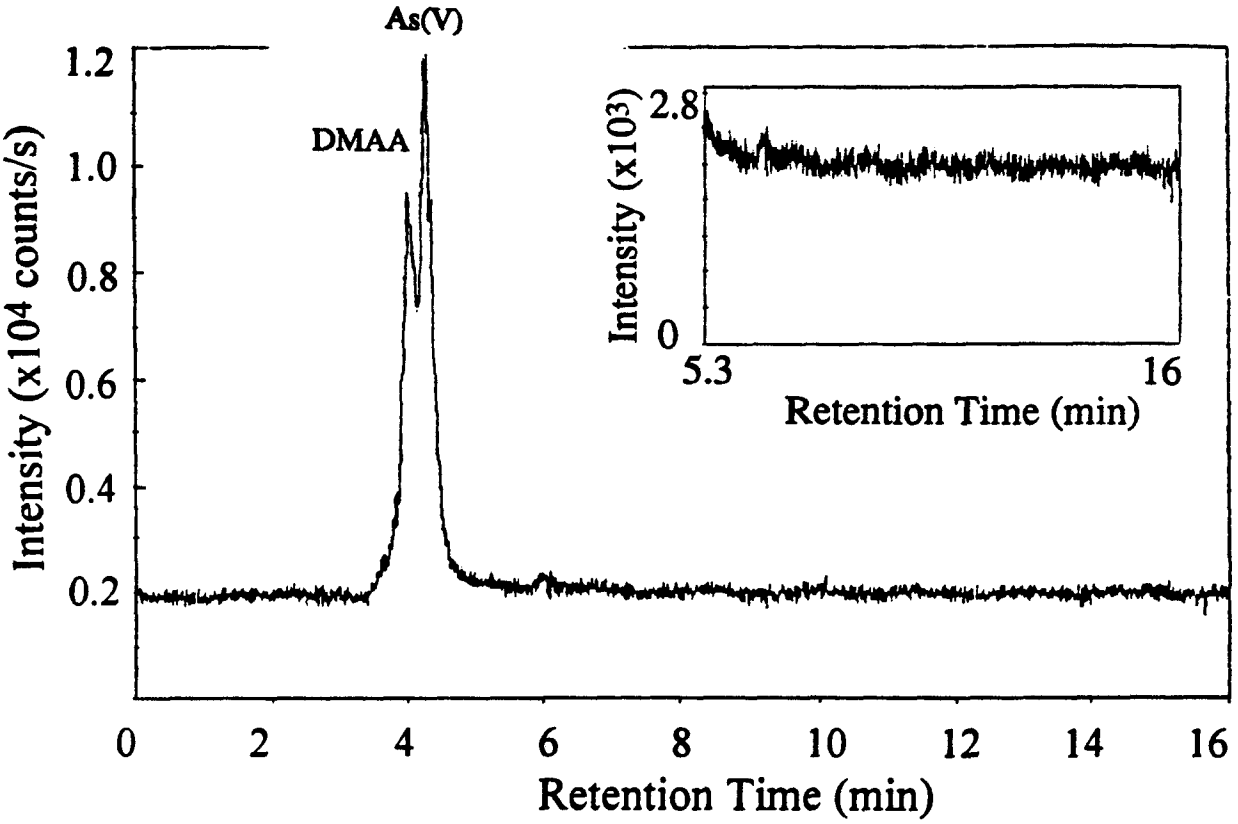


Figure 7.23. A HPLC/ICPMS trace of a urine sample from volunteer five 29.5 hr after the ingestion of 9.5 g of Nori. Same conditions as in Figure 7.3.

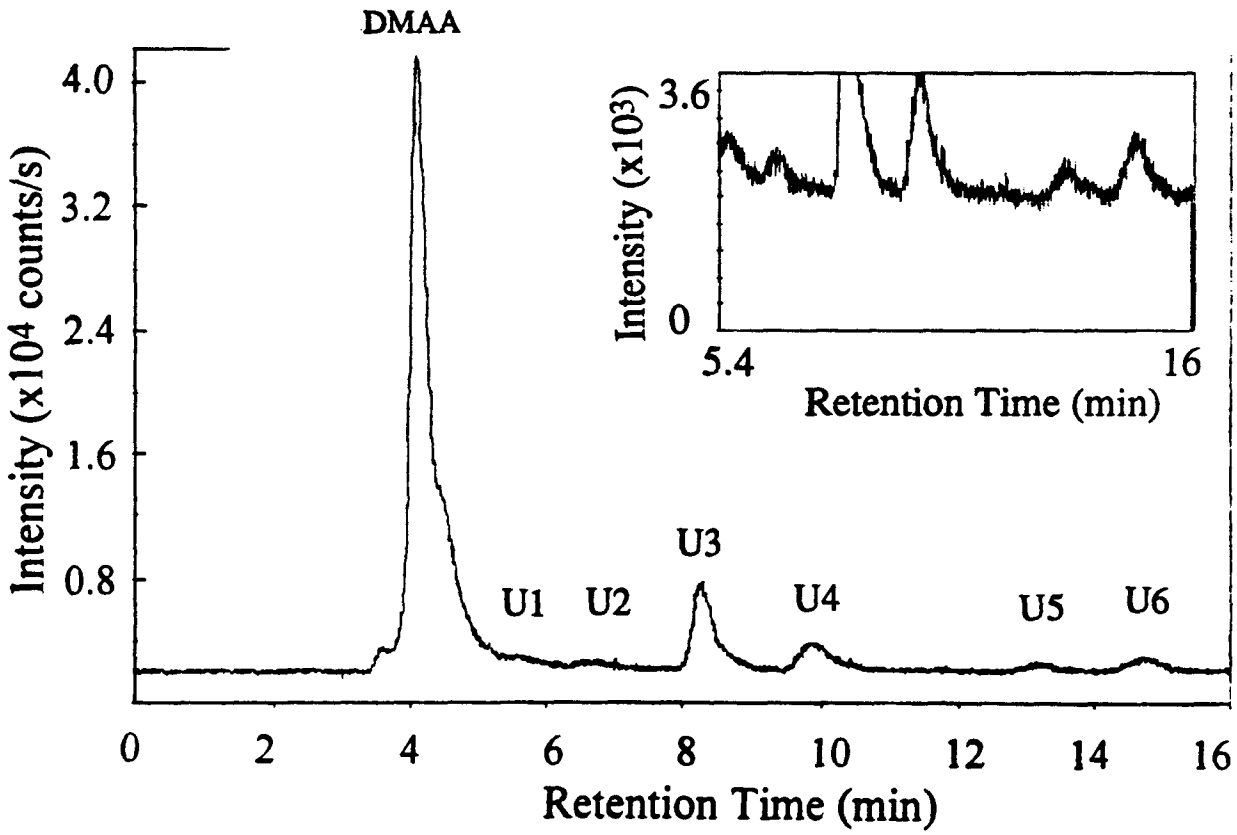


Figure 7.24. A HPLC/ICPMS trace of a urine sample from volunteer six 29 hr after the ingestion of 9.5 g of Nori. Same conditions as in Figure 7.3.

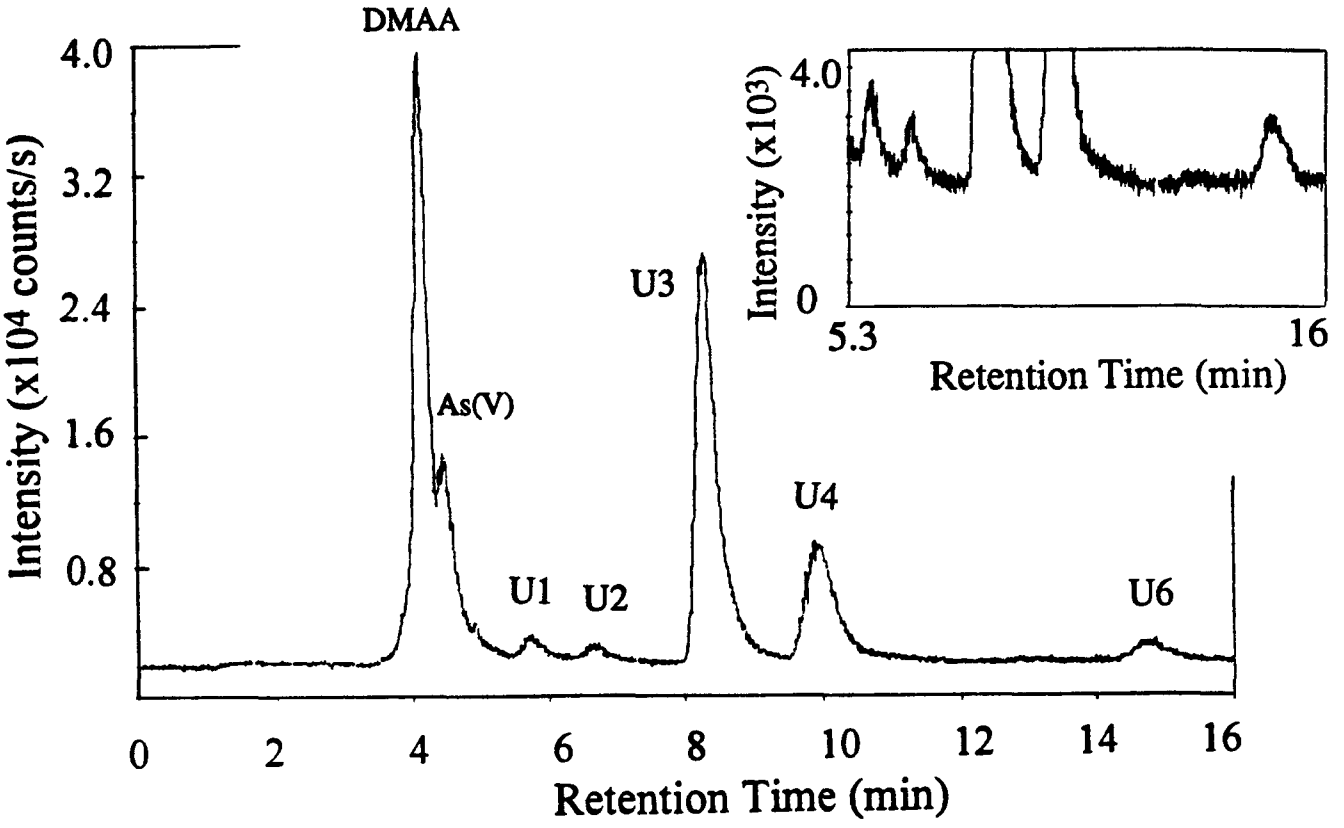


Figure 7.25. A HPLC/ICPMS trace of a urine sample from volunteer eight 15 hr after the ingestion of 9.5 g of Nori. Same conditions as in Figure 7.3.

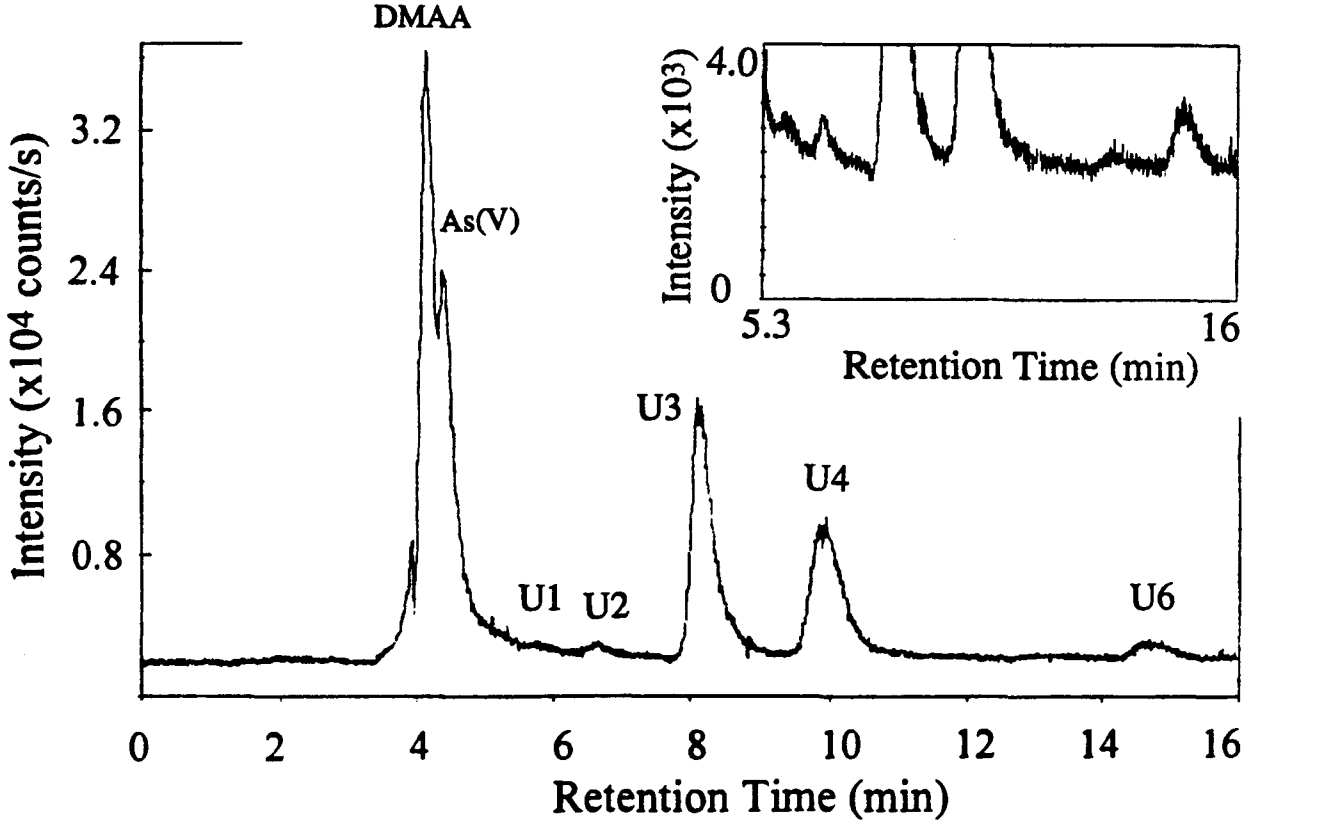


Figure 7.26. A HPLC/ICPMS trace of a urine sample from volunteer nine 19 hr after the ingestion of 9.5 g of Nori. Same conditions as in Figure 7.3.

Figures 7.27 and 7.28 show urinary arsenic excretion patterns from volunteers 3 and 4 after the ingestion of some crab meat (not the same amount). A fast excretion of arsenic compounds from the body is observed, and both volunteers clearly show a similar arsenic excretion pattern.

HPLC/ICPMS analysis indicates that arsenobetaine is excreted unchanged following the ingestion of crab. As an example, Figures 7.29 and 7.30 show the chromatograms obtained from the 26 hr urine sample (volunteer 3) and the 28 hr sample (volunteer 4), respectively. Figure 7.31 is a chromatogram of a crab meat extract showing arsenobetaine as the dominant arsenic compound. It is clear that arsenobetaine is the major arsenic compound in urine samples from both volunteers 3 and 4.

Similarly, arsenobetaine was excreted unchanged after volunteers 1, 2, 5 and 6 ingested shrimps. Examples are shown in Figures 7.32 and 7.33 and similar arsenic excretion patterns are obtained. These results are in clear contrast to those on urinary arsenic excretion patterns following the ingestion of Nori by the same volunteers as discussed previously (comparing Figures 7.7 with 7.8, and 7.9 with 7.10).

7.3.4. Percentage of excreted arsenobetaine over the total intake

In order to carry out a quantitative study, volunteers 1 and 2 were each instructed to eat a known amount of shrimps in one meal. All their urine samples were collected for 3 days following the meal, and the volume of each sample was measured. The arsenobetaine concentration in both shrimp and urine samples were determined, and the amounts of intake and excretion of arsenobetaine were obtained. For volunteer 1 who ingested 163 μg of arsenobetaine from the shrimp, the excreted arsenobetaine amounted to 120 μg (73% of the total intake) within 37 hr following the ingestion. Out of the 415 μg of arsenobetaine ingested by volunteer two, 274 μg (66%) was excreted as arsenobetaine within 37 hr. These results confirm that urinary excretion is the major pathway for the elimination of arsenobetaine from the human body.

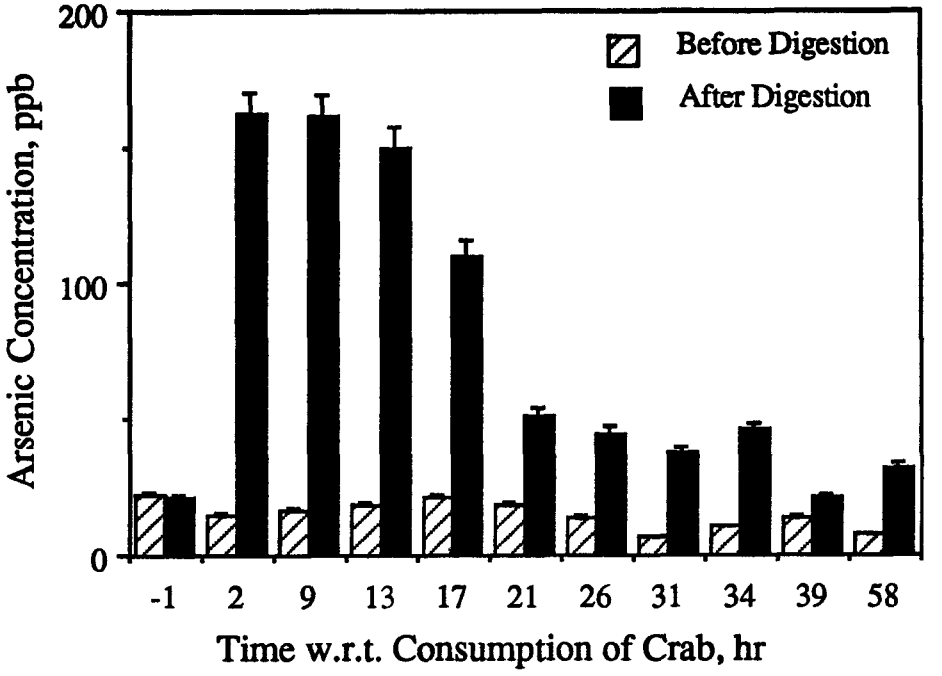


Figure 7.27. Concentration of arsenic in urine samples collected from volunteer three prior to and following the consumption of crab, determined by using FIA/HGAAS
(▨) - without digestion
(■) - with microwave assisted digestion of the sample

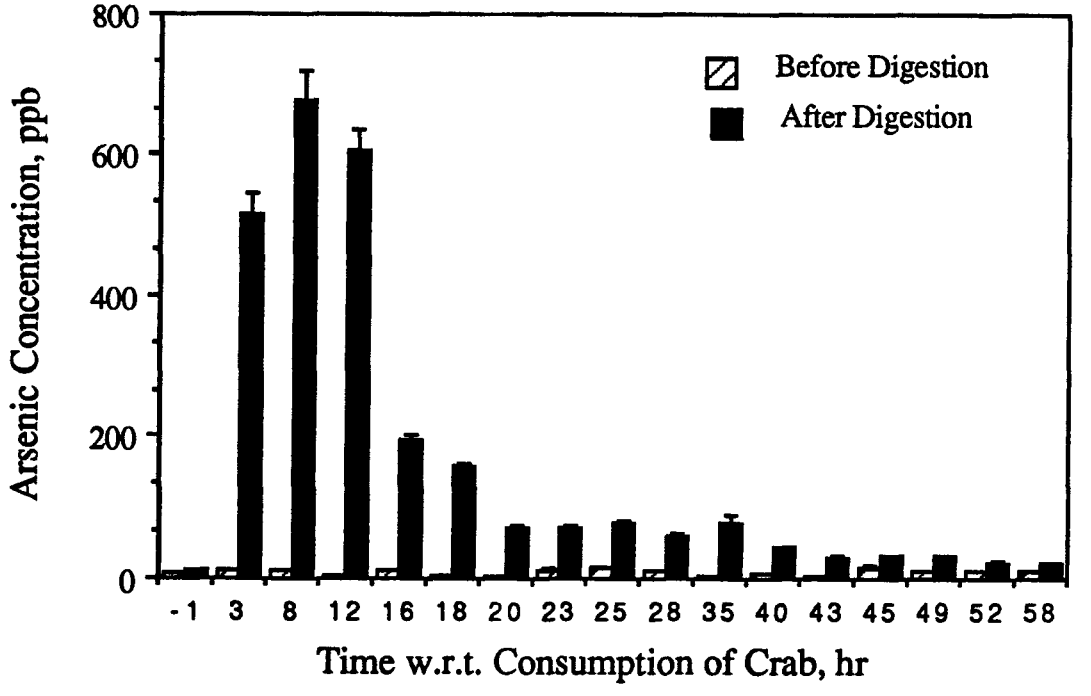


Figure 7.28. Same as in Figure 7.27 but samples from volunteer four.

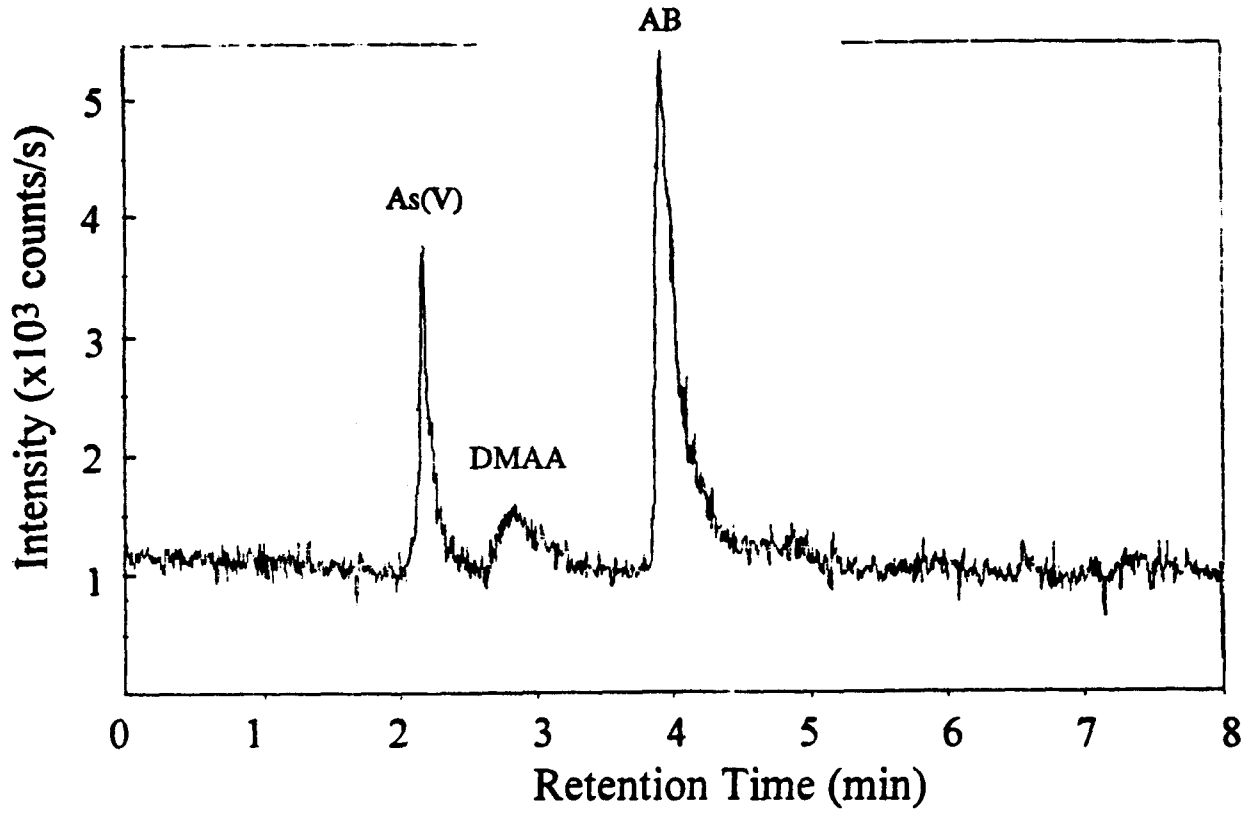


Figure 7.29. A HPLC/ICPMS trace of a urine sample from volunteer three 26 hr after the ingestion of crab. Same conditions as in Figure 7.15.

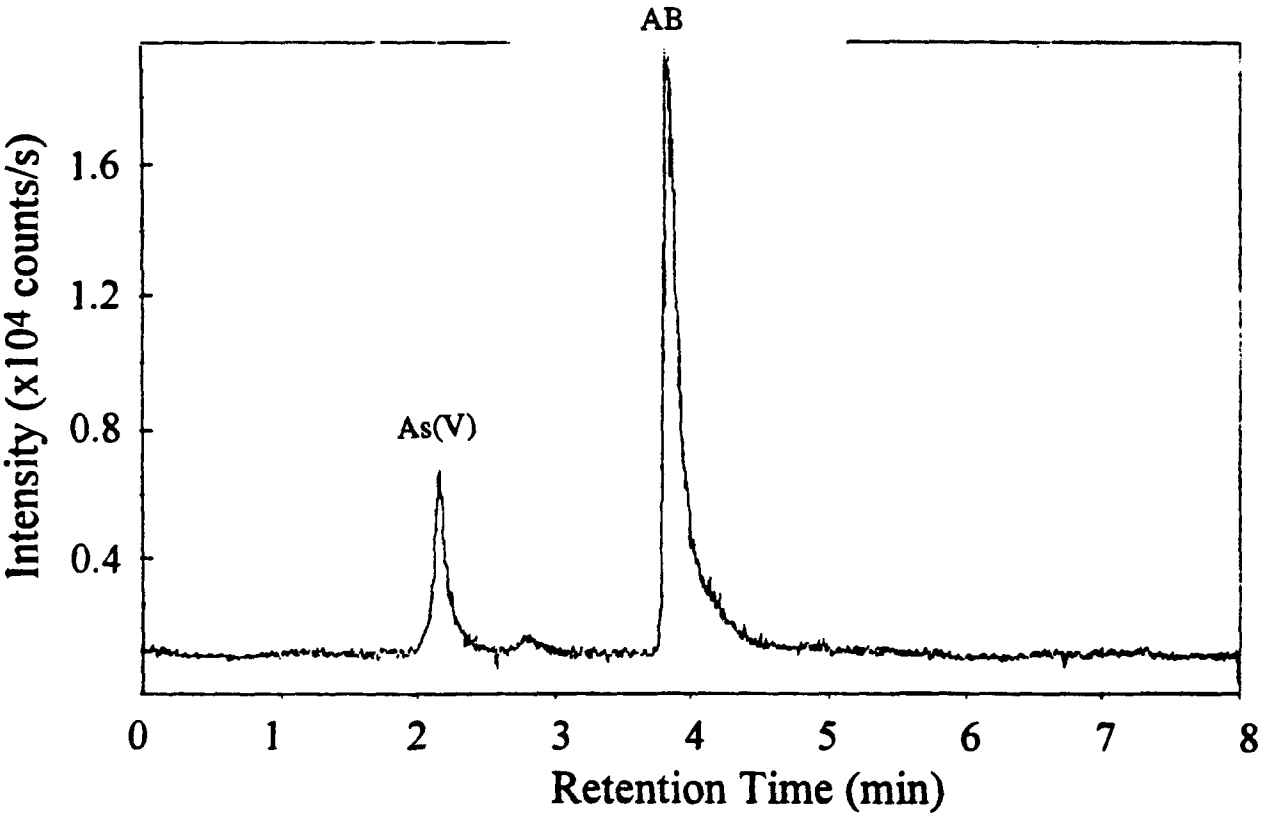


Figure 7.30. A HPLC/ICPMS trace of a urine sample from volunteer four 28 hr after the ingestion of crab. Same conditions as in Figure 7.15.

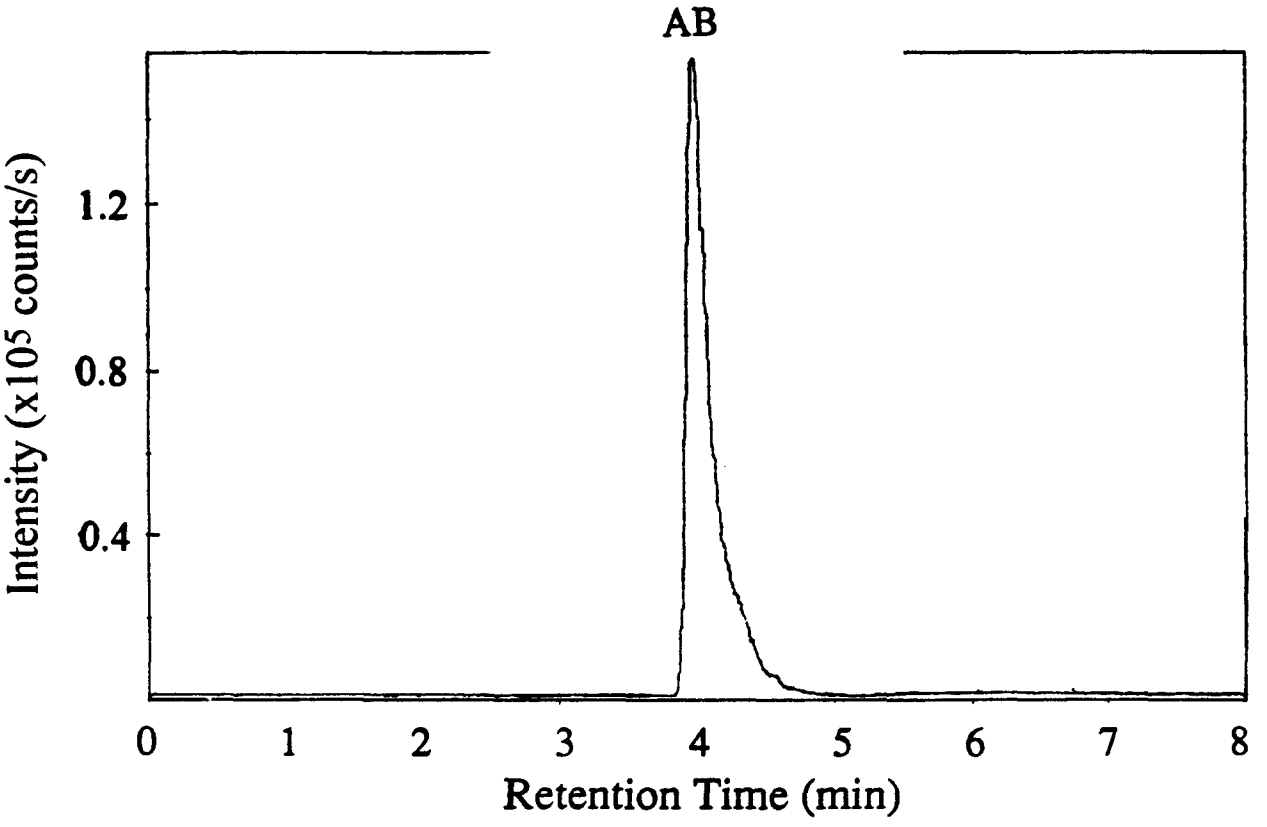


Figure 7.31. A HPLC/ICPMS trace of a crab meat extract
Same conditions as in Figure 7.15.

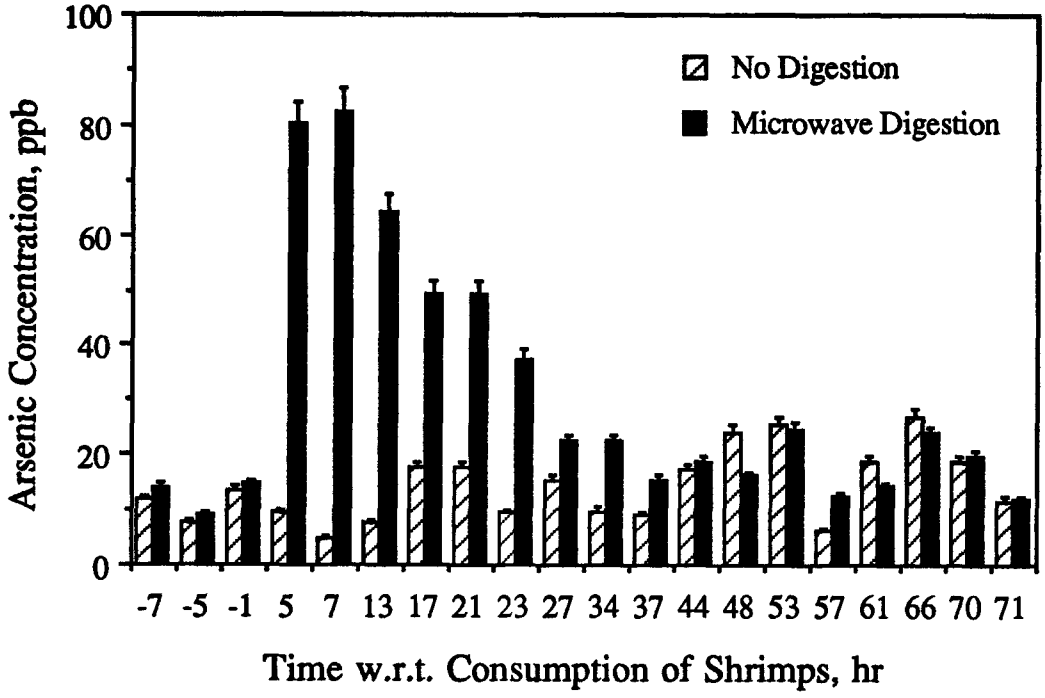


Figure 7.32. Concentration of arsenic in urine samples collected from volunteer one prior to and following the consumption of shrimps, determined by using FIA/HGAAS
(▨) - without digestion
(■) - with microwave assisted digestion of the sample

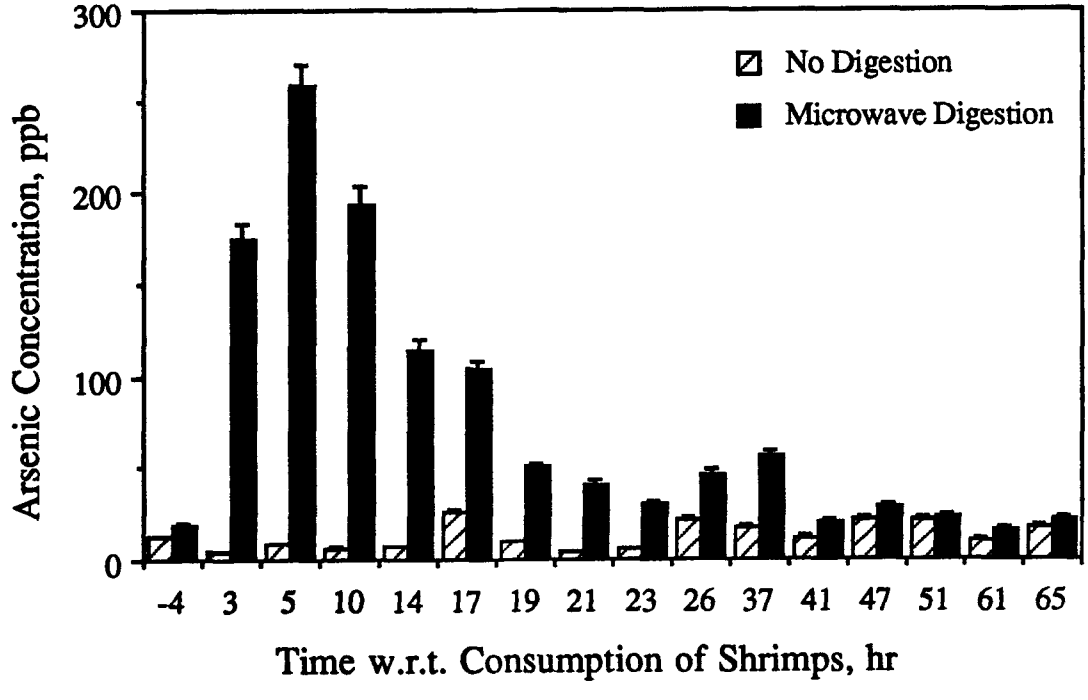


Figure 7.33. Same as in Figure 7.32 but samples from volunteer two.

7.3.5. Excretion of arsenobetaine and metabolism of arsenosugars

Arsenobetaine is a relatively stable compound, and it is generally accepted that this compound is a sink in the arsenic cycle in the marine ecosystem (5, 11, 65). Our results on the urinary excretion of arsenobetaine which is unchanged by all human subjects investigated, seems to support this view. The decomposition of arsenobetaine often requires either vigorous chemical and physical conditions, such as strong alkaline digestion with the aid of microwave energy (251, and Chapter 4) and photon energy (229), or enzymatic reactions involving microbial activities (69). The latter process is likely involved in the cycling of arsenic in the marine environment.

In contrast, arsenosugars are more labile (6, 32, 37). The present results demonstrate that they are metabolized in the human body and this is probably why different excretion patterns are observed and different arsenic species are excreted from the nine volunteers who ingested the same amount of Nori. Thus, one should be cautious when assessing the impact of seafood ingestion on urinary arsenic excretion. The type of seafood consumed, whether it is arsenobetaine-containing or arsenosugar-containing, should be specified. Otherwise, it can lead to false conclusions such as the one reached by Tamaki and Frankenberger (9), who claimed that the ingestion of seafood (crustaceans, fish and seaweed) results in arsenic excretion without any change in the chemical species.

In order to gain some information on the possibility that the acid in the human stomach is responsible for the decomposition of arsenosugars, one portion of a Nori sample was extracted with 0.01 M HCl. For comparison another portion was extracted with deionized water. Each sample, 0.5 g Nori and 10 ml of 0.01 M hydrochloric acid or deionized water was placed in a test tube (15 ml) and was sonicated for 30 min. They were then centrifuged and the extracts were analyzed by using HGAAS and HPLC/ICPMS techniques. Both HGAAS and HPLC/ICPMS analyses showed that there was no significant difference in either the total arsenic concentration or the arsenic

speciation in the two sample extracts. Similarly, when a standard reference material (NIST 1566a oyster tissue) was treated with 0.01 M hydrochloric acid or water, no difference in arsenic speciation was found from the two extracts as is revealed in Figure 7.34. This reference material is known to contain arsenobetaine and two arsenosugars X and XI as the major arsenic species (61). The chromatograms, in Figure 7.34, from the two extracts show that arsenobetaine, DMAA, and arsenosugars X and XI are present in both extracts in similar proportions. These results suggest that arsenosugars are not simply decomposed by the acid present in the stomach and that reactions involving enzymatic and/or microbial activity may be responsible for the metabolism of arsenosugars in the human body. Therefore, any difference in urinary arsenic excretion behavior towards arsenosugar ingestion observed in the nine volunteers may be attributed to a difference in individual metabolic functions.

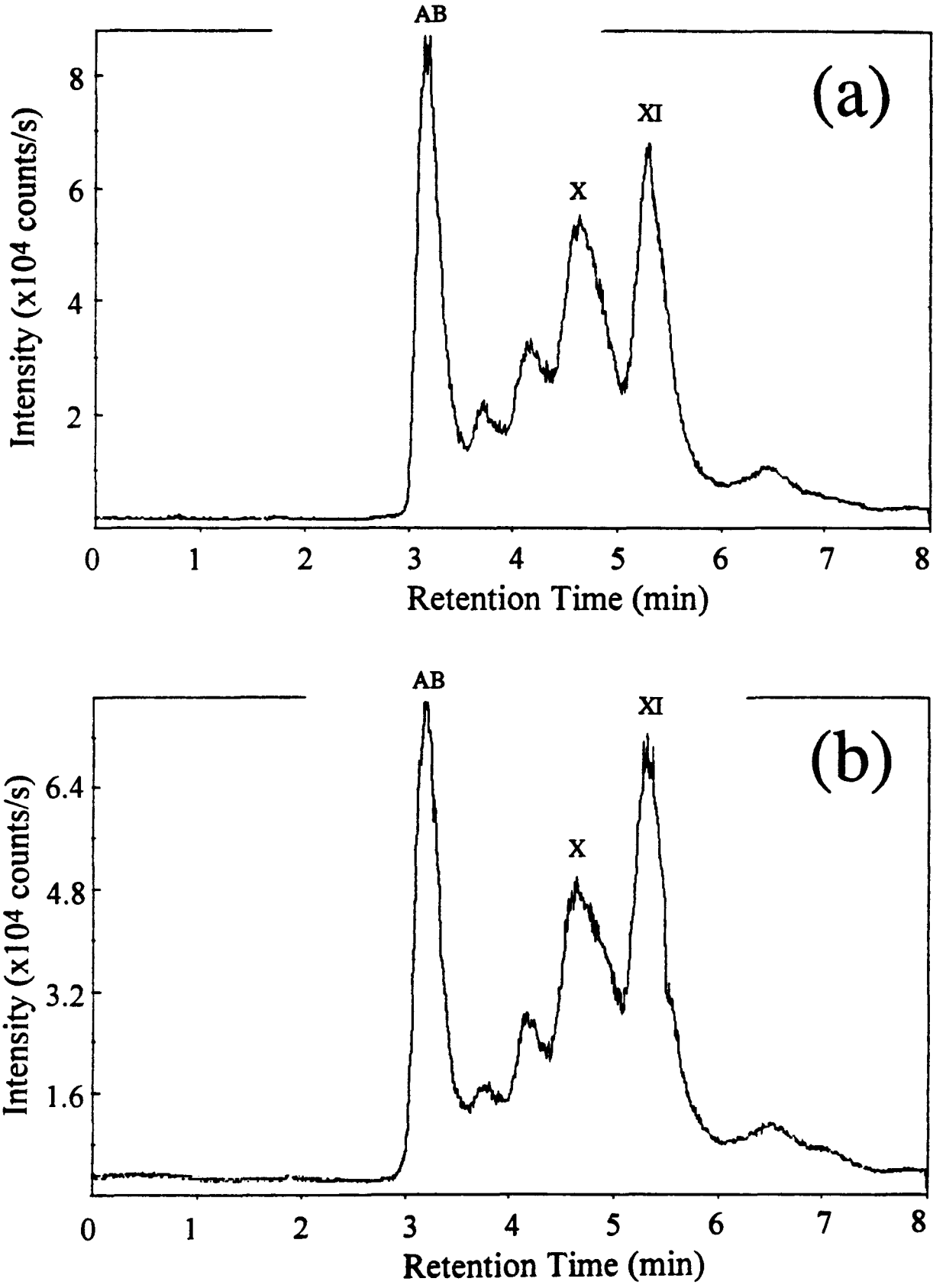


Figure 7.34. HPLC/ICPMS traces of a standard reference material (NIST 1566a oyster tissue) extracted with (a) deionized water, and (b) 0.01 M HCl
Same conditions as in Figure 7.3.

Chapter 8. CONCLUSIONS

The new hydride generators developed in this project are easy to construct and simple to operate. They show the advantages over a conventional hydride generator of improved signal-to-noise ratio and lower susceptibility to interference in the determination of arsenic, antimony, and tin. The high hydride generation efficiency (95%), measured by using radioactive tracer studies, suggests that the methodology could be applied to the determination of other hydride forming elements. The hydride generators could usefully be coupled to other more sensitive detection systems such as an ICPMS.

The use of hydride generation techniques for arsenic species is pH dependent. Identical responses cannot be obtained from arsenite, arsenate, MMAA and DMAA when the same acid and sodium borohydride concentrations are used. Thus some methods commonly used for the direct determination of total arsenic by using hydride generation methodology are subject to error. In the presence of 2% cysteine the optimum conditions for the determination of these arsenic species are in the same range, and a single arsenic species can be used for calibration. As a result of detailed studies of the effect of cysteine and thioglycerol, it is proposed that arsenate, MMAA and DMAA all in the As(+5) state, are reduced by the thiol to the As(+3) state as organo-sulfur-arsenic(III) compounds. These organosulfur derivatives of arsenic(III) easily react with sodium borohydride under similar conditions to afford the appropriate arsine. Non-thiol-containing amino acids such as methionine, glycine and histidine do not react with arsenic species in this way, and therefore they do not affect the pH dependence of the generation of arsenic hydride species. The use of cysteine also reduces common interferences caused by transition metal ions in the determination of arsenic, antimony, and tin by using hydride generation.

The microwave assisted decomposition of arsenicals to arsenate by using potassium persulfate and sodium hydroxide as digestion agents has proven to be efficient,

fast, and easy to apply. The on-line microwave oven digestion operates well with both flow injection and continuous sample introduction. A fast sample analysis (throughput 100-120 per hour) is achieved with the flow injection analysis operation. Because all arsenic compounds are converted to arsenate by the microwave assisted digestion, a single arsenic compound could be used as a standard for total arsenic measurement.

A convenient method to assess an individual's state of health is through chemical analysis of urine because urine samples are obtained readily and non-invasively. Such analysis can provide important information about exposure to many chemical species and information about many of the body's metabolic functions. Urinary excretion is the major pathway for the elimination of arsenic compounds from the body. Therefore, urinary arsenic determination is important for assessing occupational exposure to arsenic.

Occupational exposure to arsenic often involves arsenite, arsenate, MMAA, and DMAA, released through activities such as mining, smelting, glass making, and pesticide manufacturing. Dietary sources of arsenic, particularly from seafood, generally contain more complex organoarsenicals such as arsenobetaine and arsenosugars. Because arsenic compounds ingested from both occupational and dietary sources contribute to urinary arsenic, the determination of the total arsenic concentration in urine can not be used to assess occupational exposure to arsenic.

While urinary arsenic due to occupational exposure usually consists of arsenite, arsenate, MMAA and DMAA, the ingestion of crustacean type seafood results in urinary excretion of the arsenobetaine originally present as the major arsenical in the seafood. These two pools of arsenic compounds do not interchange. The analysis of a urine sample by using HGAAS technique gives the concentration of arsenite, arsenate, MMAA and DMAA, because arsenobetaine is not reducible upon treatment with sodium borohydride and therefore can not be determined by the hydride generation technique. The microwave assisted decomposition procedure using potassium persulfate and sodium hydroxide as decomposition reagents completely converts all arsenicals to arsenate and

therefore the total arsenic concentration is determined by using the HGAAS technique. The difference in arsenic concentrations obtained before and after the microwave assisted decomposition of the urine sample is a measure of the presence of arsenobetaine. Thus the present method is capable of differentiating these two pools of arsenic compounds and therefore can be used to distinguish the sources of urinary arsenic, occupational or dietary, when the seafood ingested is known to be crustacean.

However, when arsenosugar-containing seafoods such as seaweeds are ingested, the arsenosugars that are present are metabolized mainly to species such as DMAA which are excreted in urine. These arsenic species in urine samples form arsines readily with no need for prior decomposition. Therefore the source of urinary arsenic can not be distinguished, occupational exposure or seaweed ingestion, by using the same methodology.

The combination of HPLC with MD/HGAAS or HPLC with ICPMS has been demonstrated to be very useful for arsenic speciation. While the use of ICPMS as a detection system for HPLC has been well recognized, the cost (both initial purchase and operating) of such an instrument makes it one of the most expensive HPLC detectors. The HPLC/MD/HGAAS system provides an attractive alternative for routine arsenic speciation, since this system is also capable of selectively determining some arsenic compounds (for example, arsenite and arsenobetaine), which is particularly advantageous when these compounds co-elute or are not completely separated by HPLC. On the other hand, HPLC/ICPMS provides the low detection limit, which speciation of arsenic compounds in environmental and clinical applications often requires. By combining the separation power offered by HPLC and good selectivity and sensitivity obtainable by ICPMS, direct speciation of arsenic in these samples can be carried out with no need for extensive sample clean-up and pre-separation procedures.

Ion exchange chromatography is shown to be useful for the separation of some ionic arsenic species. However, other types of arsenic compounds can be separated only

by using ion pair chromatography on a reversed phase C18 column. The combination of anion pairing and cation pairing can be used to accomplish the selective separation of a variety of arsenic species. While a solvent gradient operation can be employed, the long equilibrium time required between runs makes it impractical for some applications, for example time course studies of urinary excretion; two isocratic runs of each sample under two chromatographic conditions may be more appropriate under such circumstances.

The speciation of sample extracts and defrost "juice" from previously frozen marine animal samples reveals that up to 48% of the total arsenic in the sample can be released in the "defrost juice". Clearly, appropriate sample handling procedures are very important to obtain information that reflects actual arsenic concentrations in the marine environment, and some discrepancies in the reported arsenic content of marine animals might be due to differences in sampling and storage.

Although arsenobetaine is known to be ubiquitous in marine animals, its biochemical origin is still not clear. Arsenosugars are believed to be involved in the production of arsenobetaine at some stage in the food chain. However, the exact pathway for the conversion of arsenosugars to arsenobetaine is not known. Prior to this thesis work, no arsenosugars were reported in almost all other studies on arsenic speciation in marine animals, probably because the analytical techniques used were primarily designed for the determination of arsenobetaine and not suitable for arsenosugars. The results of this thesis confirm a recent report (61) on the presence of arsenosugars and arsenobetaine as the major arsenicals in marine bivalves, suggesting that bivalves are important subjects to be studied further in order to understand at what stage in the food chain arsenosugars are transformed into arsenobetaine. Detailed studies on arsenic speciation in different families of marine animals according to their feeding habits, carnivorous or herbivorous, should also be useful.

Information on the metabolic fate of arsenosugars is also important in order to evaluate toxicological implications and health risks accompanying seaweed consumption.

Some important novel findings on this neglected aspect of arsenic metabolism are reported in this thesis as follows: (i) While arsenobetaine is excreted in urine unchanged, arsenosugars are metabolized following the ingestion; (ii) Up to six metabolites of arsenosugars are observed in urine samples; and (iii) Urinary excretion patterns of arsenic species following the ingestion of arsenosugars vary from one individual to another. During the course of this study, only nine volunteers were surveyed. A larger number of subjects is required in order to study any possible correlations between urinary arsenic excretion and a particular group of subjects. Further studies on identification of the metabolites in human urine following the ingestion of arsenosugars are necessary, and combined analytical techniques developed in this thesis such as chromatography, mass spectrometry, and spectroscopy could be important for these studies.

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