THE BIOMETHYLATION OF ARSENIC

by

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ABSTRACT

A semi-continuous hydride generation-gas chromatography-atomic absorption spectrometry (HG-GC-AA) system was developed and optimized for the determination of arsenite, arsenate, methylarsonate (MMA), dimethylarsinate (DMA), and trimethylarsine oxide (TMAO). Particularly, this system was used to study the pathway for the biomethylation of arsenicals in microorganisms and a marine alga.

The HG-GC-AA system was used to separate and identify the extracellular arsenic metabolites produced by the microorganisms *Apiotrichum humicola* (previously known as *Candida humicola*) and *Scopulariopsis brevicaulis* growing in the liquid medium enriched with arsenicals. Arsenite, MMA, DMA, and TMAO were detected following incubation with arsenate. With arsenite as a substrate, the metabolites were MMA, DMA, and TMAO; MMA afforded DMA and TMAO, and DMA afforded TMAO. Trimethylarsine was not detected in these investigations. The production of the anticipated methylated intermediates from the substrates strongly support the metabolic sequence proposed by Challenger (Challenger, F *Chem. Rev.*, 1945, 36:315).

When L-methionine-methyl-d_3 was added to the growing culture of *Apiotrichum humicola* grown in the presence of either arsenate, arsenite, MMA, or DMA, the CD_3 label was incorporated intact into the arsenic metabolites (DMA and TMAO) to a considerable extent, indicating that S-adenosylmethionine (SAM), or some related sulphonium compound, is involved in the biological methylation. Conclusive evidence of CD_3 incorporation into the arsenicals was provided by using a specially developed hydride generation-gas chromatography-mass spectrometry methodology (HG-GC-MS).

When a unicellular marine alga *Polyphysa peniculus* was grown in artificial seawater enriched with arsenicals, the arsenic metabolites produced in the cells as well as in the growth medium were identified by using HG-GC-AA methodology. Arsenite

and DMA were detected following incubation with arsenate. When the alga was treated with arsenite, DMA was the major metabolite in the cells and in the growth medium; trace amounts of MMA were also detected in the cells. With methylarsonate as a substrate, the metabolite was dimethylarsinate. *Polyphysa peniculus* did not metabolize dimethylarsinic acid when it was used as a substrate. Significant amounts of more complex arsenic species, such as arsenosugars, were not observed in the cells or medium based on the evidence given by flow injection-microwave digestion-hydride generation-atomic absorption spectrometry methodology. Transfer of the exposed cells to fresh medium caused release of most cell associated arsenicals to the surrounding environment. The alga seems to follow the biomethylation pathway proposed by Challenger for microbial process, and in the case of *P. peniculus*, DMA is the end product of this biomethylation.

When L-methionine-methyl-d_3 was added to the culture of *Polyphysa peniculus* enriched with 1 ppm of arsenate, the CD_3 label was incorporated intact in the DMA metabolite to a considerable extent. It thus confirmed that *P. peniculus* also follows the oxidation-reduction pathway involving carbonium ions originally suggested by Challenger for the alkylation of arsenic by microorganisms.

The HG-GC-MS system was also used to identify the antimony hydrides produced from the trimethylantimony compounds $Me_3Sb(OH)_2$ and Me_3SbCl_2. The possible causes of the molecular rearrangement of trimethylstibine were investigated. The extracts of plant samples collected from Kam Lake and Keg Lake (Yellowknife) were analyzed by using the HG-GC-MS system. The results provided conclusive evidence of the presence of methylantimony compounds in these samples.

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LIST OF ABBREVIATIONS

AA	atomic absorption spectrometry
AB	arsenobetaine
AC	arsenocholine
DMA	dimethylarsinate, also dimethylarsinic acid
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GFAA	graphite furnace atomic absorption spectrometry
HGAA	hydride generation atomic absorption spectrometry
HG-GC-AA	hydride generation-gas chromatography-atomic absorption spectrometry
HG-GC-MS	hydride generation-gas chromatography-mass spectrometry
i.d.	inner diameter
MMA	methylarsonate, also methylarsonic acid
MS	mass spectrometry
m/z	mass to charge ratio
NMR	nuclear magnetic resonance
ppb	parts per billion, also ng mL^{-1}
ppm	parts per million, also $\mu g\ mL^{-1}$
rpm	revolutions per minute
R.S.D.	relative standard deviation
SAM	S-adenosylmethionine
SIM	selective ion mode
TMAO	trimethylarsine oxide
TLC	thin layer chromatography

UV	ultraviolet
w/v	weight per volume

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CHAPTER 1

GENERAL INTRODUCTION

Arsenic is an element that is widely distributed in the biosphere. However, it is rarely found as the free element in nature, but occurs principally as the sulfides, realgar (As_4S_4), orpiment (As_2S_3) and arsenopyrite (FeAsS) and is usually found in association with lead, zinc, copper, and gold-bearing minerals. The average crustal concentration of arsenic is estimated to be between 1.5 and 3 ppm.[1,2] Arsenic has also been found in the atmosphere, in the aquatic environment, in soils, and in living organisms.[2] Anthropogenic input and other factors, such as weathering, volcanic, and biological activity, all contribute to the dispersion of arsenic.[3]

Arsenic compounds are notorious for their toxicity, and arsenic trioxide, known as "white arsenic", has been used for criminal purposes more than any other poison. However, arsenic compounds can also have beneficial influences on human and animal life. A paste of realgar As_4S_4 was used for the treatment of ulcers in the time of Hippocrates (460-377 B.C.).[4] The effectiveness of this tincture is unrecorded. Several organoarsenicals such as arsanilic acid, salvarsan, and neosalvarsan, have been used for the treatment of human syphilis and sleeping sickness.[4-7] Aromatic arsonic acids, such as 3-nitro-4-hydroxyphenylarsonic acid and arsanilic acid are still being used as food supplements for swine, turkeys and poultry. These compounds are believed to stimulate growth, improve feed conversion, enhance feathering, and increase egg production and pigmentation,[8] although their efficacy has never been scientifically substantiated. Arsenic compounds, such as arsenate, methylarsonate, and dimethylarsinic acid, have also found use as the active ingredients in pesticides, herbicides, fungicides, harvest

aids, and wood preservatives.[1,9-11]

Arsenic is a metalloid with a rich chemistry and can form a large number of inorganic and organic compounds. Its similarity to phosphorus and its ability to form covalent bonds with sulfur are the two main reasons for its toxicity.[12] The arsenic toxicity scale proposed by Penrose[13] follows the decreasing order: R_3As (R= H, CH_3, Cl, etc.) > As_2O_3 > $(RAsO)_n$ > As_2O_5 > $R_nAsO(OH)_{3-n}$ (n= 1,2) > R_4As^+ > As(0). Although many arsenic compounds are considered to be non-toxic,[2] the lingering memories of the criminal use of arsenic trioxide still causes the public mind to equate "arsenic" with the term "poison". Until recently, the effects of arsenic were discussed in terms of "total arsenic". However, such blanket generalizations cannot be justified since this attitude ignores the beneficial uses of some arsenic compounds and the benign nature of others. It also prevents reasonable evaluation of their impact on the environment. The fact that the toxic effects of arsenic depend not only on its concentration, but also on its speciation has led to detailed investigations of the levels and chemical structure of arsenic compounds in organisms.[2] In recent years, arsenic speciation in the environment has been extensively studied. As more attention is paid to arsenic speciation our understanding of the interactions of arsenicals with biological systems will deepen.

1.1 BIOLOGICAL TRANSFORMATION OF ARSENIC

Three major modes for the biotransformation of arsenic species have been found to occur in the environment: (1) redox transformation between arsenate and arsenite; (2) the biomethylation of arsenic; (3) the biosynthesis of more complex organoarsenicals by marine organisms.

1.1.1 Redox transformation between arsenate and arsenite

The reduction of arsenate to the generally more toxic form of arsenite has been found to occur in the environment. Aquatic bacteria, activated sewage sludge, wine yeast, mixed bacterial cultures from seawater, cultures of mixed flora from the rat stomach bacteria, fresh water algae, and marine phytoplankton can all carry out this reduction under either aerobic or anaerobic conditions.[14-22] In oxygenated seawaters arsenate is the major arsenic species, with the predominant dissolved form being $HAsO_4^{2-}$.[23,24] However, arsenite has been found to comprise significant amounts of the total arsenic in marine waters, and are in higher quantities than thermodynamic equilibrium predicts from the redox pair arsenite/arsenate.[24-26] The presence of arsenite in seawater can result from the reduction of arsenate by marine phytoplankton, bacteria, and zooplankton.[16,20-22,27] Blasco *et al.*[18,19] also reported that *Chlorella sp.*, a fresh water alga, can reduce arsenate to arsenite. The reduction of arsenate to arsenite has also been described in higher plants, and animals, including man.[5]

The oxidation of arsenite to arsenate is considered to be one of the mechanisms by which an organism protects itself against undesirable species. This process has been described for several bacterial strains isolated from cattle dipping fluids, sewage and soil.[2]

1.1.2 The biomethylation of arsenic

The biological methylation of arsenic is a common phenomenon in nature. Even though a variety of microorganisms and higher organisms including some plants, mice, monkeys and man are known to methylate inorganic arsenic compounds, the mechanism of methylation is still not fully understood.[2]

Cases of arsenic poisoning were reported to have been caused by the evolution of toxic gas from wallpaper containing such arsenic compounds as Scheele's green (cupric arsenite) and Schweinfurt green (copper acetoarsenite, Paris green). The first systematic study of this toxic gas was carried out in 1891 by Gosio who identified a number of molds that could grow on wallpaper colored with arsenite-containing pigments.[28] The pure cultures of these molds such as *Scopulariopsis brevicaulis* and *Monilia sitophila*, produced a volatile, toxic, garlic smelling arsenic species from added arsenate when grown on a potato medium.[28] Biginelli incorrectly identified this gas as $(C_2H_5)_2AsH$ based on an analysis of the precipitate obtained from passage of this gas through an acidified (HCl) mercuric chloride solution.[29] The correct characterization of this volatile gas as trimethylarsine $(CH_3)_3As$ was not achieved until 1933 by Challenger and his coworkers.[30] They grew the mold *Scopulariopsis brevicaulis* on sterile bread crumbs treated separately with As_2O_3, methylarsonic acid (MMA) $CH_3AsO(OH)_2$, and dimethylarsinic acid (DMA) $(CH_3)_2AsO(OH)$. The volatile arsine product was characterized as the mercuric chloride adduct ($(CH_3)_3As.2HgCl_2$), which precipitated out when the arsine gas stream was passed through a solution of $HgCl_2$ in hydrochloric acid (Biginelli's solution[29]). The methylarsines were also detected as hydroxytrialkylarsonium nitrates or picrates, or as benzyltrialkylarsonium picrates when they were passed through nitric acid, or alcoholic benzyl chloride.[30] Since then, several fungi isolated from soil and sewage have been found to be active in producing trimethylarsine in the presence of arsenate, arsenite, MMA, and DMA.[31-35] These fungi show selective methylation depending on the fungal species, the pH of the growth medium, the species of arsenic substrates added to the growth medium, and the concentration of phosphate in the growth medium. Bird *et al.*[34] reported that $(CH_3)_3As$ was produced from MMA, and DMA but not from arsenite by the fungi *Aerobacter*

niger, *Penicillium notatum*, and *Penicillium chrysoganum*. Cox and Alexander[32] made the first "modern" identification of $(CH_3)_3As$ produced by three fungi *Apiotrichum humicola* (originally known as *Candida humicola*), *Gliocladium roseum*, and a species of *Penicillium* when arsenate, arsenite, MMA, and DMA were added to the growing culture of these microorganisms. They identified the volatile arsine by using gas chromatography-mass spectrometry. Cullen *et al.*[33] studied *Apiotrichum humicola* extensively, and used gas chromatographic techniques to monitor trimethylarsine production. They also employed a liquid oxygen trap to cryofocus the trace amounts of volatile arsines prior to mass spectrometric identification.[33] Other alkylarsonic acids $RAsO(OH)_2$ (R = CH_3CH_2-, $CH_3CH_2CH_2$-, CH_2=$CHCH_2$-) and dialkylarsinic acids R'R"AsO(OH) (R' = CH_3CH_2-, R" = $CH_3CH_2CH_2$-) can also be metabolized by fungi to produce $RAs(CH_3)_2$ and R'R"$As(CH_3)$.[31,33-35] Using a simple method of chemofocusing and mass spectrometry, Cullen *et al.*[36] have found that *Apiotrichum humicola* methylates or reduces $PhAsO(OH)_2$, $Ph(CH_3)AsO(OH)$ and $Ph(CH_3)_2AsO$ to $Ph(CH_3)_2As$. The reduction of trimethylarsine oxide (TMAO) $(CH_3)_3AsO$ to $(CH_3)_3As$ by *A. humicola* has also been studied in detail by using gas chromatography techniques.[37] This reduction is rapid and requires biologically intact cells. The pH in the growth medium, the growth temperature, electron transport inhibitors, and uncouplers of oxidative phosphorylation such as azide and oligomycin, can all affect the reduction rate of TMAO.[37]

The first well-documented report of an arsine being produced by bacteria was made in 1971.[38] A *Methanobacterium* strain, MoH, growing in anaerobic ecosystems, such as sewage sludge, or freshwater sediment, was found to produce a volatile arsine (probably $(CH_3)_2AsH$) from arsenate. Since then, bacterial methylation of inorganic arsenic has been studied extensively. A number of nonmethanogenic bacteria are known

to synthesize CH_3AsH_2, $(CH_3)_2AsH$, and $(CH_3)_3As$ from arsenate.[39-41] A study by Cheng and Focht[39] showed that AsH_3, CH_3AsH_2, and $(CH_3)_2AsH$ were formed by soils treated separately with arsenate, arsenite, MMA, and DMA. They could not find trimethylarsine in the headspace gas. The soil bacteria *Pseudomonas* and *Alcaligenes* were shown to be responsible for the reduction. When TMAO is added to fresh water, sewage sludge, and sea sediments, $(CH_3)_3As$ can be detected and is believed to arise from bacterial action.[42]

The production of volatile arsines by algae in the presence of arsenate, arsenite, methylarsonate, and dimethylarsinate has not been reported.

Challenger[30,43] proposed a pathway for the biomethylation of arsenicals by microorganisms, involving alternating oxidation and reduction steps (Figure 1.1). Support for this sequence comes from the observation that arsenate, arsenite, MMA, DMA, and TMAO are all substrates for the production of trimethylarsine by fungi.[30-35,37] In studies connected with Challenger's mechanism, it should be noted that the species $\{CH_3As(OH)_2\}$ and $\{(CH_3)_2As(OH)\}$ probably do not exist as such; these arsenic(III) compounds are better represented as $(CH_3AsO)_n$ and $((CH_3)_2As)_2O$ in an oxic environment.[44] Cullen *et al.*[45] incubated the organoarsenic(III) derivatives $(CH_3AsO)_n$ and $(CH_3AsS)_n$ with active cultures of *Apiotrichum humicola*, *Scopulariopsis brevicaulis*, and other organisms such as *Veillonella alcalescens*. Both compounds are metabolized by *A. humicola* and *S. brevicaulis* to $(CH_3)_3As$ and $(CH_3)_2AsH$. Moreover, $(CH_3)_2AsO(OH)$ was found in the culture medium incubated with $(CH_3AsO)_n$.[45] This was the first time that a methylated arsenic metabolite had been isolated from the culture medium, a result which could be predicted from a consideration of Figure 1.1.

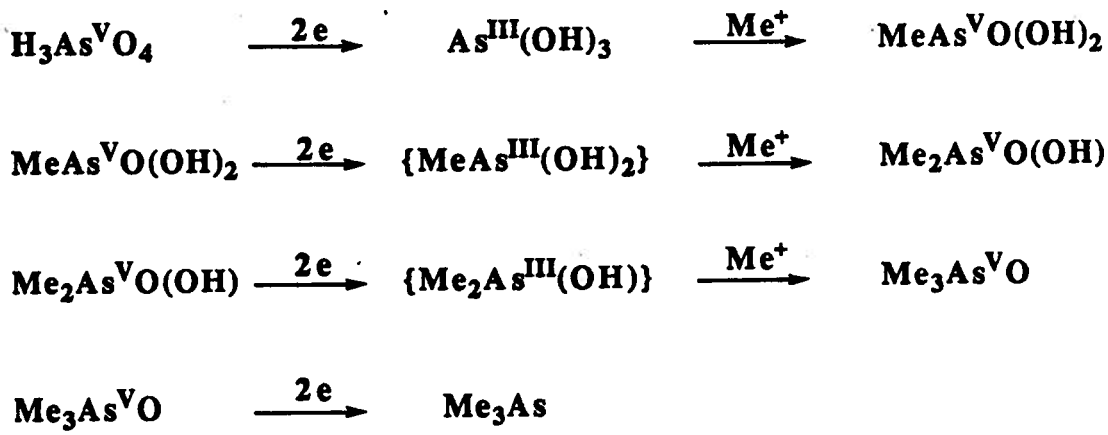


Figure 1.1 Challenger's mechanism for the biomethylation of arsenic.[30,43] The intermediates in {} are unknown as monomeric species. They can be isolated as $(CH_3AsO)_n$ and $(CH_3As)_2O$, respectively, when prepared by conventional methods.

In Challenger's mechanism, each methylation step is preceded by a reduction step. This two-electron reduction can be carried out *in vitro* by using lipoic acid and a range of thiols and dithiols, including cysteine, glutathione, mercaptoethanol, and dithiothreitol.[46,47] It is possible these are the reducing agents for the biological process. A possible mechanism for this reaction is shown in Figure 1.2.

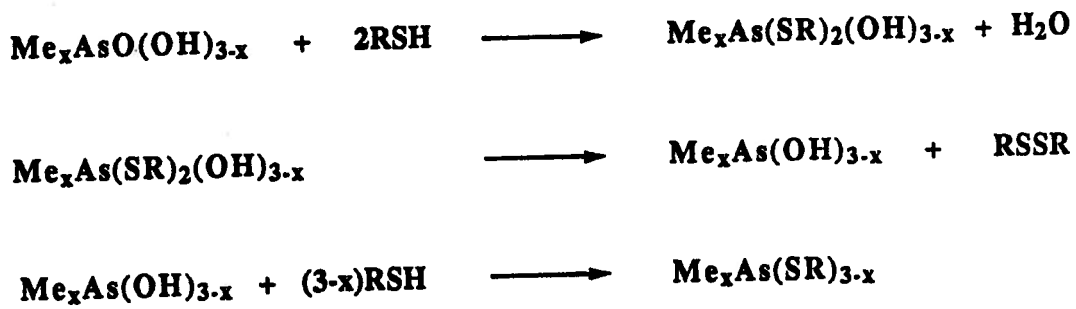


Figure 1.2 The mechanism for arsenical reduction by thiols.[46,47]

The methyl groups added to the arsenic atom during metabolism come from a methyl donor. In his 1945 review,[30] Challenger favored the hypothesis that the methylation of arsenic involved the transfer of a methyl group from some methyl containing compounds such as betaine, methionine, or a choline derivative. To investigate the possible methyl donor, Challenger *et al.*[48] added ^{14}C labeled methionine ($^{14}CH_3SCH_2CH_2C(NH_2)HCOOH$) to the culture medium and was able to detect ^{14}C labeled trimethylarsine. Based on these results it was proposed that "active methionine", later characterized as S-adenosylmethionine (SAM),[49] is possibly involved in the transfer of the methionine methyl group to arsenic during mycological methylation.[48] The structure of SAM is given in Figure 1.3.

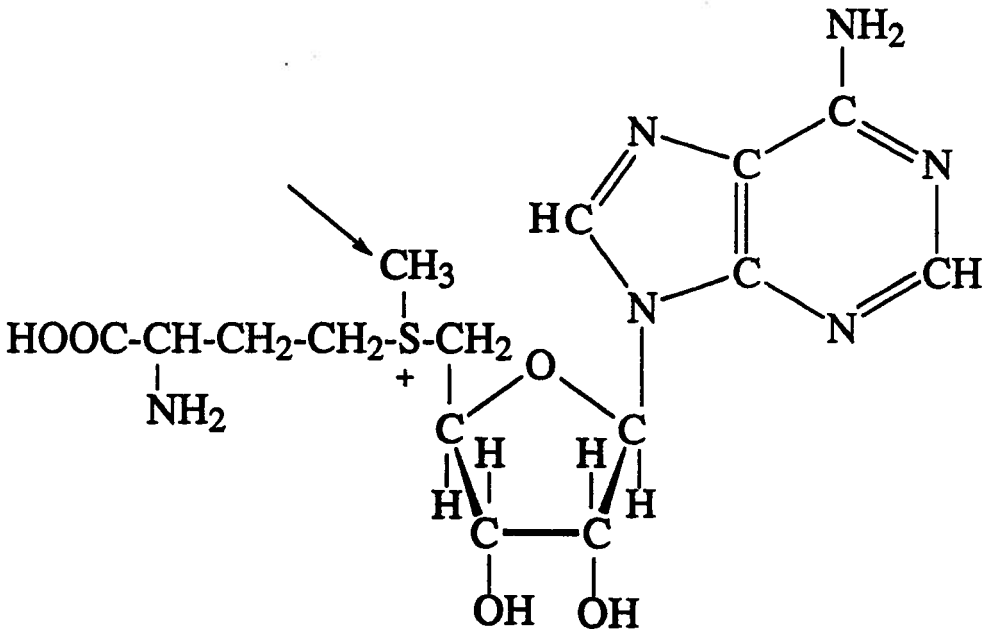


Figure 1.3 Structure of S-adenosylmethionine (SAM).[49] The arrow indicates the methyl group that is used for methylation.

Cullen *et al.*[33] grew cultures of *Apiotrichum humicola* and *Scopulariopsis brevicaulis* aerobically in the presence of L-methionine-methyl-d_3 and found that CD_3 was transferred intact to arsenite, arsenate, MMA, and DMA. The presence of the label was established by using mass spectrometry, and a high incorporation of the CD_3 group, 80-90%, was found in the isolated trimethylarsine. For example, the arsine produced from As_2O_3 consisted of 83% $(CD_3)_3As$, 13% $(CD_3)_2AsCH_3$, 3% $CD_3As(CH_3)_2$, and 1% $(CH_3)_3As$. These results reinforce the suggestion that S-adenosylmethionine or related sulphonium compounds are the source of the $[CH_3]^+$ shown in Figure 1.1. In order to develop these ideas further, broken-cell homogenates of *A. humicola* were incubated with arsenate, arsenite, MMA, DMA, SAM, and NADPH.[51] The methylated arsenic metabolites in the supernatant were confirmed by using a combination of electrophoresis analysis and column chromatography. Yet it is still unclear if SAM is the only *in vivo* alkylating source for fungi or whether other methylated sulfur compounds will cause the same reaction to occur.[4]

For bacteria, the source of the methyl donor in the arsenic biomethylation process is still not well understood.[2] Cell-free extracts of the *Methanobacterium* strain MoH produced dimethylarsine when incubated anaerobically with arsenate, CH_3-B_{12} (methylcobalamin, a derivative of vitamin B_{12}), H_2, and ATP.[38] CH_3-B_{12} was added because at that time CH_3-B_{12}, usually a donor of the carbanion CH_3^-, was believed to be involved in methane production as well as in the methylation of mercury[52]. The true methane precursor was later identified as $HSCH_2CH_2SO_3^-$ (coenzyme-M, HS-CoM).[38,53,54] Although CH_3-B_{12} served as a precursor in the production of dimethylarsine, the oxidative addition of CH_3^- to arsenic(III) is unlikely.[33] Cullen *et al.*[2] suggested three possibilities for the role of CH_3-B_{12} in the arsenic biomethylation process: (a) CH_3S-CoM is produced from CH_3-B_{12}, and this becomes involved with the

methylation of arsenic; (b) CH_3-B_{12} reacts with the arsenicals in a purely chemical or biological process; (c) CH_3-B_{12} provides the methyl group to a component in the cell extract, e.g., methionine, which is the ultimate methyl donor to the arsenic. So far, the available data do not allow any distinctions to be made among these possibilities, and without any strong evidence, there seems little need to invoke a different mechanism for arsenic methylation by bacteria from that discussed above for fungi.[2] The only major difference between fungi and bacteria seems to be that reduction of methylarsenic(V) species to arsines, $(CH_3)_nAsH_{3-n}$ (n=0-2), is a more common response by bacteria.[2]

The possible source of the methyl donor for arsenic methylation by algae has not been investigated previously, although it has been speculated that SAM is the primary methyl donor in the production of the MMA and DMA detected in marine algae.[9]

1.1.3 Organoarsenic compounds in the marine environment

1.1.3.1 Arsenic compounds in marine algae

Algae, located at the bottom of the aquatic food web, have often been the subject of arsenic metabolism studies because of their ecological and nutritional importance. Through bioaccumulation, algae exhibit concentrations of arsenic that are much higher than those of the surrounding water.[55-57] Arsenic concentrations in a range of marine algae are shown in Table 1.1.[58-64] Studies of the interaction of marine algae with arsenicals are relevant because arsenic compounds produced by algae are generally believed to be the source of the arsenic compounds found in marine animals, although it is not well established how and when these transformations take place.

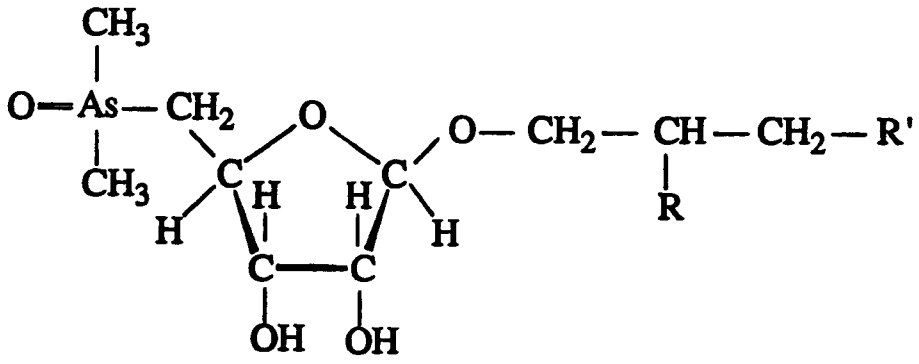
Table 1.1 Arsenic concentrations (μg/g) in some marine algae

Type of algae (No. of species)	Location	Arsenic concentration (μg/g) (dry weight) Range	Mean	References
Brown (8)	India	8-68	30	58
Red (5)		0-5.0	1.5	
Green (5)		0.1-6.3	2.2	
Brown (7)	Norway	15-109	44	59
Red (2)		10-13	12	
Brown (3)	UK	26-47	39	60
Red (2)		11-39	25	
Brown (15)	Japan	<1-230	46	61
Red (18)		<1-12	4.4	
Green (3)		<1-8	3.8	
Brown (24)	USA	1.06-31.6	10.30	62
Red (15)		0.43-3.16	1.43	
Green (16)		0.17-23.3	1.54	
Brown (14)	Canada	40.8-92.4	57.0	63
Brown (14)	Australia	21.3-179	62.0	64
Red (10)		12.5-31.3	19.2	
Green (9)		6.3-16.3	10.7	

In seawater, arsenate is the predominant arsenic species and is present at approximately 1.0-2.0 ppb.[2,20,23-25] However, significant amounts of arsenite, monomethylarsenicals, probably as MMA, and dimethylarsenicals, probably as DMA, have also been observed and are believed to be a consequence of the biological activity of marine algae. It is believed that arsenate, being chemically similar to phosphate, is readily taken up by algae from the water via phosphate transport systems located in the algal cell membranes.[65-67] In highly productive environments, oceanic phosphate levels, reduced at the surface by phytoplankton and bacterial consumption, approach those of arsenate, and are sometimes even less than those of arsenate.[24,68] This creates a favorable environment for algae to take up arsenate. Once inside the algal cell, arsenate can remain unaltered or it can be reduced and transformed to a variety of organic arsenic compounds. The algal reduction of arsenate, as well as its methylation leading to various water- and lipid-soluble compounds, is considered as a detoxification process.[69-80]

Most of the arsenic in marine macroalgae exists in complex forms including a variety of arsenosugar derivatives which have been isolated and characterized. The first successful isolation of arsenosugars was achieved from the brown kelp *Ecklonia radiata* by Edmonds and Francesconi.[81,82] The arsenicals were extracted by using methanol, subsequently isolated by using column purification and preparative TLC, and finally characterized by using microanalysis and spectroscopic techniques such as ^{1}H- and ^{13}C-NMR spectrometry. These methodologies provided convincing evidence for the suggested structures of two arsenic containing ribofuranosides, 4a and 4b (Figure 1.4). These two compounds accounted for 81% of the total arsenic present in this brown kelp.[81,82] Since then a variety of arsenosugars and their derivatives has been reported to exist in macroalgae.[83-89] Methanol extracts of the edible brown seaweed *Laminaria*

japonica showed the existence of three arsenic sugar derivatives, namely 4b, 4c, 4d.[83] Another edible seaweed, *Hizikia fusiforme*, which belongs to the fucales, contains essentially half of its total arsenic as sugar derivatives, as 4a, 4c, 4d and 4e, and half as arsenate.[84] Arsenosugars were also isolated from the kidney of the giant clam, *Tridacna maxima*, in western Australia;[90] these sugars were proposed to be the metabolic products of symbiotic, unicellular green algae living in the clam.



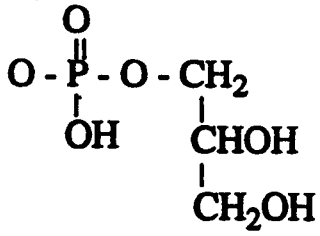
	R	R'
4a	- OH	- OH
4b	- OH	- SO_3H
4c	- OH	- OSO_3H
4d	- NH_2	- SO_3H
4e	- OH	

Figure 1.4 Structure of arsenosugar derivatives.

Edmonds and Francesconi[80] have proposed a pathway for the biotransformation of arsenate by marine algae (Figure 1.5). This pathway initially follows the mechanism outlined by Challenger (CH_3^+ donation indicated by A in figure 1.5).[30,43] However, in the final steps the adenosyl group (indicated by B in Figure 1.5) of the methylating agent SAM is transferred to the arsenic atom of dimethylarsinate, ultimately to form arsenosugars and arsenolipids. Enzymatic hydrolysis of the resulting intermediate (7) would lead to (8) which could form the arsenosugars (4a-e) by reaction with available algal metabolites.[80] Recently, the proposed key intermediate (7), an arsenic containing nucleoside, has been isolated from the kidney of giant clam, *Tridacna maxima* by Edmonds and Francesconi.[91]

Although arsenosugars have been isolated from seaweeds, there are only a limited number of reports that describe the biotransformation of arsenicals by macroalgae grown in culture media. The marine macroalgae *Fucus spiralis* (L) and *Ascophyllum nodosum* (L) assimilate arsenate to produce both water-soluble and lipid-soluble organoarsenicals,[92,93] although these compounds were not positively identified as arsenosugars. Sanders and Windom[20] used arsenate, arsenite, and DMA as substrates for cultures of a marine macroalga *Valonia macrophysa*. Digestion of the cells in dilute nitric acid led to an increase in methylated arsenicals, suggesting that more complex arsenic compounds were produced.

Studies on arsenic biotransformation in marine phytoplankton in cell culture experiments have demonstrated the ability of some marine phytoplankton to produce alkylated arsenic compounds and more complex water-soluble and lipid-soluble organoarsenicals in the presence of inorganic arsenic.[20,69-79] However, there has not been any strong evidence for the production of arsenosugars in marine phytoplankton. Investigations of arsenate uptake by marine phytoplankton have established that the

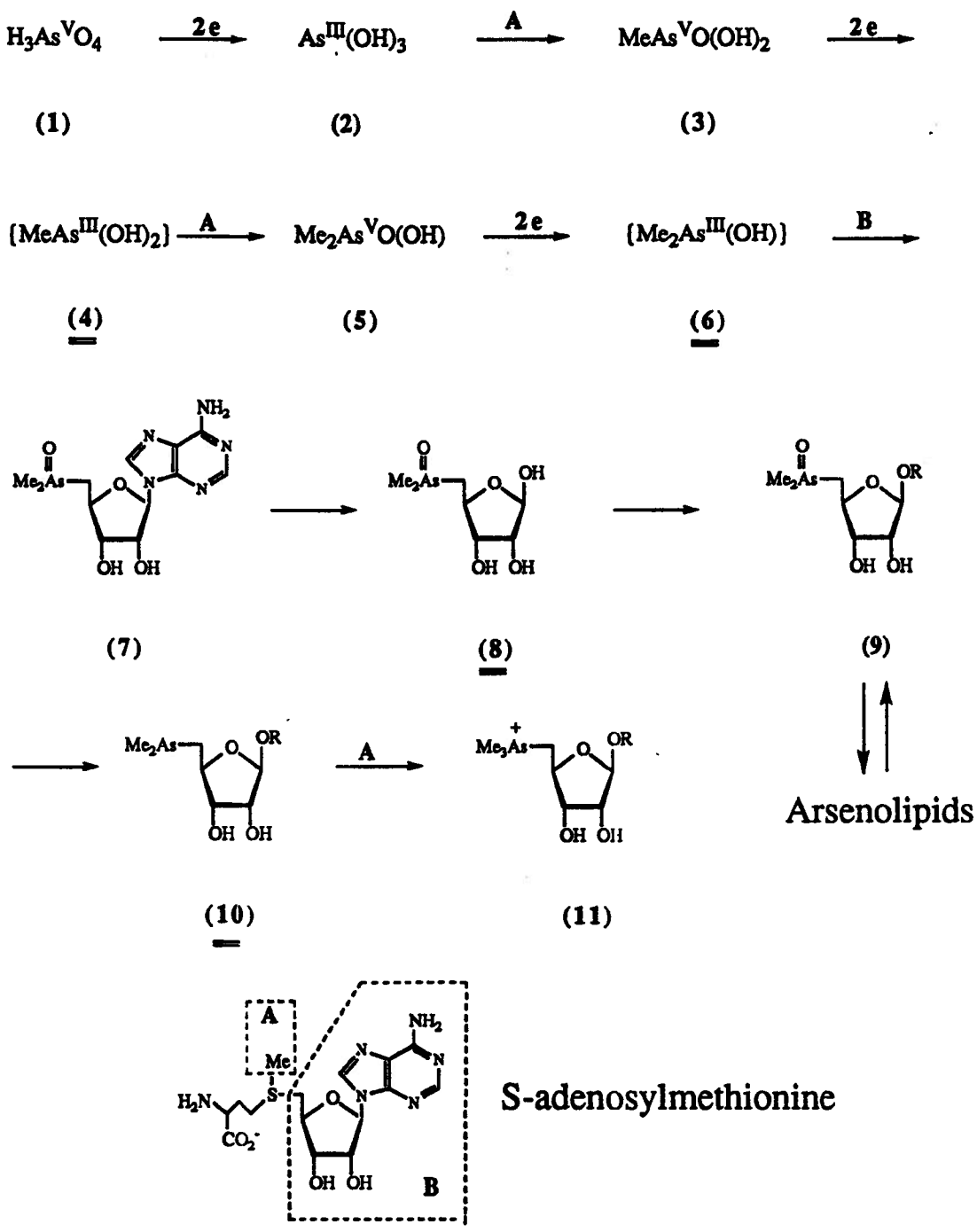


Figure 1.5 Proposed mechanism for transformation of arsenic compounds in marine algae.[80] Unidentified compounds are underlined. The **A** represents Me^+ from SAM, and **B** is the adenosyl group from SAM.

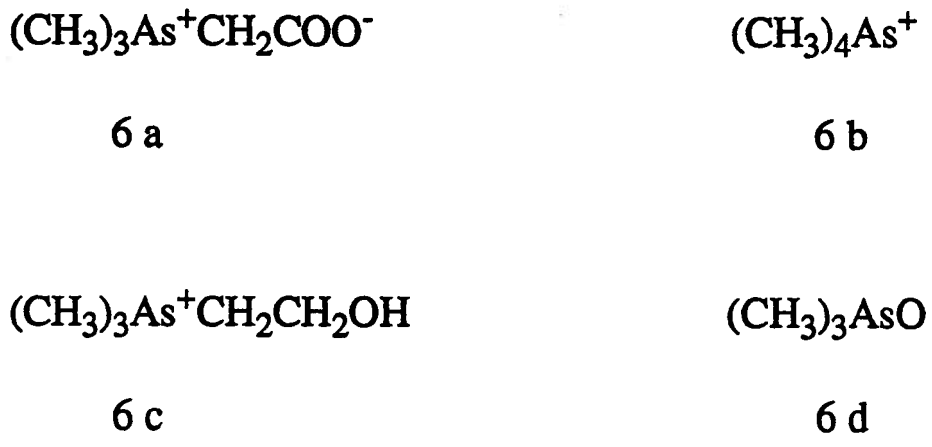
arsenic is distributed between the $CH_3OH/CHCl_3$ extractable fraction and insoluble components of the cells, although no individual arsenic compound has been positively identified apart from arsenite, MMA, and DMA. Lunde[75] reported that the marine unicellular algae *Chlorella ovalis, Phaedactylum tricornutum,* and *Skeletonema costatum* incorporated [^{74}As]arsenate and [^{74}As]arsenite from seawater into various water-soluble and lipid-type fractions. Arsenate was the preferred substrate for the biosynthesis of organoarsenic compounds by these algae. Irgolic *et al.*[74] grew 12 species of marine unicellular algae at arsenate levels ranging from 500 to 50,000 ppb (in arsenic), but were not able to characterize the lipid-type arsenicals that were produced. Andreae *et al.*[70] showed that four classes of marine phytoplankton: the diatoms, coccolithophorids, dinoflagellates, and green algae (*Prasinophyceae*) can transform arsenate to arsenite and subsequently to MMA and DMA. A portion of the arsenate was retained within the cell. An increase in methylated arsenicals was observed after base digestion of the aqueous extracts, suggesting the presence of more complex organic compounds. Cooney *et al.*[69] incubated the marine diatom *Chaetoceros concavilornis* with [^{74}As]arsenate, and found that a large amount of complex water-soluble arsenic compounds (62% of the total arsenic) and lipid-soluble arsenicals (33% of the total arsenic) could be extracted with water or $CHCl_3$. This water-soluble compound was initially characterized as trimethylarsoniolactate, but the author retracted the identification later in favor of an arsenosugar.[78] The complexity of arsenic biotransformation in marine phytoplankton has been revealed in studies of arsenate uptake by the unicellular alga *Dunaliella tertiolecta.*[72,79] Wrench and Addison[72] found that three arsenolipids, which are not related to arsenosugars, were produced when *D. tertiolecta* was treated with 0.2 MBq of [^{74}As]arsenate for 45 min. They suggested that one of these is a complex between arsenite and phosphatidyl inositol, the second a

neutral or zwitterionic complex between arsenite and a glycolipid, and the third an unidentified phospholipid-like arsenical. However, other workers[79] have reported that about 47% of the arsenic in the same alga is present as a phospholipid (O-phosphatidyl trimethylarsoniumlactate, later recharacterized as an arsenosugar derivative[80]) and as an unknown lipid (48% of the total arsenic in cells) following exposure for 48 hours to [^{74}As]arsenate.

1.1.3.2 Organoarsenic compounds in marine invertebrates and fish

It has been known for many years that the concentration of arsenic species in marine and freshwater animals is considerably higher than the background concentrations in the surrounding water.[2] The "fish arsenic" found in marine animals is chemically and physiologically different from arsenate and arsenite (Figure 1.6).[94-100] Evidence indicates that a large proportion of this arsenic is present as organoarsenic compounds which are non-toxic.[18,20,97,100] It was not until 1977 that arsenobetaine (6a) was isolated from the rock lobster *Palinurus cygnus*.[101,102] Since then arsenobetaine has been shown to be the most abundant arsenical in most marine animals so far investigated, including lobster, fish, clam, crab, mussels and scallops.[103-108] In most of the studies, organoarsenicals in marine animals were isolated and identified following solvent extraction, ion-exchange chromatography, HPLC, and TLC. Other organoarsenicals have also been found in marine animals. The tetramethylarsonium ion (6b) has been found in the clam *Meretrix lusoria* along with arsenobetaine and small amounts of two unidentified arsenicals.[109] Similar results are observed for other bivalves, clams, mussels and scallops.[108,110,111] Arsenocholine (6c) has also been claimed to be found in shrimps,[112-114] but this result could not be confirmed by

others.[106,115] TMAO (6d) has been found as a minor component in a number of fish species and in clams.[108] It has been suggested that $(CH_3)_3AsO$ is the breakdown product of some unidentified arsenicals present in the fish since its concentration in frozen perch is much higher than in fresh perch.[116]



6 a 6 b

6 c 6 d

Figure 1.6 Structure of organoarsenicals isolated from marine invertebrates and fish.

It is important to establish at what stage in the food chain the interconversion of the organoarsenicals takes place. This can be achieved only by closely controlled experiments. Limited experimentation has indicated that marine animals acquire their arsenic burdens through the food chain rather than directly from ambient water.[80,91] The organoarsenicals found in the marine organisms are believed to result from the accumulation of compounds that have been synthesized from arsenate at low trophic levels.[2] Edmonds *et al.*[80,91,117] suggested that arsenosugars are likely to be converted to arsenobetaine within the food chain. The facile transformation of arsenosugars present in the brown algae *Ecklonia radiata* into dimethyloxarsylethanol under

anaerobic conditions supports this view.[117] Two routes are possible to produce arsenobetaine via dimethyloxarsylethanol as shown in Figure 1.7. Such processes are most likely to occur (probably microbially mediated) in marine sediments.[80] However, it is not obvious where, or if, any of these transformations would occur in a natural ecosystem.

Recently Shibata and Morita[118] isolated a trimethylarsenoriboside (compound 11 in Figure 1.5) from the marine alga *Sargassum thunbergii*. Anaerobic degradation of this compound could directly produce arsenocholine which would be readily converted into arsenobetaine in fish or elsewhere. Cullen and Nelson[119] reported the probable existence of arsenobetaine in seawater.

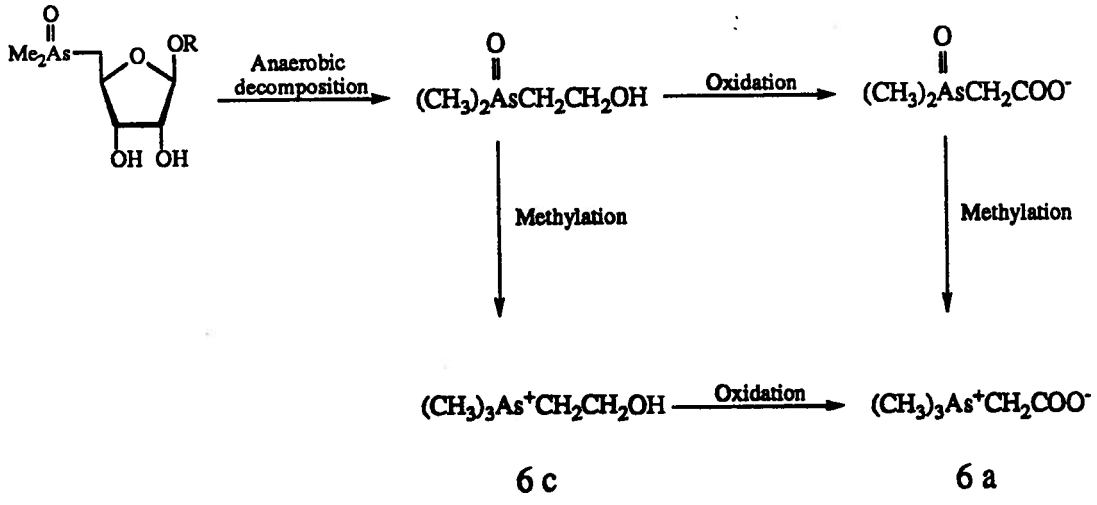


Figure 1.7 Possible pathways for the production of arsenobetaine from arsenosugar derivatives.

1.2 SCOPE OF WORK

This thesis is concerned with the interaction of arsenic with the microorganisms *Apiotrichum humicola* and *Scopulariopsis brevicaulis* as well as a marine unicellular alga *Polyphysa peniculus*. The effect of several arsenicals on cell cultures as well as the mechanism of biomethylation of arsenicals has been examined.

A continuous hydride generation-gas chromatography-atomic absorption analytical system is described in Chapter 2. This system was optimized to determine arsenate, arsenite, MMA, DMA, and TMAO in the cell extracts and in the growth medium of the microorganisms *Apiotrichum humicola* and *Scopulariopsis brevicaulis* and the marine alga *Polyphysa peniculus*. Graphite furnace atomic absorption spectrometry was also optimized for the determination of total arsenic in the samples.

The biotransformation of arsenate, arsenite, methylarsonate, and dimethylarsinate by the microorganisms *Apiotrichum humicola* and *Scopulariopsis brevicaulis* is described in Chapter 3. The separation, identification, and quantification of extracellular arsenical metabolites in the growth medium was carried out by using the hydride generation-gas chromatography-atomic absorption spectrometry system described in Chapter 2.

In Chapter 4, a hydride generation-gas chromatography-mass spectrometry technique is described. This system was used to characterize the extracellular arsenicals in the media of microorganisms grown in the presence of arsenate, arsenite, methylarsonate, dimethylarsinic acid, and L-methionine-methyl-d_3. The nature of the methyl donor in the biomethylation of arsenic compounds was investigated.

Chapter 5 reports on the effect of adding arsenate, arsenite, methylarsonate and dimethylarsinic acid to an unicellular marine alga *Polyphysa peniculus*. The arsenic accumulation, methylation and excretion by the alga was investigated. In the presence of

L-methionine-methyl-d_3 and arsenate, the arsenic metabolites excreted by the alga in the growth medium were characterized by using hydride generation-gas chromatography-mass spectrometry.

In Chapter 6, a continuous type hydride generation system coupled with GC-MS is described. This system was used to identify the antimony hydrides produced from the trimethylantimony compounds $Me_3Sb(OH)_2$ and Me_3SbCl_2. The possible causes of the molecular rearrangement of the trimethylstibine were investigated. The HG-GC-MS system was also used to analyze the extracts of plant samples collected from Yellowknife.

CHAPTER 2

ANALYTICAL METHODOLOGY

2.1 INTRODUCTION

Since the introduction of atomic absorption spectrometry, applications of this analytical technique have become routine in laboratories through the world.[120-122] Graphite furnace atomic absorption spectrometry (GFAA) has become one of the most widely used analytical methods because of its high sensitivity and applicability for smaller sample volumes. It has been used for total arsenic determination in a variety of samples such as drinking water, seawater and brines, biological fluids, soils, industrial products, and wastes.[120-126] Hydride generators coupled to atomic absorption spectrometers have also found wide application in trace analysis because of the 10-1000 fold increase in sensitivity compared to other liquid sample nebulization procedures.[127-131] Hydride generators coupled to a range of detectors have been used to determine the total amounts of arsenic compounds that can be transformed into hydride-forming species through acid digestion[102,108,132-136] or base digestion.[113,137-139] The speciation analysis of arsenate, arsenite, MMA, DMA and TMAO that are precursors to the volatile arsines AsH_3, CH_3AsH_2, $(CH_3)_2AsH$ and $(CH_3)_3As$, can be achieved by using a hydride generator coupled to a gas chromatograph, with an atomic absorption spectrometer as the detector.[139-144] The major components of such a hydride generation system are shown in Figure 2.1.

In the present work, optimum conditions for determination of arsenic compounds by using GFAA were established. In addition, continuous hydride generation-gas chromatography-atomic absorption spectrometry methodology was developed and optimized for applications to the work outlined in the introduction (Chapter 1).

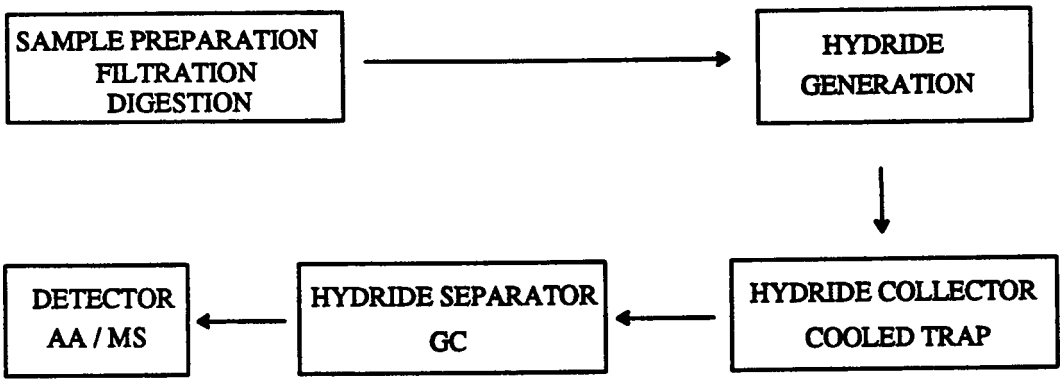


Figure 2.1 Scheme of the major components of a hydride generation system.

2.2 EXPERIMENTAL

2.2.1 Chemicals and reagents

All chemicals used were of reagent grade. Deionized water was used for dilution. Glass and plasticware were cleaned by soaking overnight in 2.0% Extran solution, followed by a water rinse, a soak in dilute hydrochloric acid, and finally a deionized water rinse.

Arsenic standards were freshly prepared each day by serial dilution from stock solutions (1000 ppm of elemental arsenic) of the following compounds: sodium arsenate, $Na_2HAsO_4.7H_2O$ (Baker); sodium arsenite, $NaAsO_2$ (Baker); disodium methylarsonate, $CH_3AsO_3Na_2.6H_2O$ (Alfa); dimethylarsinic acid, $(CH_3)_2AsO(OH)$ (Alfa); trimethylarsine oxide, $(CH_3)_3AsO$, which was synthesized according to the

literature.[145] Solutions of 2.0% (w/v) $NaBH_4$ prepared in 0.1% (w/v) NaOH, 1.0 M HCl, and 4.0 M acetic acid were freshly made daily.

2.2.2 Apparatus

2.2.2.1 Graphite furnace atomic absorption spectrometry (GFAA)

A Varian Techtron Model AA 1275 Atomic Absorption Spectrometer was used for the arsenic determination. It was equipped with a Varian Spectra AA hollow cathode lamp set at 8 mA, a deuterium background corrector, and was connected to a Hewlett-Packard 82905A printer. The monochromator was set at 193.7 nm, and the slit width at 1 nm. A Varian Techtron GTA-95 atomizer accessory fitted with a pyrolytically coated graphite tube was used to achieve graphite furnace atomization. Argon was used as the purge gas.

2.2.2.2 Hydride generation-gas chromatography-atomic absorption spectrometry (HG-GC-AA)

The hydride generation system used in most of the work is shown in Figure 2.2. A Gilson Minipuls 2 four-channel peristaltic pump was used to deliver and mix sample, acid, and sodium borohydride solution. The sample solution first met with a continuous flow of acid and then borohydride. Hydride generation took place in an 18-turn reaction coil, producing both arsines and hydrogen. The gases and spent solutions were separated in a gas-liquid separator.

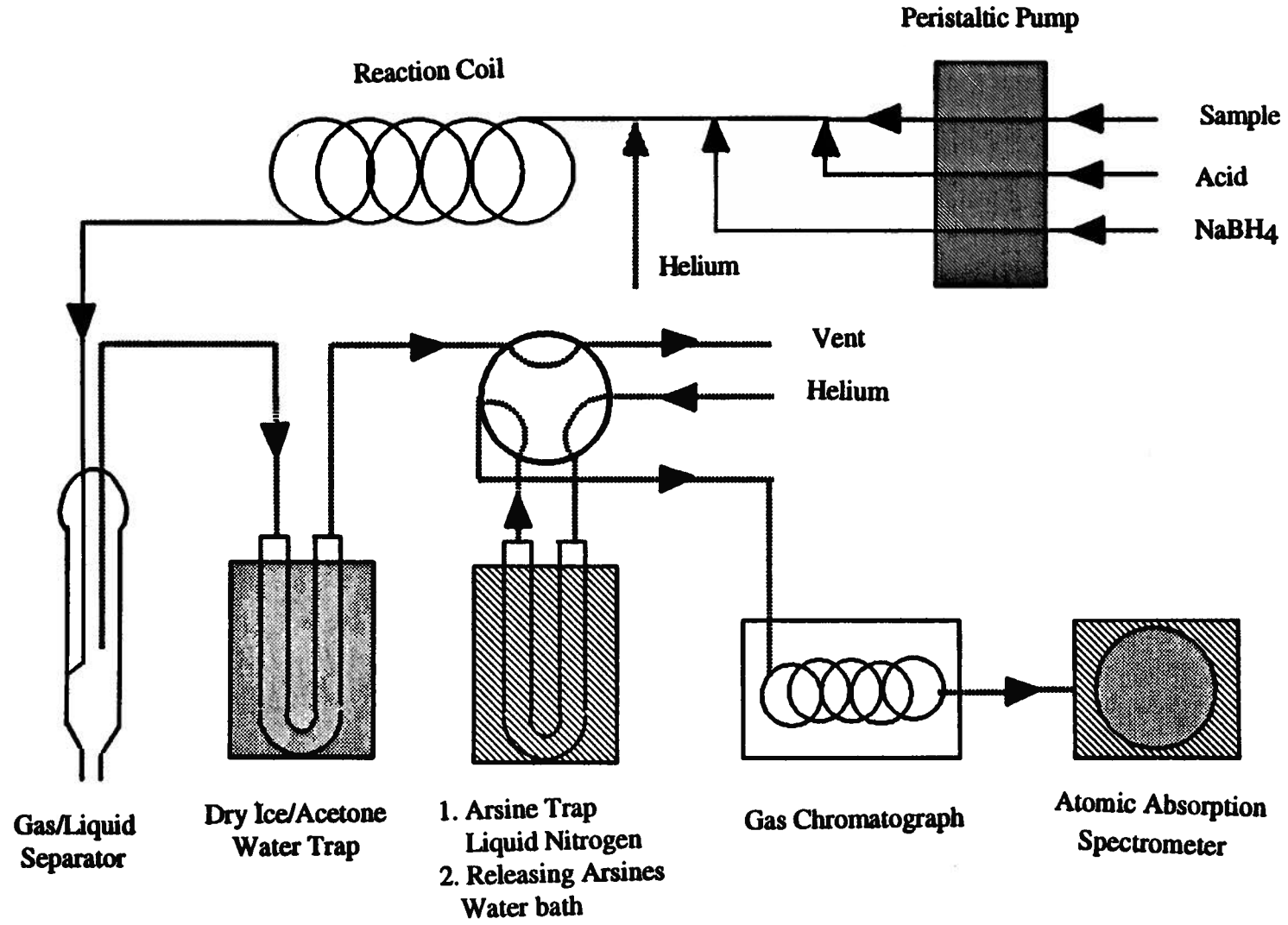


Figure 2.2 Hydride generation-gas chromatography-atomic absorption spectrometry experimental setup. Dotted lines represent gas lines.

The effluent gases were passed through two U-tube traps connected in series. The first U-tube was a moisture trap made of Teflon (30 cm x 0.8 cm i.d.) which was cooled by a dry ice-acetone slurry (-78^{o}C). The second trap (Teflon, 30 cm x 0.4 cm i.d.) was immersed in liquid nitrogen (-196^{o}C) and was used to collect the volatile arsines. A six-way valve was interfaced between the arsine trap and a gas chromatograph. The valve could be switched from the stripping gas stream of the generating apparatus, to the carrier stream of the gas chromatograph. The interior parts of the six-way valve were made of stainless steel.

A 5830A Hewlett Packard Gas Chromatograph was used to separate the produced volatile arsines. The GC column consisted of a Teflon tube (50 cm x 0.4 cm i.d.) hand packed with Porapak-PS (80-100 mesh, Chromatographic Specialties).

Atomic absorption measurements were performed by using a Jarrell-Ash Model 810 atomic absorption spectrometer equipped with a Varian Spectra arsenic hollow-cathode lamp set at 10 mA. A hydrogen-air flame in a quartz cuvette mounted on the spectrometer was used to atomize the volatile arsines. Light from the arsenic hollow cathode lamp was aligned to pass through the hydrogen/air flame. The monochromator was set at 193.7 nm, and the slit width at 1 nm. A Hewlett-Packard 3390A integrator was connected to the AA spectrometer to record the absorbance values as peak areas.

2.2.3 Analytical procedures

2.2.3.1 GFAA

Operating parameters such as temperature, time, and gas flow need to be optimized in order to achieve good analytical sensitivity in GFAA analysis. These

parameters have been optimized by other workers in this research group for the purpose of determination of arsenic in standard solutions and in the extracts of plant cells. Therefore, only minor changes in the furnace operating parameters were made when the acid digestates of the algal cells were analyzed.

The GTA-95 accessory was used to program the furnace operating parameters such as temperature and time for drying, ashing, and atomization stages. In the optimization process, the arsenic standard solution (volume 20 μL) was injected into the graphite furnace by using the automatic delivery system of the GTA 95 accessory. Palladium solution (20 μL), prepared as palladium nitrate (100 ppm) in citric acid (2.0% w/v), was used as a modifier. By using the single parameter variation method, temperature and heating time were optimized to give the maximum absorbance signal (peak area) for arsenic in a standard solution. These optimized parameters are shown in Table 2.1.

Table 2.1 Furnace operating parameters for the determination of arsenic

Step #	Temperature (^{o}C)	Time (sec)	Gas Flow (L min^{-1})	Comment
1	70	5	3.0	dry
2	120	30	3.0	dry
3	1200	20	3.0	ash
4	1200	1.0	0	ash
5	2300	1.0	0	atomize
6	2300	1.0	0	atomize
7	2300	2.0	3.0	clean

The standard additions technique[159] was used to compensate for matrix interferences when the cell digestates were analyzed. The sample (volume 10 μL) and the standard solution (volume 3-15 μL) were mixed with palladium modifier by using the automatic delivery system of the GTA 95 accessory. The mixture was then injected into the graphite tube before the furnace was heated. The parameters established for the standard additions are given in Table 2.2.

Table 2.2 Typical sampling parameters for Standard Additions Method for GFAA analysis

Solution	Standard Volume (μL)	Sample Volume (μL)	Blank Volume (μL)	Modifier Volume (μL)
Blank	---	---	25	20
Addition 1	3	10	12	20
Addition 2	6	10	9	20
Addition 3	9	10	6	20
Addition 4	12	10	3	20
Addition 5	15	10	0	20
Sample	---	10	15	20

2.2.3.2 HG-GC-AA

When HG-GC-AA analysis was performed, the sample solution, 1.0 M hydrochloric acid or 4.0 M acetic acid, and 2.0% (w/v) sodium borohydride solution were pumped from their respective containers by using a peristaltic pump and mixed continuously in an 18-turn reaction coil (~ 2.5 m) with the help of a carrier gas (helium)

and the evolved hydrogen. The solution introduction rate was 3 mL/min. After the sample solution (typically between 1-5 mL) was taken up, the probe in the sample cup was transferred into a wash solution of deionized water for another 2 minutes to clean out the apparatus. The mixed reagents were carried into the gas/liquid separation apparatus where the waste solution constantly drained out.

After trapping the volatile arsines in the U-tube immersed in liquid nitrogen, the liquid nitrogen bath was removed and the U-tube was immersed in a 70°C water bath. The gas-sampling valve was switched, and the arsines were carried by a stream of helium gas to the GC column, where they were separated by means of careful temperature programming (Table 2.3). The effluents from the GC were then carried to the atomic absorption spectrometer, where they were combusted in a hydrogen/air flame in a quartz cuvette, and quantified. The experimental conditions are summarized in Table 2.3.

2.3 RESULTS AND DISCUSSION

2.3.1 Graphite furnace atomic absorption spectrometry (GFAA)

The GFAA technique permits the direct analysis of liquid samples. A measured volume of the sample solution (5-70 μL) is placed in the furnace and electrically heated in a series of stages so that the analyte element is freed from as many of the impurities as possible before it is atomized.[146] The first stage is the *drying* step where the solvent is evaporated from the sample solution by holding the temperature of the furnace slightly above the boiling point of the solvent. Arsenic is not likely to be lost during this cycle unless arsenic trichloride is present.[147] The formation of arsenic trichloride is

Table 2.3 Operating conditions for the HG-GC-AA System

AA spectrometer	810 Jarrell-Ash	
Wavelength	193.7 nm	
Slit Width	1 nm	
Lamp Current	10 mA	
Flame	Air/Hydrogen	
Air Flow Rate	120 mL min^{-1}	
Hydrogen Flow Rate	75 mL min^{-1}	
Carrier Gas	Helium	
Carrier Gas Flow Rate	30 mL min^{-1}	
(reaction coil)		
Carrier Gas Flow Rate (GC)	20 mL min^{-1}	
Size of Quartz Burner	10 cm x 0.8 cm i.d.	
Trapping Time	3 min	
Sample Size	1-5 mL	
Pump Rate	3 mL min^{-1}	
GC Temperature Program	Temperature 1	50°C
	Time 1	0
	Temp. Rate	30°C min^{-1}
	Temperature 2	150°C
	Time 2	2 min

favored by a high-chloride medium. This occurs particularly when a hydrochloric acid solution is present. In the second *ashing* stage, the temperature is raised to a value that is sufficient to remove all organic materials by thermal decomposition as gaseous substances, but not sufficient to vaporize the arsenicals. This cycle was carried out at temperatures ranging from 120°C to 1200°C. The final step is the *atomization* stage, in which the temperature is rapidly increased to a point at which gaseous atoms of the arsenicals are formed. The absorption of the analyte is measured during this stage. A stream of argon gas is usually used to protect the furnace and the analyte from oxidation, and to transport impurities vaporized during thermal pretreatment steps out of the furnace.

Samples were analyzed for arsenic by using methods involving either simple calibration curves or standard additions. Typically, calibration curves were obtained daily by plotting absorbance (peak area) against concentration for 20 μL injections of a series of standard arsenic solutions. A linear relationship was obtained for arsenic concentrations up to 200 ng/mL. The relative standard deviation for twenty injections of 20 μL of 20 ng/mL standard arsenic solution was calculated to be 6.9%. The limit of detection is defined as the analyte concentration that gives a signal equal to the blank plus three standard deviations of the blank, and was determined to be 5 ng/mL.

When samples containing different arsenic compounds are analyzed, an additional complication arises: arsenic compounds do not all have the same volatility at a particular temperature, and some volatile arsenic compounds may be lost during the *ashing* stage. To overcome these difficulties in the direct determination of total arsenic by GFAA, matrix modifiers were added.[152,153] Chemical modification of the sample is essential for the analysis of many biological samples. The aim of chemical modification in GFAA is to prevent the loss of elemental analyte at the high pyrolysis temperatures

required to remove the bulk of the matrix components during thermal pre-treatment of the sample.[148,154] Among the chemical modifiers, nickel nitrate is commonly used in the analysis of arsenic, where the added nickel forms a stable arsenide that atomizes at a high temperature.[148,149,152,155] Lately, palladium has been suggested as a very effective chemical modifier for arsenic and many other elements.[156,157] Typically, 20 μL of palladium modifier, prepared as palladium nitrate (100 ppm) in citric acid (2.0% w/v), was added to the sample inside the graphite tube. Palladium nitrate needs to be reduced to Pd(0) metal by citric acid in order to act as an effective modifier. Palladium was found to form a stable intermetallic compound with arsenic in the graphite furnace.[158] Under these conditions, the ashing temperature can be increased up to 1450°C without any loss of arsenic by volatilization. A temperature of 1200°C was used as the ashing temperature in our studies.

Other matrix interferences can be minimized by using the standard additions technique which consists of taking a number of replicate aliquot portions of the sample solution, and adding to them known increasing quantities of the arsenic standard solution.[159] Since all calibration solutions have the same composition with the exception of their analyte contents, the influence of impurities will be the same. The standard additions technique was used in the work to determine the total amount of arsenic accumulated in cells of *P. peniculus* described in Chapter 5.

The presence of background interferences due to the incomplete isolation of the radiation absorbed by the analyte element from other radiation, or radiation absorption due to the interferent, can be overcome by using a background correction device such as a deuterium lamp. In this system, the radiation from the line source (arsenic hollow cathode lamp) and a continuous source (deuterium lamp) is passed alternately through the graphite furnace. The measured absorbance with the line source is due to both the

analyte and background interference. When the continuous beam of radiation passes through the cell, the absorbance is almost entirely the result of background absorption. After the subtraction of the background absorption from the total absorbance, the absorbance due to the analyte can be obtained.

2.3.2 Hydride generation-gas chromatography-atomic absorption spectrometry (HG-GC-AA)

The hydride generation procedure involves the reduction of the analyte to a volatile covalent hydride that is subsequently swept into a detector where quantitative measurements can be made. Holak, in 1969, was the first to use hydride generation for the determination of arsenic using atomic absorption spectrometry.[160] He generated arsine by adding zinc to the sample solution acidified with hydrochloric acid and collected the arsine in a trap cooled in liquid nitrogen. Finally the arsine was atomized in an argon-hydrogen flame for quantification. This procedure proved to be extremely useful because the analyte is separated from the sample matrix, and therefore, matrix interferences are substantially reduced, and even eliminated.

There are two main reactions used for the generation of arsine. The first involves a metal-acid reducing system such as zinc-hydrochloric acid.[160] The reaction can be written as below:

$$4Zn + 8H^+ + As(OH)_3 = 4Zn^{2+} + AsH_3 + 3H_2O + H_2$$

The major disadvantages of this method are that the time for complete reaction can be as long as 10 minutes and it is possible to introduce contamination from the

metal. As a consequence, this process has not gained general acceptance for analytical purposes.

The second and more effective method for the production of arsine involves the use of sodium borohydride (sodium tetrahydroborate, $NaBH_4$) and acid. The reaction is written as follows:

$$6BH_4^- + 3H_2O + 3H^+ + 5As(OH)_3 = 5AsH_3 + BO_3^{3-} + 5H_3BO_3 + 9H_2$$

The $NaBH_4$-acid system can afford better yield, involves a shorter reaction time, and less contamination is present in the analysis. Initial applications involved the use of $NaBH_4$ as pellets dropped into a reaction flask containing an acid solution of arsenic.[164-166] It is now customary to use a solution of $NaBH_4$: the recommended concentration ranges from 0.5 to 10%.[117-139] Since 1969, the method has been extended to the determination of other elements such as Hg, Ge, Sn, Pb, Sb, Bi, Se, and Te.[127-139,161-163]

Braman *et al.*[162] noted that the reduction of arsenic compounds with sodium borohydride was pH dependent and also related to the pK_a of the individual arsenic acids (Table 2.4). This allowed for a selective reduction of arsenate or arsenite. To separate arsenate (or arsenite) from MMA and DMA, Braman *et al.*[162] and Andreae[140] established cold-trapping procedures that effectively pre-concentrate several arsines derived from the hydride-forming arsenicals present in 10-50 mL sample volumes (<1 ppb). As a result of the volatility differences of the arsines, slow warming of the trap facilitates their sequential determination with a suitable detector. Alternatively, GC

separation has been successfully used to separate AsH_3, CH_3AsH_2, $(CH_3)_2AsH$ and $(CH_3)_3As$.[139-144]

Table 2.4 Reaction conditions for the conversion of inorganic and methylarsenic compounds to volatile arsines

Arsenic Compounds	pK_{a1}	Reduction pH	Product	b.p. °C
arsenite	9.23	< 7	AsH_3	-55
arsenate	2.20	> 4.0	no reaction	
		1	AsH_3	-55
MMA	4.10	> 5.0	little reaction	
		1-3	CH_3AsH_2	2
DMA	6.19	1-4	$(CH_3)_2AsH$	36
TMAO	---	1	$(CH_3)_3As$	52

2.3.2.1 The hydride generation system

Two hydride generators, batch type and continuous type, have been widely used for hydride production. In the batch type hydride generator, the acidified sample is first purged by a helium gas stream in a reaction vessel, and then reacted with the reducing agent ($NaBH_4$) which is injected by means of a syringe. The hydrides produced are carried by the helium gas, either directly into a detector or into a collection trap before being introduced to a detector. In general, the batch type hydride generator is suitable for samples containing a low arsenic level (usually < 1 ppb). The disadvantages of this batch-type hydride generator are: (1) a large volume of sample (>10 mL) is needed to obtain enough hydride for analysis; (2) the experimental results may critically depend on

the experience and skill of the operator; (3) the procedure can be time consuming, one sample analysis taking as long as 20-30 minutes.

The continuous type hydride generator coupled with an arsine trap and GC-AA detector is simple to operate and easy to automate.[167,168] Better precision can be achieved since a peristaltic pump is used for introducing reactants, and operating inconsistency can therefore be eliminated. However, the arsenic concentrations required for successful analysis are relatively high, usually above 5 ppb in arsenic. In spite of this limitation, we have chosen to use a continuous type hydride generator for most of our sample analysis because of its fast sample introduction rate and good reproducibility. The time for one sample analysis can be reduced to less than 7 minutes. Only a small volume of sample (< 5 mL) is needed for an analysis. This system has proved to be efficient and gives reproducible results in dealing with a large number of samples of relatively high arsenic concentration (5 ppb-10 ppm).

Volatile arsines produced in the continuous mode hydride generator (Figure 2.2) are carried by an inert gas and the produced hydrogen into a cooled trap, where they are collected. This step concentrates the arsenic species before detection. Arsines are then volatilized by warming the trap, separated by using a gas chromatograph, and then detected by using an atomic absorption spectrometer. Carbon dioxide and water that accompany the arsine production can cause clogging of hydride trap (Teflon U-tube, 30 cm x 0.4 cm i.d.), and affect the column efficiency. Sodium hydroxide, calcium chloride, potassium carbonate, silica gel, and magnesium perchloride have been used for removing water and carbon dioxide.[140,162] However, these reagents also absorb some of the arsines. To achieve the maximum trapping of water and the minimum loss of arsines, we used a Teflon tube immersed in dry ice-acetone slurry as the water trap. It was found that the diameter of the tube is very critical. When the diameter is 0.4 cm, the

water trap is easily clogged by moisture produced during hydride generation. If the evolved gases are passed through a Teflon tube with a diameter of 2.0 cm, the water trap could not remove moisture efficiently: clogging of the hydride trap is soon observed. A Teflon U-tube, 30 cm x 0.8 cm i.d., was chosen as the water trap because it is effective in removing moisture carried out of the reaction system by the helium gas and the evolved hydrogen. The efficiency of this water trap was judged by the absence of water accumulated in the hydride trap immersed in liquid nitrogen. Although the water trap was efficient in trapping moisture and easy to assemble, it should be noted that the U-tube needed to be cleaned after 8 to 10 runs because of clogging from the accumulated water. The possible loss of arsines in the water trap was assayed by comparing the arsine response signals with and without the presence of moisture trap. No significant difference was observed. Other water traps such as the U-tube packed with NaOH or glass wool were also investigated: both water moisture and arsines are absorbed by these traps.

Different cold traps have been used for arsine collection following hydride generation. Odanaka *et al.*[169] trapped the arsines in n-heptane cooled with dry ice-acetone. Aliquots of the heptane solutions were then injected into the GC-MS for arsenic analysis. The liquid nitrogen-cooled trap, filled with different packing materials such as glass beads,[140,144,162] and glass wool (silanized),[140] are by far, the most frequently used trapping techniques. Reimer[170] used a Teflon U-tube (30 cm x 0.4 cm i.d.) immersed in liquid nitrogen (-196°C) as a hydride trap. In the present work , this trap was found to collect the arsines efficiently and to give reproducible results, and was therefore adopted for general use.

After all of the arsines are collected, the liquid nitrogen Dewar is removed and the trap is warmed in a water bath (70°C) to volatilize the arsines. The arsines can be fed directly into the detector where they arrive in the order of increasing boiling point and molecular mass.[147] In order to separate the arsines, Braman *et al.*[162] packed the hydride U-trap with glass beads and wrapped it with Nichrome wire connected to a variable transformer which was used to elevate the tube's temperature. Quartz wool and different chromatographic packing have also been used in the U-trap to increase the retention time.[140] If a better and more reliable separation of the arsines is desired, a gas chromatograph can be placed between the liquid nitrogen trap and the detector.[139,141,170,172] Chromosorb 101[172] with 16.5% silicone oil DC-550,[171] or Chromosorb W (AW DMCS)[139,140] have served as stationary phases. Reimer[170] reported that a column packed with Porapak-PS (80-100 mesh) gave a good separation of arsines. In the present work, several packing materials such as chromosorb W, silanized quartz wool, and Porapak-PS were investigated. Only Porapak-PS provides a good separation of the arsines and this packing was adopted for general use.

2.3.2.2 Optimization

The acid concentration, sodium borohydride concentration, and carrier flow (in reaction coil) were optimized by using the single parameter variation method. The peak area of the signals was chosen as the response to be optimized.

The effect of hydrochloric acid concentration on the determination of 60 ng each of arsenite, arsenate, MMA, DMA, and TMAO is shown in Figure 2.3. The results indicate that the concentration of hydrochloric acid is more critical in the determination of DMA and TMAO than it is for arsenate, arsenite, and MMA. The responses of

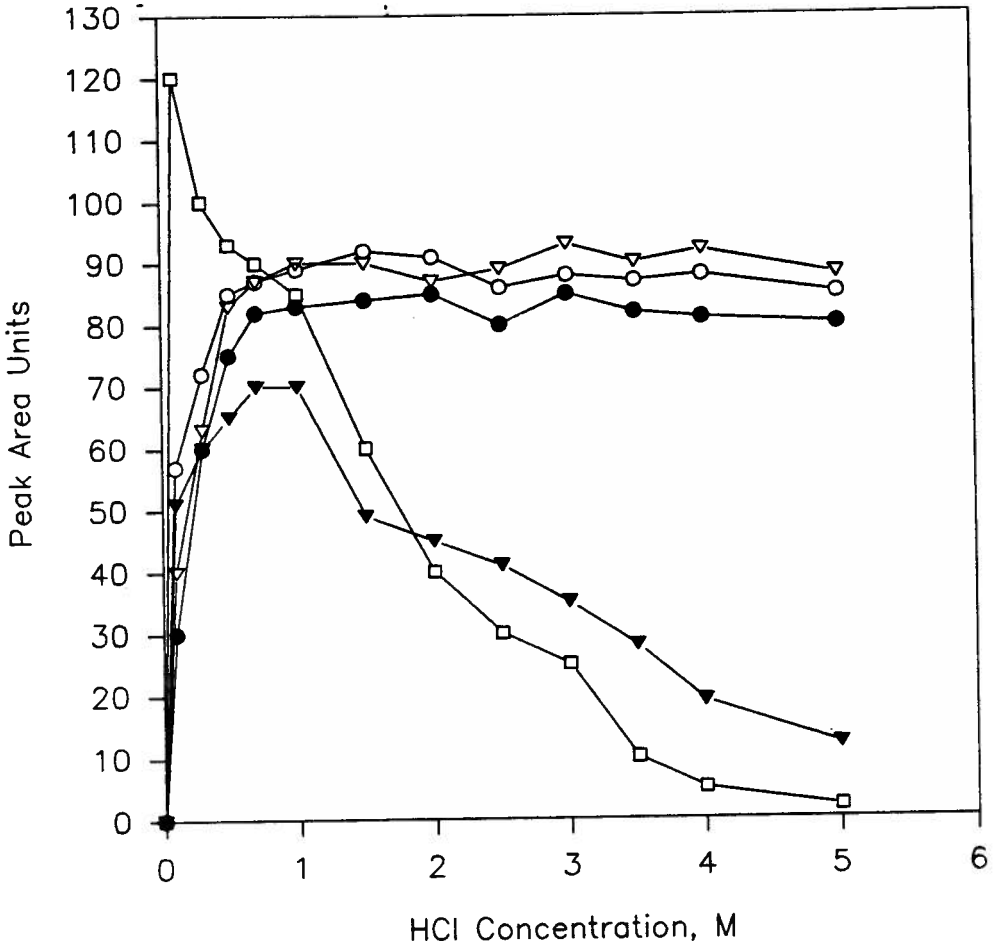


Figure 2.3 Effect of the concentration of hydrochloric acid on the determination of hydride-forming arsenicals. The reductant was a 2.0% (w/v) $NaBH_4$ aqueous solution. (O) arsenite (●) arsenate (▽) MMA (▼) DMA (□) TMAO

arsenite, arsenate, and MMA increase rapidly with increasing acid concentration, reaching a constant value at concentrations above 0.5 M. The response for DMA is shown to reach a maximum at 0.75-1.0 M HCl, but falls thereafter, with the absorbance approaching 30% of its maximum absorbance in 4.0 M hydrochloric acid. The production of trimethylarsine is not favored by the higher HCl concentrations: the highest response for TMAO is achieved in the presence of 0.1 M HCl. Similar results were reported by other workers using automated systems for the determination of "total" arsenic.[143,144,173] The reasons for the difference in responses are not known, although kinetic factors or mixing dynamics may be responsible.[143] Optimum ranges of hydrochloric acid concentration for maximum sensitivity are 0.5-5 M for arsenate, arsenite, MMA; 0.75-1.0 M for DMA; and 0.1 M for TMAO. A solution of 1.0 M of hydrochloric acid concentration was adopted for the work, and was primarily used for the determination of total inorganic arsenic (arsenate and/or arsenite), MMA, and DMA.

The selective reduction of arsenic species has been used for arsenic speciation analysis by a number of researchers.[140,142-144,162,168,173] Most of the studies were carried out by using a batch type hydride generator. Acetate, KHP, citrate, Tris-Tris maleate, and Tris-HCl were used to give a sample pH value of 5 before injection of the reductant into the sample. Under these conditions, arsenite, but not arsenate, was reduced to arsine by sodium borohydride. We have studied the effects of Tris-HCl (1.0 M, pH 6.2), acetate (2.0 M, pH 5), citric acid/citrate (1.0 M, pH 5.5), and acetic acid (4.0 M, pH 4.0) on the production of arsines in the continuous type hydride generator. They all gave satisfactory results for arsenate and arsenite separation. We chose acetic acid for routine use because it is economical and easy to obtain. The effects of acetic acid on arsine production are shown in Figure 2.4, where it can be clearly seen that the responses from arsenite, DMA, and TMAO are at constant levels over almost the entire

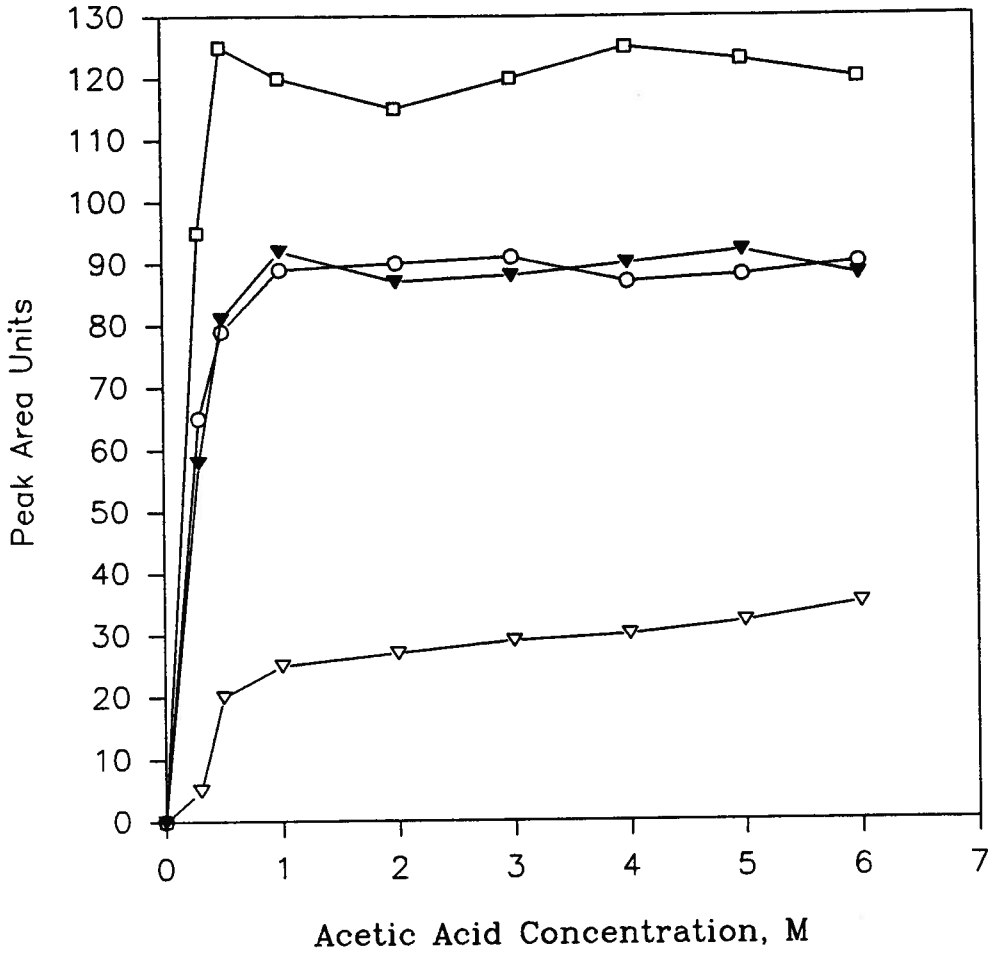


Figure 2.4 Effect of the concentration of acetic acid on the determination of hydride-forming arsenicals. The reductant was a 2.0% (w/v) $NaBH_4$ aqueous solution.
(O) arsenite (▽) MMA (▼) DMA (□) TMAO

concentration range studied (1.0-6.0 M). The response for arsenate is almost negligible and so not shown on the graph (Figure 2.4). The signal from MMA is largely suppressed in almost the entire range studied. Since acetic acid provides a wider optimum range for the production of dimethylarsine and trimethylarsine, it was used not only to separate arsenite from arsenate but also to quantify arsenite, DMA and TMAO. A solution of 4.0 M acetic acid was chosen for the work.

The effects of the sodium borohydride concentration on the absorbance of arsenate, arsenite, MMA, DMA, and TMAO in the presence of hydrochloric acid and acetic acid are shown in Figure 2.5 and Figure 2.6, respectively. When 1.0 M hydrochloric acid is used, the results indicate that the concentration of sodium borohydride is less critical in the determination of all the arsenicals studied except TMAO. The optimum concentration of sodium borohydride falls in the range of 1.0%-4.0% (w/v). The small decrease in the responses of all the arsenicals at higher concentration (> 4.0%) of sodium borohydride (Figure 2.5) is probably due to poor decomposition of the borohydride; a large amount of hydrogen gas is produced from the reaction of the sodium borohydride with the hydrochloric acid. If 4.0 M acetic acid is used, the optimum range of sodium borohydride concentration for maximum sensitivity is 1.0-6.0% for arsenite, DMA, and TMAO (Figure 2.6). Therefore, a solution of 2.0% of sodium borohydride was adopted in the determination of arsenicals when either acid was used.

The effect of the flow rate of the carrier gas (used for carrying reactant into liquid/gas separator) on the peak area of signals was also investigated (Figure 2.7). When flow rates are between 10 mL/min and 60 mL/min the arsenic atomic absorption responses are not changed significantly. When the flow rate is above 80 mL/min, the responses of the arsines declines. This is probably due to a lowering of the time the

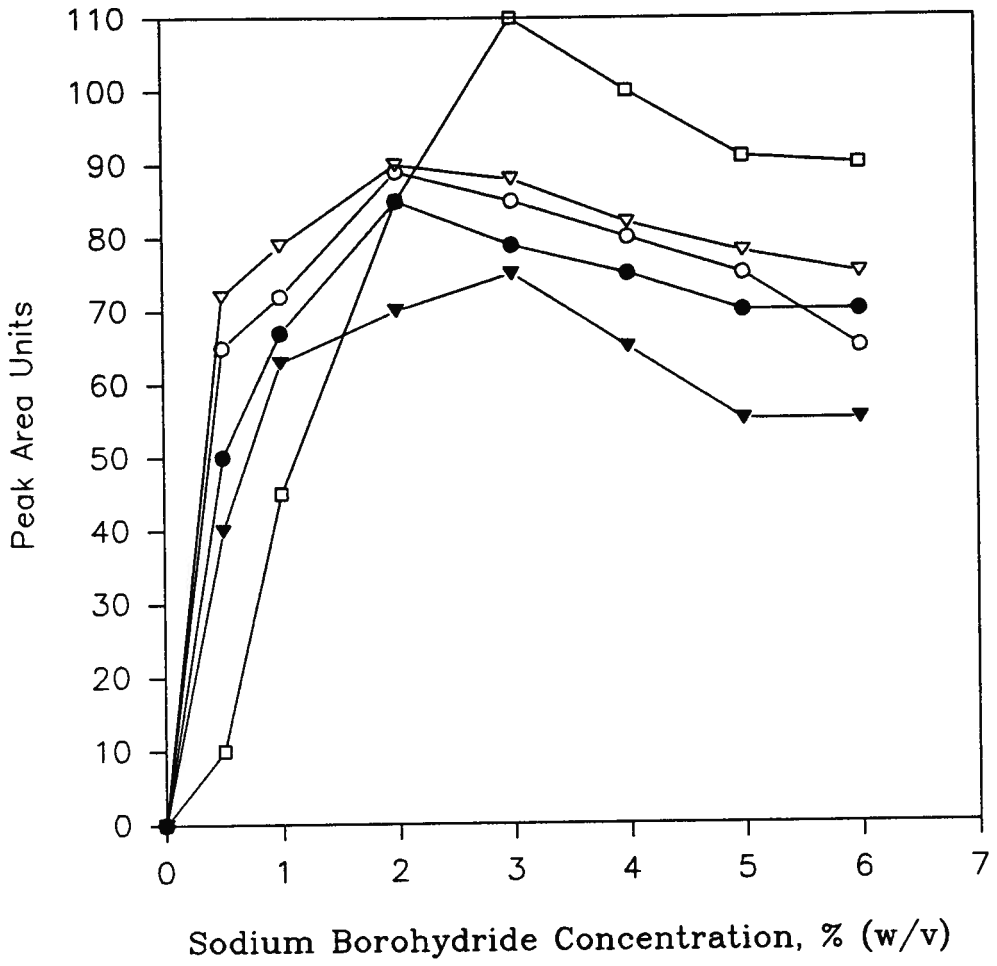


Figure 2.5 Effect of the concentration of $NaBH_4$ on the determination of hydride-forming arsenicals. The acid medium was a 1.0 M hydrochloric acid solution. (O) arsenite (●) arsenate (▽) MMA (▼) DMA (□) TMAO

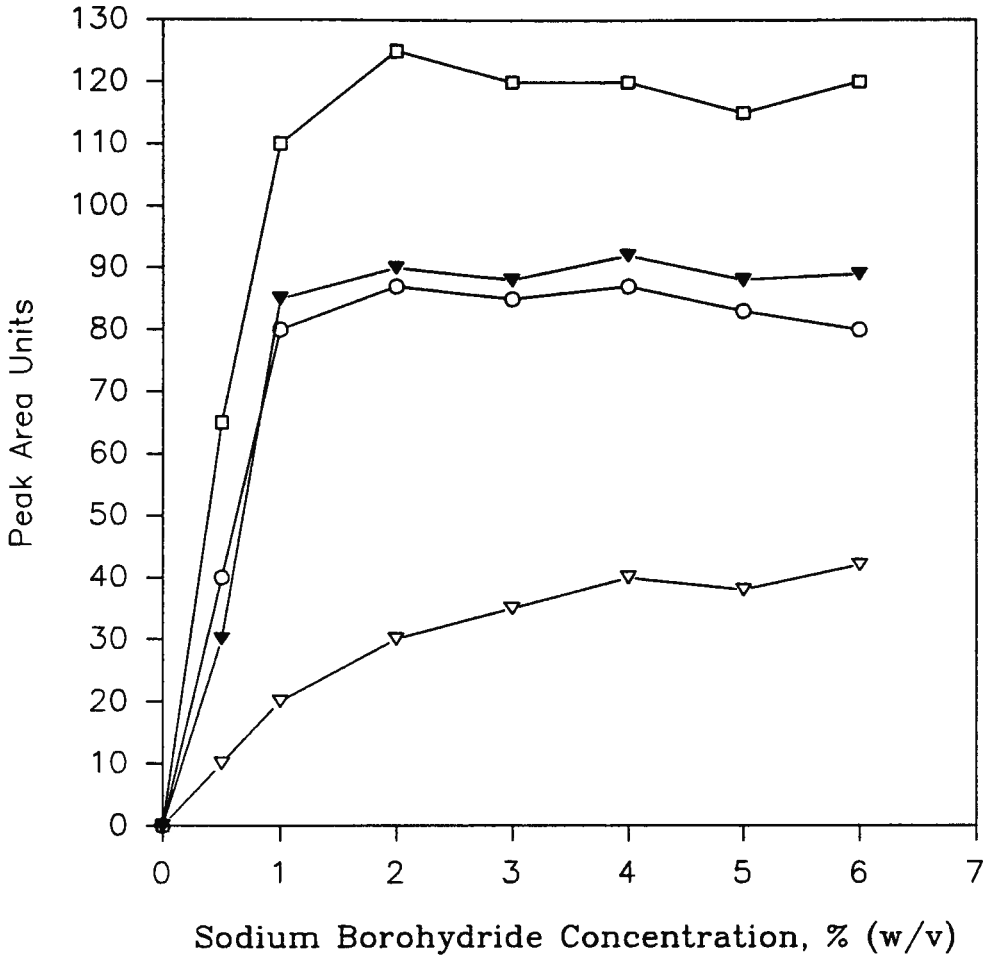


Figure 2.6 Effect of the concentration of $NaBH_4$ on the determination of hydride-forming arsenicals. The acid medium was a 4.0 M acetic acid. (O) arsenite (∇) MMA (▼) DMA (□) TMAO

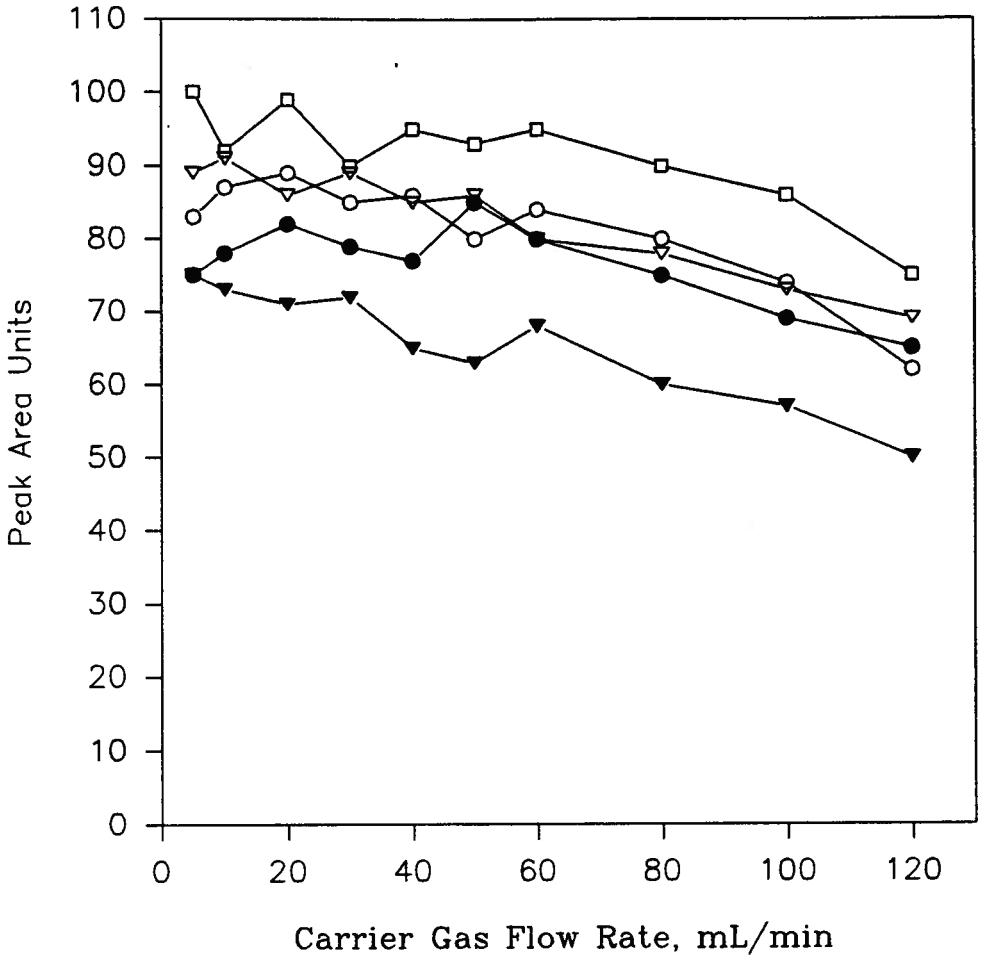


Figure 2.7 Effect of the carrier gas flow rate (in reaction coil) on the determination of hydride-forming arsenicals. (O) arsenite (●) arsenate (∇) MMA (▼) DMA (□) TMAO

reactants spend in the reaction coil. The high flow rate can also carry more water vapor to the moisture trap and the hydride trap, causing clogging of both traps. The slow flow rate does not affect the response of the arsines, but increases the operation time. The optimum flow rate of the carrier gas that does not alter arsenic response, and provides a reasonable operation time without clogging the moisture and hydride traps is 30 mL/min. The flow rate of the second carrier gas, used as the GC carrier gas, could affect the separation of AsH_3, CH_3AsH_2, $(CH_3)_2AsH$, and $(CH_3)_3As$. If the flow rate is above 70 mL/min, the elutants are not separated completely. When the flow rate is reduced to 10 mL/min, the elution time can be as long as 6 min. A good separation of arsines as well as a reasonable retention time can be achieved when the carrier gas flow rate is 20 mL/min, and so this flow rate is adopted in the work.

2.3.2.3 Interference studies

Interferences with the arsenic determination may be encountered during the formation of arsine in the hydride generation vessel, during transport to the detector, or in the detector. It has been reported that the presence of certain anions[173-175] and cations[143,144,173,176-182] can influence the production of arsine. Some transition metal ions can suppress arsine evolution during the reduction stage of hydride generation.[143] Part of these interferences can be avoided by adding complexing agents to the arsenic-containing analyte. Complexing agents that have been used include thiosemicarbazide, 1,10-phenanthroline, EDTA, tartaric acid, thiourea and potassium iodide.[144,176-182]

Possible interference effects were assessed by comparing the result of standard arsenic solutions in the absence or presence of Fe^{3+} ($FeCl_3$), Zn^{2+} ($Zn(NO_3)_2$), Mn^{2+}

($MnCl_2$), Cu^{2+} ($Cu(NO_3)_2$), Co^{2+} ($CoCl_2$), and Ni^{2+} ($NiCl_2$). In the present work, the concentrations of each arsenic species and the interfering metal ions used were 20 ppb, and 10 ppm, respectively. A 3 mL of sample was used for most of the studies. Table 2.5 shows the effect of various metal ions on the response of arsenic species in two acidic systems. The effects are expressed as percentage deviations from the interference-free response. Differences of less than 5.0% may not be significant. Deviations greater than 10% are considered to be the result of metal ion interference.

In the hydrochloric acid reaction medium, the metal ions interfere with the reduction of all the arsenic species. Nickel has the greatest effect: it suppresses the signal responses of arsine, CH_3AsH_2 and $(CH_3)_2AsH$ by almost 40%. When acetic acid is used for arsenic speciation analysis, the interference is quite significant in the presence of Fe^{3+}, Zn^{2+}, Cu^{2+}, and Ni^{2+}. These results are consistent with those found previously.[143,177]

The chelating agent EDTA was added in an attempt to remove the metal ion interferences in the two acid systems. It was found that mixing 2 mL of 10% (w/v) EDTA (disodium salt) with the samples containing one of the five metals (10 ppm) 3 min before the reduction, effectively prevents interference from the metal ions. A quantitative recovery (90-100%) of arsenic was achieved under these conditions.

2.3.2.4 Calibration, limit of detection and precision of HG-GC-AA analysis

Quantification of arsenicals can be easily achieved through calibration curves derived from different standard arsenicals. Typically, calibration curves are obtained daily for the arsenic compounds by plotting absorbance (peak area) against the amount of arsenicals in the standard arsenic solutions. The responses of arsenate and arsenite are

Table 2.5 Percentage deviations of the response of arsenic species in the presence of interfering ions (10 ppm)

Interfering ions	arsenite	arsenate	MMA	DMA	TMAO
			in the presence of HCl		
Fe^{3+}	+7	+12	-10	-13	-21
Zn^{2+}	-12	-5	-7	-12	-10
Mn^{2+}	+7	+5	-10	+5	-21
Cu^{2+}	-17	-22	-12	-15	-35
Co^{2+}	-15	-12	-17	-11	-23
Ni^{2+}	-38	-32	-41	-29	-45
			in the presence of acetic acid		
Fe^{3+}	-15	-10	-17	-12	-15
Zn^{2+}	-23	-10	-15	-23	-33
Mn^{2+}	+5	+7	-8	-14	-12
Cu^{2+}	-28	-33	-35	-50	-45
Co^{2+}	+5	+10	-3	+15	-15
Ni^{2+}	-70	-55	-62	-71	-80

similar when 1.0 M hydrochloric acid is used (Figure 2.3). Therefore, the calibration curves for arsenate and arsenite can be used interchangeably to calculate the total amount of inorganic arsenic (arsenate and arsenite). The quantification of MMA can be achieved by using the calibration curve obtained in 1.0 M hydrochloric acid. When 4.0 M acetic acid is used, the hydride production from arsenate is completely suppressed, and under these conditions arsenite, DMA and TMAO can be quantified. The calibration curves are linear to 200 ng for arsenate, arsenite and MMA, and 250 ng for DMA and TMAO when 1.0 M HCl is used. Under 4.0 M acetic acid conditions, the calibration curves are linear to 200 ng for arsenite, DMA and TMAO, and 400 ng for MMA.

On the basis of 8 replicate runs of standards containing 60 ng of arsenic, the analytical precision for arsenate and arsenite are 7% relative standard deviation (% RSD), 5% RSD for MMA, 9% RSD for DMA, and 9% RSD for TMAO.

The limits of detection, defined as the amount of analyte which gives a signal equal to the blank plus three standard deviations of the blank, are 0.20 ng (of arsenic) for arsenate and arsenite, 0.15 ng for MMA, and 0.30 ng for DMA and TMAO in the presence of 1.0 M hydrochloric acid. When 4.0 M acetic acid is used, the detection limits are 0.20 ng for arsenite, DMA and TMAO, and 0.40 ng for MMA.

CHAPTER 3

THE IDENTIFICATION OF EXTRACELLULAR ARSENICAL METABOLITES IN THE GROWTH MEDIUM OF MICROORGANISMS

3.1 INTRODUCTION

The first investigation of the biological production of an arsine was published in 1891 and was concerned with the formation of "Gosio gas" (Section 1.1.2).[28] It was only after Challenger started to work on the problem that the metabolic product was correctly identified as trimethylarsine.[30] A metabolic pathway was proposed for the biomethylation of arsenicals to trimethylarsine (Figure 1.1).[30,43] This pathway is made up from two basic steps: (1) reduction of the arsenic(V) species to arsenic(III) species, possibly oxides; and (2) subsequent oxidative methylation to methylarsenic(V) moieties.

Evidence supporting Challenger's pathway has come from studies on *Scopulariopsis brevicaulis* and *Apiotrichum humicola* (previously known as *Candida humicola*).[30-35,37] Experiments demonstrated that arsenate, arsenite, MMA, DMA, and TMAO served as precursors for trimethylarsine synthesis. These arsenicals are likely intermediates in the biomethylation reactions and are known to be present in the environment and the biosphere. However, the presence of arsenite, MMA, DMA and TMAO as metabolic intermediates in the biomethylation of, for instance, arsenate, has rarely been reported.[2] Challenger stated that arsenic intermediates from the proposed metabolic pathway (Figure 1.1) were not found in the culture medium of *S. brevicaulis*, although no details were given regarding the methodology used to support this conclusion.[43] Baker *et al.*[183] incubated arsenite and arsenate, respectively, in a nutrient medium containing sediment collected from a small acidic, oligotrophic lake. By using

HG-GC-AA techniques, they reported that 0-0.7% of the total arsenic was transformed to MMA and DMA and a variety of microorganisms are believed to have contributed to these biological methylation processes. Cullen *et al.*[51] incubated labeled arsenicals with broken-cell homogenates of *Apiotrichum humicola* in order to look for metabolic intermediates in the growth medium. They employed a combination of molecular sieving, anion exchange, and electrophoresis to separate the arsenicals from each other. Arsenite, MMA and DMA were found to be metabolites of [^{74}As]arsenate, and TMAO was a metabolite of [^{14}C]methylarsonate and [^{14}C]dimethylarsinate. In addition, a demethylation product, [^{14}C]methylarsonate, was observed when [^{14}C]dimethylarsinate was used. Replacement of the cell preparation by buffer failed to bring about any transformations. Hence, the various compounds identified represent probable intermediates in the biosynthesis of trimethylarsine. When *S. brevicaulis* and *A. humicola* were treated with the model arsenic(III) intermediate $(CH_3AsO)_n$, the production of DMA, together with trace amounts of MMA and trimethylarsine was observed.[45] This was the first time that non-volatile methylated intermediates had been identified in the growth medium of a pure culture.

In the present work we report on the effect of adding low levels of four arsenic compounds, arsenate, arsenite, MMA and DMA to cultures of the microorganisms *A. humicola* and *S. brevicaulis* growing aerobically in a liquid medium. Hydride generation-gas chromatography-atomic absorption spectrometry (HG-GC-AA) was used to identify the extracellular arsenic metabolites present in the culture medium.

3.2 EXPERIMENTAL

3.2.1 Reagents

All chemicals and reagents are as previously described in Section 2.2.1.

3.2.2 Microorganism cultures

Apiotrichum humicola was obtained from the American Type Culture Collection (ATCC 26699) and *Scopulariopsis brevicaulis* was provided by the "Fungus Culture Collection" of the Chemistry Department at U.B.C. The cultures were grown aerobically in a synthetic inorganic liquid medium at pH 5 as described by Cox and Alexander (Table 3.1).[32]

Table 3.1 Composition of the growth medium (1 L)[32]

$(NH_4)_2SO_4$	2.0	g
KH_2PO_4	0.1	g
$MgSO_4.7H_2O$	0.05	g
$FeSO_4.7H_2O$	0.0018	g
thiamine hydrochloride	0.01	g
glucose	10	g
succinic acid buffer (pH 5)	0.05	M

3.2.3 Hydride generation-gas chromatography-atomic absorption spectrometry (HG-GC-AA)

The HG-GC-AA system described in Section 2.3.2 was used for arsenic analysis. A peristaltic pump was used to mix the sample solution (1-3 mL) with an acid (1.0 M

HCl or 4.0 M acetic acid) and 2.0% (w/v) $NaBH_4$ solutions, and the arsines produced were collected in a U-tube immersed in liquid nitrogen. After warming the hydride trap in a water bath (70°C), these arsines were separated in a Porapak-PS GC column by using a Hewlett Packard Model 5830A gas chromatograph. An 810 Jarrell-Ash atomic absorption spectrometer was used as the arsenic detector at 193.7 nm. The arsines were atomized in an air/hydrogen flame in a quartz cuvette. The signal was recorded on a Hewlett Packard 3390A integrator.

3.2.4 Experimental procedures

Aqueous solutions of the appropriate arsenical were filter sterilized (0.2 μm membrane) separately and added to the autoclaved culture medium in the flask. Typically, a 10 mL aliquot of an actively growing culture of *A. humicola* or a 2 mL aliquot of an actively growing culture of *S. brevicaulis* was added to 250 mL of the medium which contained 1 ppm of arsenic. During the growth period the cultures were maintained at 21-22°C and were agitated by using a rotary shaker running at 130 rpm. Once each day for 2 weeks, a 6 mL culture aliquot was removed and stored frozen prior to HG-GC-AA analysis. After the first 2 weeks of incubation, a glass microfiber paper pre-soaked in 5.0% mercuric chloride was suspended in the head-space of cultures to trap the volatile arsines (chemofocusing) as shown in Figure 3.1.[36] The medium (6 mL) was then collected once each week for another 2 to 3 weeks. The experiment was terminated after 4 weeks incubation of *A. humicola* and 5 weeks incubation of *S. brevicaulis*. Terminal cultures were centrifuged, and the cells were washed and freeze-dried for future analysis.

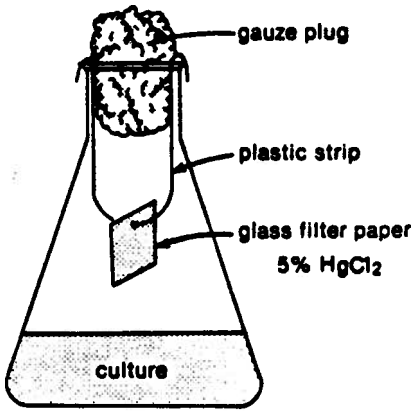


Figure 3.1 The growth flask fitted with the mercuric chloride trap.

To determine the arsenic species in the cells, the freeze-dried samples were weighed and transferred into an Erlenmeyer flask (250 mL) containing 30 mL of mixed solvent $CHCl_3$/MeOH/H_2O (1/1/1). The mixture was sonicated for 2 hours and then agitated on a mechanical shaker for 24 hours. The extracts were centrifuged to separate the aqueous fraction from the organic fraction. The aqueous layer was kept at -4°C prior to analysis. The organic extract and the residue were air dried, digested in 4 mL of 2.0 M NaOH in a water bath at 95°C for 3 hours, then neutralized with 6.0 M hydrochloric acid prior to HG-GC-AA analysis.

The hydride generation system described in Section 2.3.2 was used to identify hydride-forming arsenicals in growth media as well as in the cells. To determine the arsenic species in the growth medium, the sample was diluted to an appropriate volume before it was subjected to HG-GC-AA analysis. Usually, 0.2 mL of the sample in the growth medium was diluted to 3 mL and mixed with 1.0 M HCl or 4.0 M acetic acid and 2.0% (w/v) $NaBH_4$. To monitor the production of arsenite from arsenate by

microorganisms, the hydrochloric acid solution was replaced by 4.0 M acetic acid. Under this condition, arsenate is not reduced to arsine by sodium borohydride solution.

During culture incubations, the presence of trimethylarsine was assessed by using two methods: one based on odor and the other on chemofocusing. An intense and distinctive garlic-like odor has been used as qualitative evidence of arsine production.[184-188] The odor threshold for $(CH_3)_3As$ now appears to be 2 pg. g^{-1} in dilute aqueous solution.[189] This allows qualitative evaluation of arsine production by cautious sniffing of the gas in the culture head-space. The chemofocusing method has also proved to be an effective means to trap the volatile arsines.[36] If trimethylarsine is produced, crystals of the $HgCl_2$ adduct are formed on a glass fiber filter soaked in 5.0% mercuric chloride solution that is suspended in the head-space of cultures (Figure 3.1). Subsequent heating of the filter decomposes the mercuric chloride adduct and the volatile arsines are released for mass spectrometric analysis.[36]

3.3 RESULTS

A. humicola and *S. brevicaulis* grown in the presence of each of the four substrates arsenate, arsenite, MMA, and DMA produced a number of compounds; therefore each substrate will be discussed separately.

3.3.1 Transformation of arsenate

The growth medium was analyzed by using HG-GC-AA. Selective reduction of arsenicals can be accomplished by varying the pH, and this was used to distinguish arsenate from arsenite in the samples. Thus when 1:0 M HCl is used, both arsenate and

arsenite in these samples are reduced to AsH_3 by 2.0% (w/v) sodium borohydride. In the presence of 4.0 M acetic acid, arsenite forms arsine, but arsenate does not.

A. humicola exposed to 1 ppm arsenate reduced more than 90% of the substrate to arsenite (detected as AsH_3) within 2 days of incubation as shown in Figures 3.2 and 3.3. The chromatograms of AsH_3 in Figure 3.2 were obtained when 1.0 M HCl was used to assist the hydride production. The AsH_3 was derived from arsenate in the sample on day 0, and from a mixture of arsenate and arsenite in the sample on day 2. Figure 3.3 shows that only traces of arsenite, detected as AsH_3 following reduction in the presence of acetic acid, were in the sample on day 0. However, a strong AsH_3 signal was observed on day 2 under the same conditions, indicating the production of arsenite from arsenate. Oxidation of the arsenite to arsenate was not observed during the rest of the growth period although the concentration of arsenite in the growth medium further decreased with time, and only background levels were detected after 4 weeks of incubation (Figure 3.4). In addition to arsenite, small amounts of DMA and TMAO were detected as $(CH_3)_2AsH$ and $(CH_3)_3As$, respectively, in the culture medium collected on day 5. The concentration of DMA increased from 0 to 0.02 ppm by day 7, but a further increase was not observed. The TMAO concentration in the growth medium increased rapidly with time, reaching 0.75 ppm at the end of the fourth week. The formation of the CH_3AsH_2 from MMA is largely suppressed when the hydride generation is carried out in acetic acid as described in Chapter 2, and trace amounts of MMA (if present in the growth medium) would not be detected. However, when HCl was used instead the acetic acid, trace amounts of MMA were detected as CH_3AsH_2 in the culture medium after 2 weeks of incubation, but the concentration remained constant for a further 2 weeks. The change in the concentrations of the arsenic species as a function of incubation time is shown in Figure 3.5. It should be noted that adsorption of

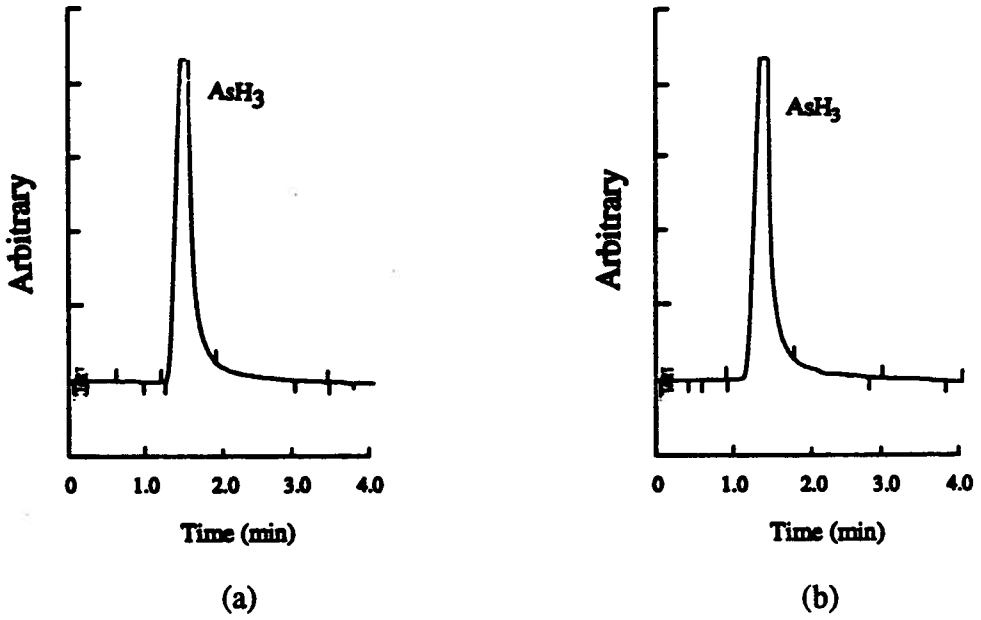


Figure 3.2 Chromatograms of arsenic compounds in the growth medium of *A. humicola* (enriched with 1 ppm arsenate) were obtained by using HG-GC-AA with 1.0 M HCl and 2.0% (w/v) $NaBH_4$. (a) the growth medium collected on day 0, (b) the growth medium collected on day 2

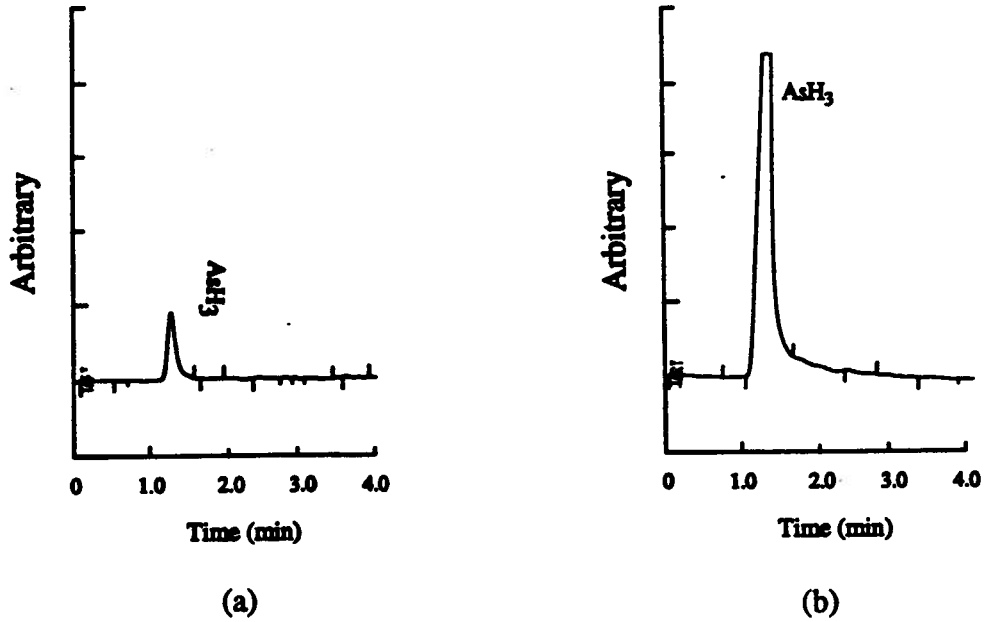


Figure 3.3 Chromatograms of arsenic compounds in the growth medium of *A. humicola* (enriched with 1 ppm arsenate) were obtained by using HG-GC-AA with 4.0 M acetic acid and 2.0% (w/v) $NaBH_4$. (a) the growth medium collected on day 0, (b) the growth medium collected on day 2

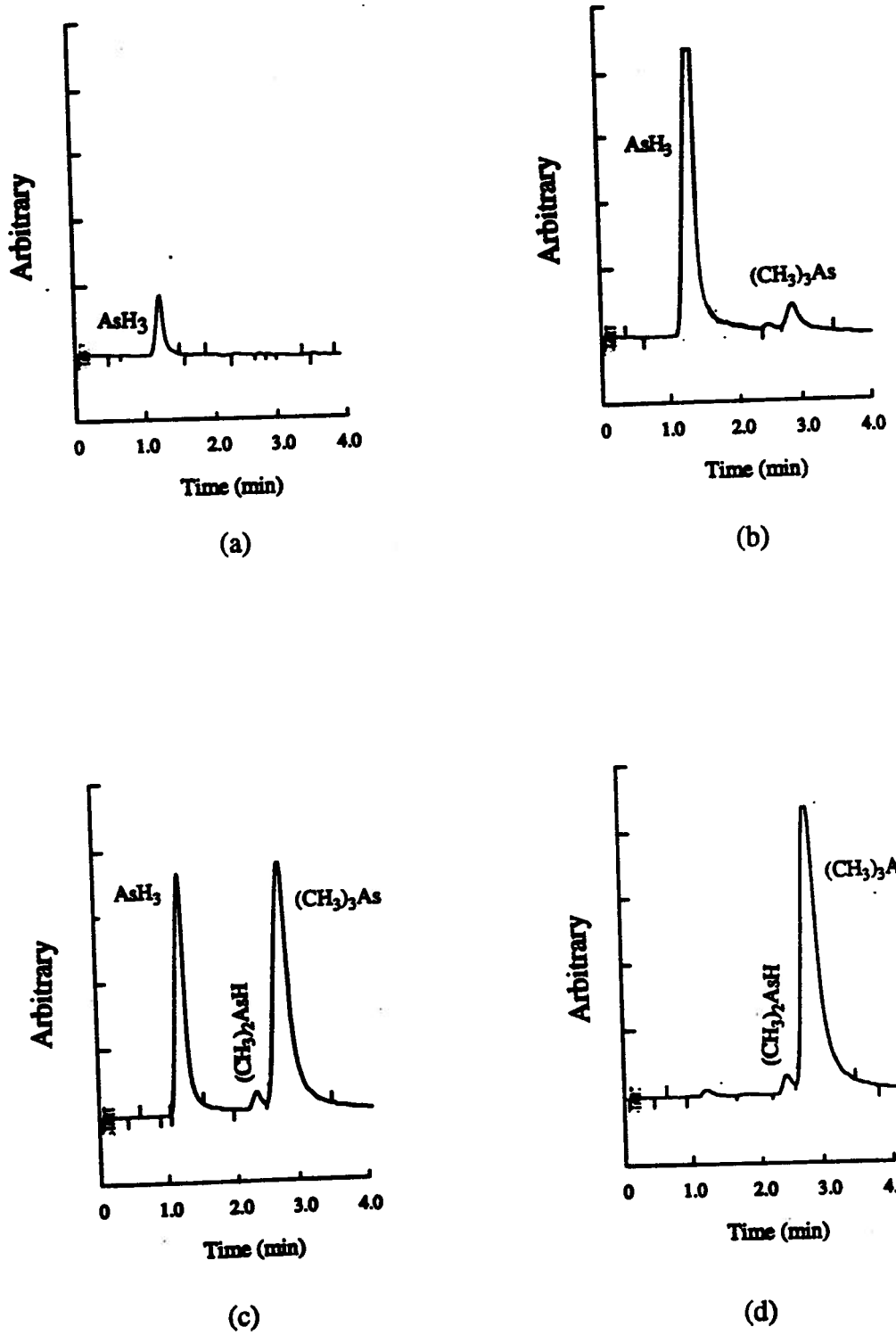


Figure 3.4 Chromatograms of arsenic compounds in the growth medium of *A. humicola* (enriched with 1 ppm arsenate) were obtained by using HG-GC-AA with 4.0 M acetic acid and 2.0% (w/v) $NaBH_4$. (a) the growth medium collected on day 0, (b) the growth medium collected on day 5, (c) the growth medium collected on day 15, (d) the growth medium collected on day 28

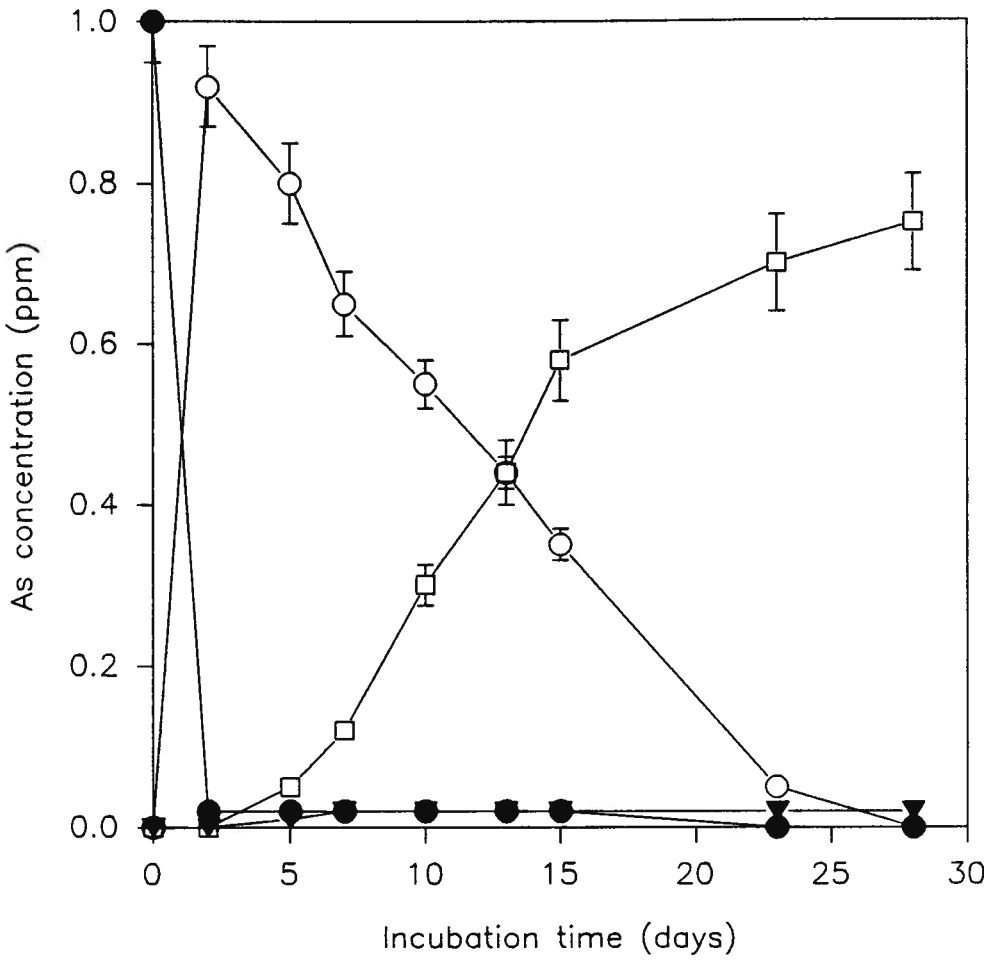


Figure 3.5 The change in arsenic concentrations in the growth medium of *A. humicola* enriched with 1 ppm arsenate. (O) arsenite (●) arsenate (▼) DMA (□) TMAO
****The values are averages of four replicate determinations by HG-GC-AA of separate aliquots (0.2 mL) from the 6 mL sample taken at each time interval. The error bars represent 90% confidence intervals, and are smaller than the size of the symbols when they are not evident on the graph.**

TMAO on the surface of the cells can also take place; the concentration of TMAO in the growth medium decreases dramatically if the sample is not shaken before analysis (Figure 3.6).

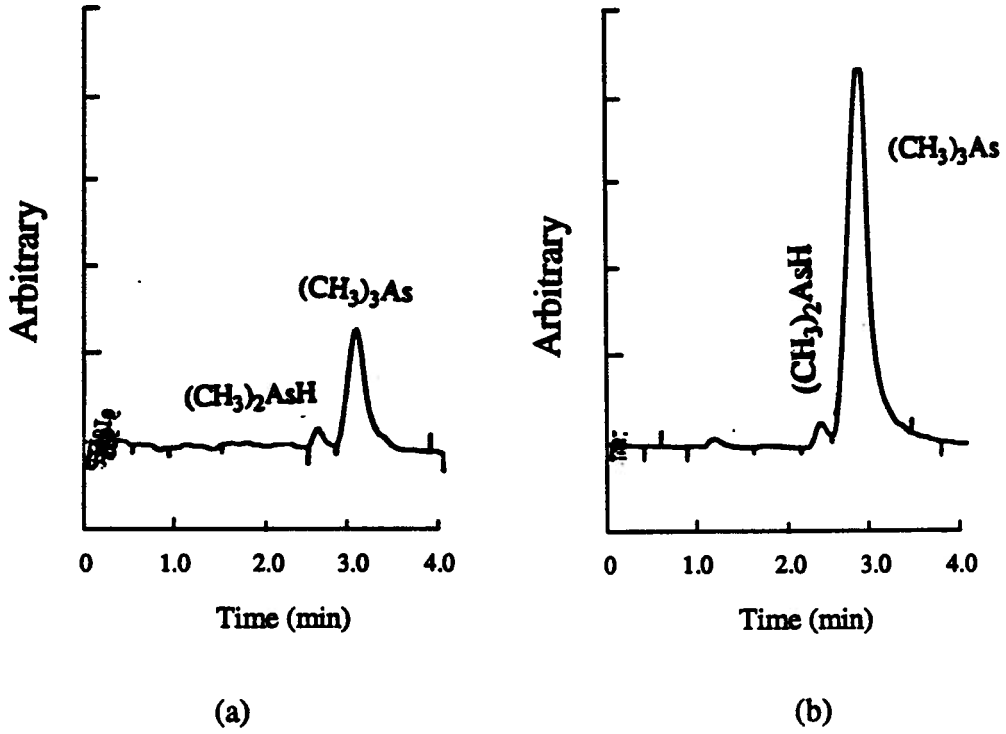


Figure 3.6 The effect of adsorption by the cells of *A. humicola* on the concentration of TMAO in the growth medium. (a) without agitation before analysis, (b) with agitation before analysis.

The change of arsenic speciation in the growth medium of *S. brevicaulis* is less dramatic. The total reduction of arsenate to arsenite was achieved 4 days after inoculation of the stock culture. The quantity of arsenite (~ 1 ppm) in the medium did

not change significantly over a 5 week incubation period. TMAO (0.01 ppm) and trace amounts of DMA were detected on day 5. The amount of TMAO had increased slowly to approximately 0.04 ppm at the end of the experiment. During this period, the quantity of DMA did not change significantly. No MMA was detected in these samples.

Neither *A. humicola* nor *S. brevicaulis* produced trimethylarsine as judged by the odor test or by the use of the chemofocusing trap. In the latter case no arsine was detected by mass spectrometry when strips of the filter were analyzed.

No significant amounts of arsenic were found in cells of *A. humicola* and *S. brevicaulis* at the end of the incubation time by using HG-GC-AA.

3.3.2 Transformation of arsenite

Actively growing cultures of *A. humicola* (10 mL) and *S. brevicaulis* (2 mL) were inoculated with 250 mL of liquid media containing arsenite (1 ppm) and grown aerobically at 21-22°C for 4 or 5 weeks, respectively.

The concentration of arsenite in the culture medium of *A. humicola* decreased rapidly from 1 ppm to 0.29 ppm in 2 weeks, and reached a background level after 4 weeks of incubation (Figure 3.7). The rate of arsenite disappearance was similar to that discussed in Section 3.3.1. The oxidation of arsenite to arsenate in the growth medium was not observed. Both TMAO and DMA were detected as $(CH_3)_3As$ and $(CH_3)_2AsH$, respectively, in the growth medium on day 5. The DMA concentration increased from 0 to 0.02 ppm in 2 weeks of incubation, and no further change was found thereafter. The TMAO concentration increased from 0.05 ppm on day 5 to 0.69 ppm at the end of the fourth week. Trace amounts of MMA were also detected after 2 weeks of incubation, but the quantity of MMA did not increase significantly over a further 2 weeks

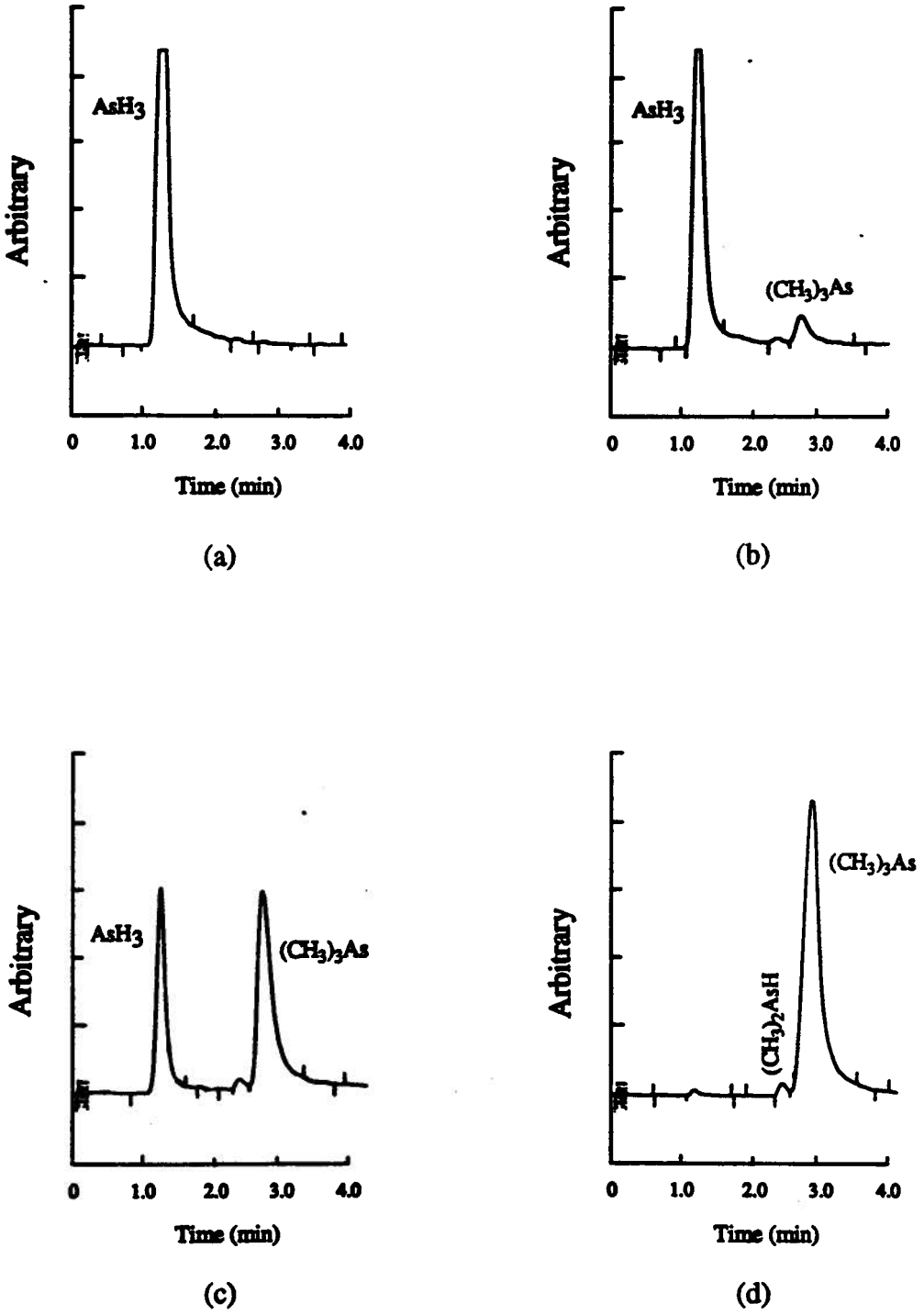


Figure 3.7 Chromatograms of arsenic compounds in the growth medium of *A. humicola* (enriched with 1 ppm arsenite) were obtained by using HG-GC-AA with 4.0 M acetic acid and 2.0% (w/v) $NaBH_4$. (a) the growth medium.collected on day 0, (b) the growth medium collected on day 5, (c) the growth medium collected on day 15, (d) the growth medium collected on day 28

incubation. The change in concentrations of arsenite, DMA and TMAO in the growth medium is shown in Figure 3.8. The adsorption of TMAO on the surface of the cells of *A. humicola* was also observed.

In the growing culture of *S. brevicaulis*, the substrate arsenite was not oxidized to arsenate, and the change in its concentration was minimal. TMAO and trace amounts of DMA were detected after 8 days of incubation. TMAO in the medium increased slowly with time, reaching about 0.04 ppm at the end of 5 weeks of incubation. There was no further increase in the concentration of DMA over the same period. No MMA was detected in the culture medium.

No significant accumulation of arsenic in cells of *A. humicola* and *S. brevicaulis* was found by using HG-GC-AA.

No trimethylarsine was produced by the two microorganisms in the experiments.

3.3.3 Transformation of methylarsonate

Following the addition of MMA, DMA (0.02 ppm in the medium) and TMAO (0.01 ppm in the medium) were detected in the *A. humicola* culture on the second day of incubation (Figure 3.9). The concentration of DMA and TMAO increased to 0.15 ppm and 0.33 ppm, respectively, at the end of the experiment. The MMA concentration in the medium decreased from 1 ppm to 0.5 ppm at the end of the incubation. The change in arsenic concentration is shown in Figure 3.10. TMAO adsorption on the surface of cells of *A. humicola* was observed as judged by a significant increase in TMAO concentration in the sample after it was agitated a few times.

For *S. brevicaulis*, TMAO and trace amounts of DMA were detected in the growth medium after 2 weeks of incubation. TMAO concentration had increased from 0

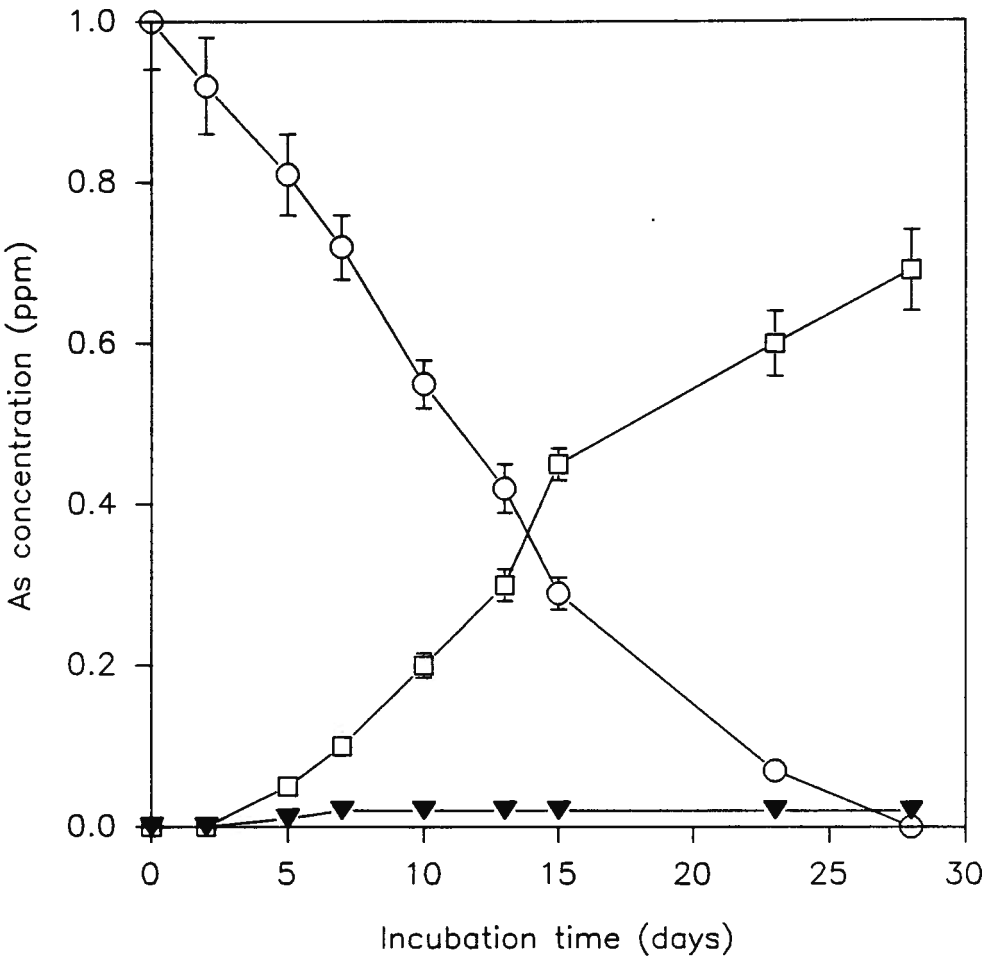


Figure 3.8 The change in arsenic concentrations in the growth medium of *A. humicola* enriched with 1 ppm arsenite. (O) arsenite (▼) DMA (□) TMAO

****The values are averages of four replicate determinations by HG-GC-AA of separate aliquots (0.2 mL) from the 6 mL sample taken at each time interval. The error bars represent 90% confidence intervals, and are smaller than the size of the symbols when they are not evident on the graph.**

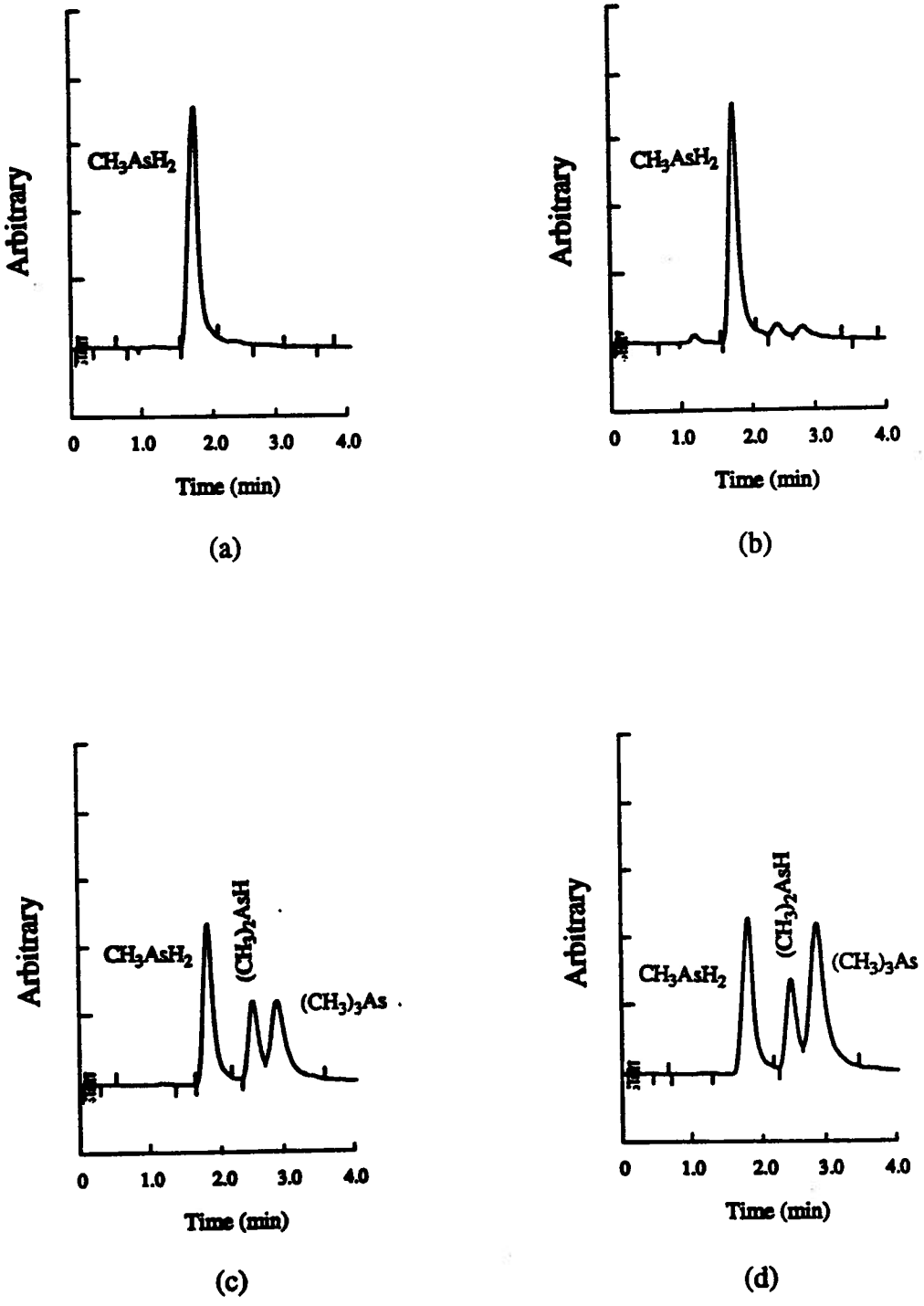


Figure 3.9 Chromatograms of arsenic compounds in the growth medium of *A. humicola* (enriched with 1 ppm MMA) were obtained by using HG-GC-AA with 4.0 M acetic acid and 2.0% (w/v) $NaBH_4$. (a) the growth medium collected on day 0, (b) the growth medium collected on day 2, (c) the growth medium collected on day 15, (d) the growth medium collected on day 28

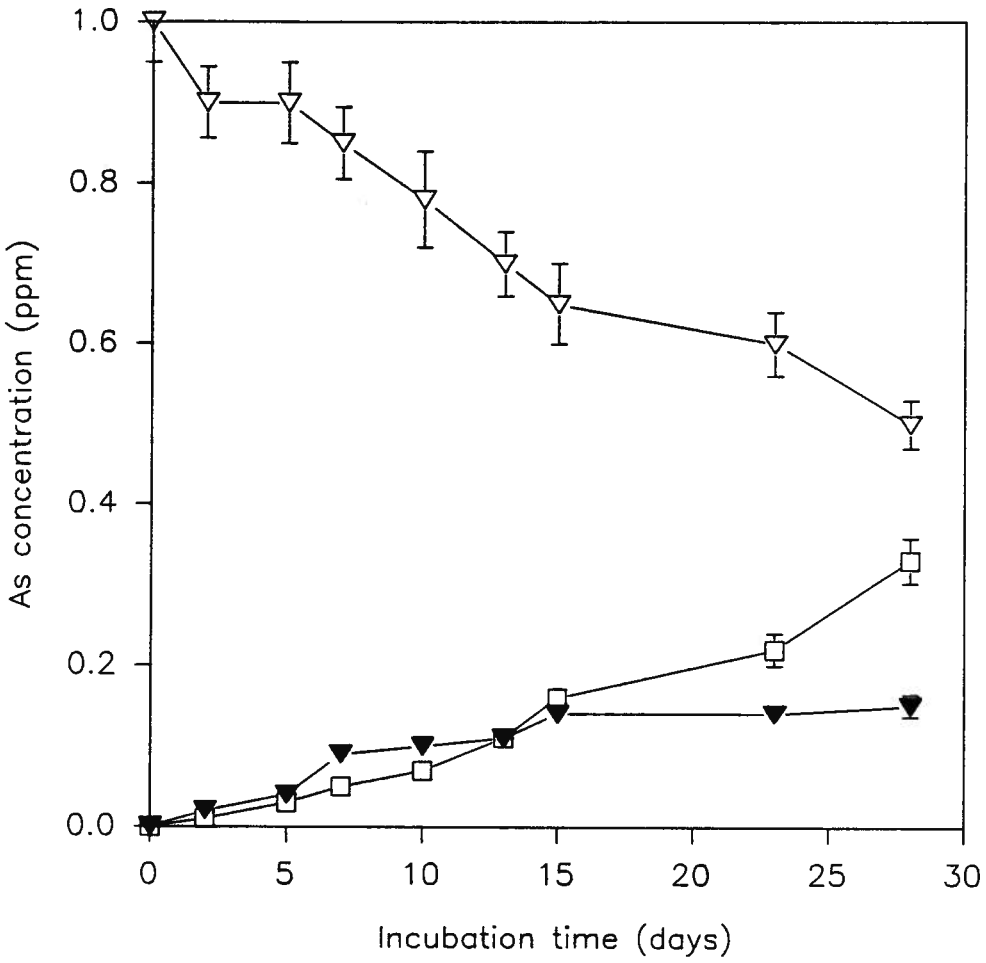


Figure 3.10 The change in arsenic concentrations in the growth medium of *A. humicola* enriched with 1 ppm MMA. (∇)MMA (▼) DMA (□) TMAO

****The values are averages of four replicate determinations by HG-GC-AA of separate aliquots (0.2 mL) from the 6 mL sample taken at each time interval. The error bars represent 90% confidence intervals, and are smaller than the size of the symbols when they are not evident on the graph.**

to 0.02 ppm when the experiment was ended. The MMA concentration in the medium did not change significantly in the time period of the experiment.

No trimethylarsine was detected when MMA (1 ppm) was incubated with *A. humicola* or *S. brevicaulis*. The possible demethylation products, arsenate and/or arsenite were not observed.

Accumulation of arsenicals in the cells of both microorganisms was not observed.

3.3.4 Transformation of dimethylarsinate

Following the addition of DMA, trace amounts of TMAO were detected in the growing culture of *A. humicola* after 15 days of incubation, and the decrease in the DMA concentration was insignificant.

TMAO was detected in the culture medium of *S. brevicaulis* but not until day 10. The concentration of TMAO increased with time and was about 0.07 ppm in the growth medium after 5 weeks of incubation.

Incubation of *A. humicola* and *S. brevicaulis* with DMA (1 ppm) did not produce detectable amounts of trimethylarsine or demethylation products, such as MMA.

The accumulation of arsenicals in cells of *A. humicola* and *S. brevicaulis* was not detected by using HG-GC-AA.

3.4 DISCUSSION

A combination of molecular sieving, anion exchange, and electrophoresis has proved to be a reliable method to isolate and identify the arsenicals present in biological

material.[45,51] These procedures, however, are lengthy and time consuming especially when dealing with large numbers of samples; there may also be problems with detection when the concentration of analytes is low. The HG-GC-AA system used in this study can minimize the time for analysis (less than 10 min per sample) and can be used to detect low levels of arsenate, arsenite, MMA, DMA and TMAO in samples provided that it is assumed that these are the precursors to the arsines that are ultimately formed and quantified. On this basis, the technique is capable of determining the concentration of these extracellular arsenic metabolites at concentrations which are too low to be detected by other convenient techniques. However, a possible disadvantage of the method is that the separation and identification of the arsenic species is based on the properties of derivatives of the arsenicals in the medium rather than on the properties of the arsenicals themselves.

Exposure of *A. humicola* and *S. brevicaulis* to arsenate yields arsenite, DMA and TMAO in the growth medium, and MMA is found in the growth medium of *A. humicola* but not *S. brevicaulis*. The substrate arsenite is metabolized by *A. humicola* to produce MMA, DMA and TMAO and by *S. brevicaulis* to produce DMA and TMAO. Both microorganisms transform MMA to DMA and TMAO, and DMA to TMAO.

The identification of these arsenicals is noteworthy as it is the first time that any of the non-volatile arsenic intermediates (shown in Figure 1.1) have been identified in the growth medium of a pure culture spiked with either arsenate, arsenite, MMA or DMA. As was mentioned in the introduction, methylated intermediates have only been isolated from broken-cell extracts of *A. humicola*[51] and from the growth medium of cultures of *A. humicola, S. brevicaulis, V. alcalescens* and *L. brevis* when methylarsine oxide [$(CH_3AsO)_n$] was used as a substrate.[45]

The reduction of arsenate to arsenite is the first step towards methylation in Challenger's mechanism.[30,43] Our results support this assertion, as most of the arsenate is reduced to arsenite prior to the detection of any methylated arsenicals in the medium. The production of the anticipated methylated intermediates from the substrates, the absence of oxidation of arsenite to arsenate, and the lack of demethylation products strongly support the metabolic sequence of Figure 1.1 proposed by Challenger.[30,43]

Cells of *A. humicola* take up and metabolize arsenate to arsenite, and then excrete the arsenite into the medium. The arsenate is believed to be reduced inside the cells by thiols or dithiols as a detoxification process.[2] The uptake of arsenate by the cells probably involves an active transport system because when the phosphate concentration is equimolar with arsenate, the rate of arsenate uptake by *Apiotrichum humicola* is reduced to 18% of that observed in the absence of phosphate.[190] The uptake of MMA and DMA probably occurs by means of slow passive diffusion.[190]

Cullen *et al.*[51] reported that only trace amounts of MMA are produced from arsenate by broken cell homogenates of *A. humicola* and that MMA is the least transformed arsenical by the broken-cell homogenates. They speculated that MMA would not be found as a free intermediate in Challenger's arsenate-to-trimethylarsine pathway. The present results show that MMA is a metabolite of arsenate and arsenite in whole cell cultures of *A. humicola*, but it is produced in a much lower concentration than either DMA or TMAO. We also find that the production of DMA in the growth medium of *A. humicola* is a rapid process when MMA is used as a substrate since DMA and TMAO are detected within 2 days of incubation. This result indicates that MMA, if it exists as an intermediate in the arsenate/arsenite-trimethylarsine methylation process, would be metabolized rapidly inside the cells. Therefore, little MMA would be excreted and detected as an extracellular metabolite of the cells. Cullen and Nelson[191-193] have

studied the biomobility of MMA and DMA by measuring the rate at which these arsenicals diffuse through the walls of liposomes, regarding these as models for biological membranes. It was found that the membranes are less permeable to MMA than DMA. The diffusion coefficient of MMA is 10 times lower than DMA. Because of the low diffusion coefficient, it is possible that the cells metabolize the endocellular MMA to DMA and TMAO faster than MMA can diffuse into the growth medium. The DMA thus produced has a higher permeability coefficient, and can diffuse into the growth medium. This premise seems to be consistent with what we have observed.

In our work, the transformation of DMA to other arsenic metabolites is a slow process in cultures of *A. humicola*. This is a surprising result as DMA has been found to be a better precursor to trimethylarsine than is arsenate or MMA.[32] We suggest that these latter experiments were carried out with high concentrations of arsenicals (> 100 ppm) and that this may be necessary to trigger the methylation process from DMA to $(CH_3)_3As$, as is discussed next. Other factors may also affect the low production of TMAO, and they are discussed in the proposed methylation model.

Apart from the substrate DMA, which is hardly metabolized, the concentrations of all other arsenic substrates decrease with time in the growing culture of *A. humicola*, corresponding to an increase in the concentrations of arsenic metabolites. Only trace amounts of inorganic arsenic are detected in the growth medium at the end of incubations when arsenate or arsenite is used as a substrate: the major transformation product is TMAO. The total concentration of arsenicals in the two growing cultures is relatively constant during the growth period (approximately 0.8-0.9 ppm), indicating that the uptake, the methylation, and the excretion of arsenic proceed simultaneously. Since neither the production of the volatile trimethylarsine is observed during the growth period nor significant amounts of arsenic are found in the cells harvested at the

end of the incubations, the small decrease in the total arsenic concentration of the two growth media may be due to the adsorption of TMAO on cell surface of *A. humicola*. When MMA is used as a substrate, the arsenic speciation in the growth medium is changed dramatically while the total concentration of arsenic is kept relatively constant (0.9-1.0 ppm). This also indicates that the accumulation of arsenic and the production of volatile trimethylarsine by the cells are limited.

The detection of TMAO in the growing culture medium of microorganisms is a very significant observation. Not only is this the first time that it has been found as an extracellular metabolite of fungi, but also it is the major arsenic metabolite of the microorganism. It is well known that both *A. humicola* and *S. brevicaulis* methylate arsenate, arsenite, MMA and DMA to trimethylarsine.[30-35] However, this volatile arsine was not detected during the present experiments. It is unlikely that this volatile metabolite is lost during sampling since the chemofocusing trap is an effective arsine collection device.[36] Pickett *et al.*[37] have demonstrated that TMAO can be reduced to trimethylarsine rapidly by whole cells of *A. humicola*. The rate of arsine production from TMAO increases linearly with the TMAO concentration, and is considerably faster than from arsenate or DMA. Because of this rapid reduction of TMAO, it was suggested that TMAO would not be detectable as an intermediate in cultures of *A. humicola* and that it would be unlikely to be found in the environment.[37] However, in the present experiments TMAO is found to be the major methylation product and seems to be the end product of the methylation process. It now seems likely that low concentrations of TMAO (< 1 ppm) do not greatly affect the living fungal system. Therefore, further detoxification by reducing TMAO to trimethylarsine is not necessary. Previously, much higher concentrations (> 100 ppm) of the arsenicals including arsenate, arsenite, MMA, DMA and TMAO, were added to the growing culture of *A. humicola* to produce

trimethylarsine.[30-35,37] These high concentrations were used because of analytical expedience and not for any scientific reason. The observation of TMAO is not without precedent. Kaise *et al.*[194] incubated arsenobetaine (100 ppm) in an inorganic medium containing bottom sediment collected from coastal waters. After 100 hours of incubation, arsenobetaine was degraded into TMAO completely. This degradation is believed to be caused by indiginous microorganisms. Trimethylarsine was not detected in these experiments.[194]

The limited metabolism of the arsenic substrates by *S. brevicaulis* in the growth medium is not surprising. This liquid medium was optimized by Cox and Alexander for the production of trimethylarsine by *A. humicola*: it has since been shown to support the growth of *S. brevicaulis* but no comparative growth studies were made.[32] Bread cultures of *S. brevicaulis* may be more productive than cultures in liquid media, even though the reported rates of arsine production are not great.[2]

Although our findings lend general support to Challenger's proposed pathway, they indicate that it is an over-simplification of the many processes involved. Therefore, an extended model is proposed in Figure 3.10, for the methylation of arsenate by growing cells of *Apiotrichum humicola.* At first, the cells take up arsenate from the medium through a phosphate transport system, reduce the arsenate to arsenite inside the cells probably by using thiols and/or dithiols, and excrete most of the arsenite into the growth medium, again probably by using an active transport system. This result can be achieved within 2 days. Any arsenite in the cells can be methylated to MMA by S-adenosylmethionine, SAM, but because of its low passive diffusion coefficient, most of the endocellular MMA does not diffuse into the growth medium. Rather, the endocellular MMA is more likely to be reduced and methylated to DMA and then TMAO. It is also possible that two methyl groups from SAM can be transferred to the

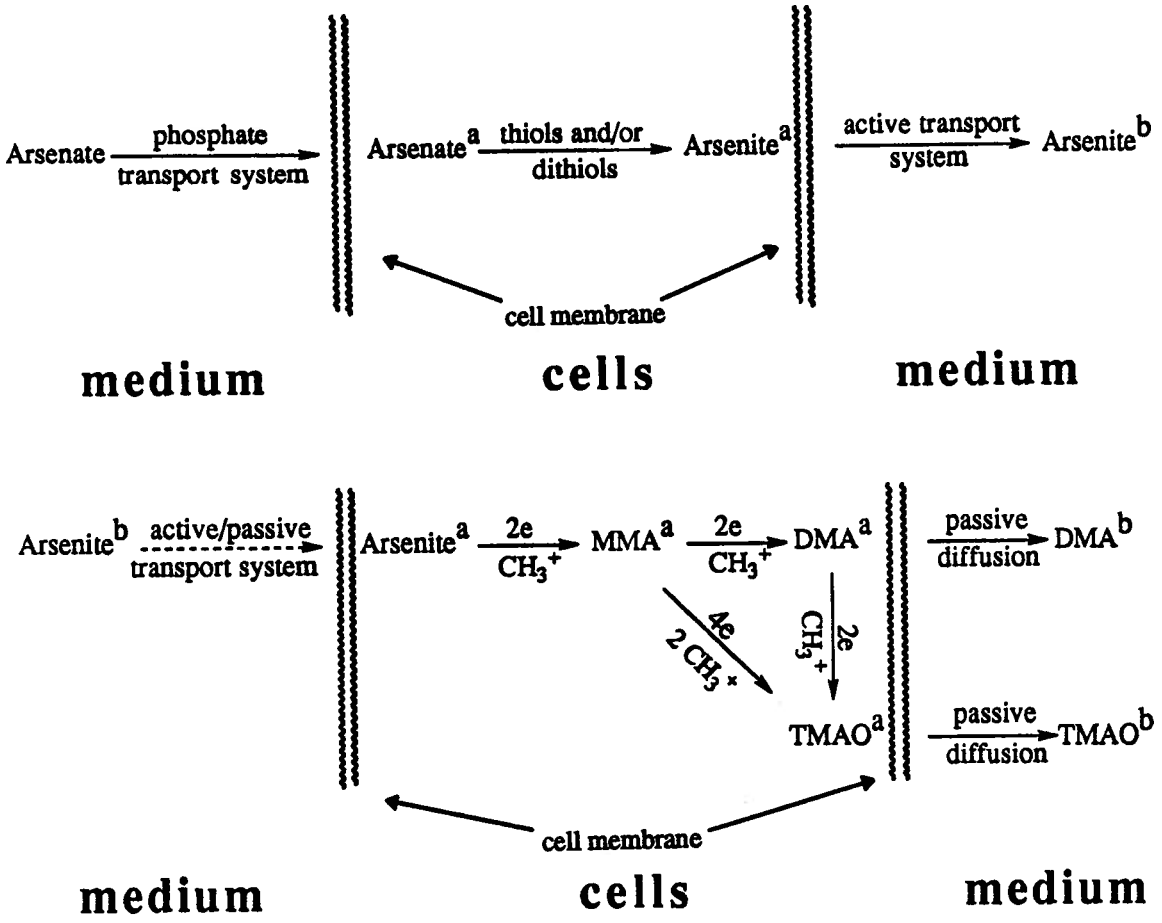


Figure 3.11 Proposed biotransformation model of arsenate in *A. humicola*.
[a] endocellular arsenicals [b] extracellular arsenicals

intermediate MMA sequentially to form TMAO, but without the formation of DMA as a free intermediate. This is indicated by the low and constant level of extracellular DMA in the growth medium and by the slow methylation process observed when DMA is used as a substrate. Both DMA and TMAO are able to diffuse into the growth medium. The excreted or added arsenite enters the cells of *A. humicola* by means of an active

transport system and/or by passive diffusion, and is metabolized in the same sequence to form MMA, DMA and TMAO. When MMA and DMA are used as substrates, their uptake is achieved by means of passive diffusion and the methylation process is similar to that presented above.

Although this model can explain some of the results of our work, the biotransformation process is undoubtedly more complex in reality.

CHAPTER 4

THE BIOMETHYLATION OF ARSENICALS BY A MICROORGANISM *APIOTRICHUM HUMICOLA* IN THE PRESENCE OF L-METHIONINE-METHYL-D_3

4.1 INTRODUCTION

Since the first systematic investigation of the biotransformation of arsenic conducted by Gosio in 1891,[28] many studies have shown that the biological methylation of arsenic is a ubiquitous phenomenon in nature;[2] however, the process is still not fully understood.[2]

Challenger showed that *Scopulariopsis brevicaulis* produced trimethylarsine from arsenate and proposed a biosynthetic pathway as outlined in Figure 1.1.[30,43] Evidence supporting the Challenger pathway has come from studies of microorganisms.[30-35,37] This work demonstrated that arsenate, arsenite, MMA, DMA, and TMAO are precursors to trimethylarsine.

Challenger suggested that some methyl containing compounds such as betaine, methionine, or a choline derivative could be the possible methyl donor to arsenic.[30] To further investigate this hypothesis, Challenger *et al.*[48] added these compounds, labeled with ^{14}C, to cultures of *S. brevicaulis* growing on bread crumbs enriched with arsenite. These experiments showed that only ^{14}C labeled methionine $^{14}CH_3SCH_2CH_2C(NH_2)HCOOH$ was able to transfer its label to arsenite to a significant extent. The maximum methylation percentage observed was 28% after 5 days of incubation. Cullen *et al.*[33] demonstrated that the CD_3 group in L-methionine-methyl-d_3 was incorporated into the trimethylarsine that was evolved from cultures of

Apiotrichum humicola and *S. brevicaulis* grown in the presence of arsenite, arsenate, MMA, and DMA. In these experiments, the arsine was collected by cryofocusing in liquid oxygen, and characterized by using mass spectrometry.[33] These results strongly indicate that "active methionine", identified as S-adenosylmethionine (SAM),[49] is involved in the transfer of the methionine methyl group to arsenic during mycological methylation.[48]

In Chapter 3, we reported that extracellular arsenic metabolites, mainly dimethylarsenic species (probably DMA) and trimethylarsenic species (probably TMAO), were found in the growth medium of pure cultures of *Apiotrichum humicola* and *Scopulariopsis brevicaulis* grown in the presence of arsenate, arsenite, MMA or DMA. These results reinforce the validity of the mechanism proposed by Challenger; however, it has not been verified that SAM is the methyl donor for the extracellular arsenic metabolites produced by *A. humicola.*

In this chapter we report on the effect of adding L-methionine-methyl-d_3 and each of the four arsenic substrates arsenate, arsenite, MMA, and DMA, separately, to the growing culture of *Apiotrichum humicola.* The incorporation of the deuterated methyl groups from methionine in the extracellular arsenic metabolites was monitored by using hydride generation-gas chromatography-mass spectrometry (HG-GC-MS). The mass spectra obtained by using this technique provided conclusive evidence of CD_3 incorporation in the arsenic metabolites.

4.2 EXPERIMENTAL

4.2.1 Reagents

All chemical reagents were prepared as previously described in Chapters 2 and 3. A solution of L-methionine-methyl-d_3, $CD_3SCH_2CH_2CH(NH_2)COOH$ (Aldrich) was prepared by dissolving the compound in deionized water.

4.2.2 Microorganism cultures

The source of the cultures and the growth medium is as described in Section 3.2.

4.2.3 Hydride generation-gas chromatography-mass spectrometry

A continuous type hydride generation apparatus connected to a gas chromatograph-mass spectrometer system was used to generate, separate, and characterize arsines (Figure 4.1). The hydride generator was thoroughly described in Chapter 2 and 3. A peristaltic pump was used to introduce and mix the sample solution (1-3 mL) with 4.0 M acetic acid and 2.0% (w/v) sodium borohydride solutions. In the presence of acetic acid, arsenite, MMA, DMA, and TMAO can all be reduced to their corresponding arsines, but arsenate does not react. These conditions were optimized for efficient hydride production of $(CH_3)_2AsH$ and $(CH_3)_3As$. The arsines produced were separated on a Porapak-PS column (80-100 mesh) by using a gas chromatograph (Varian, Vista 6000 GC) with a pre-set temperature program (Table 4.1), and were characterized by using a quadrupole mass spectrometer (Nermag R10-10C). Data acquisition and processing were performed by using a PC based data system (Teknivent,

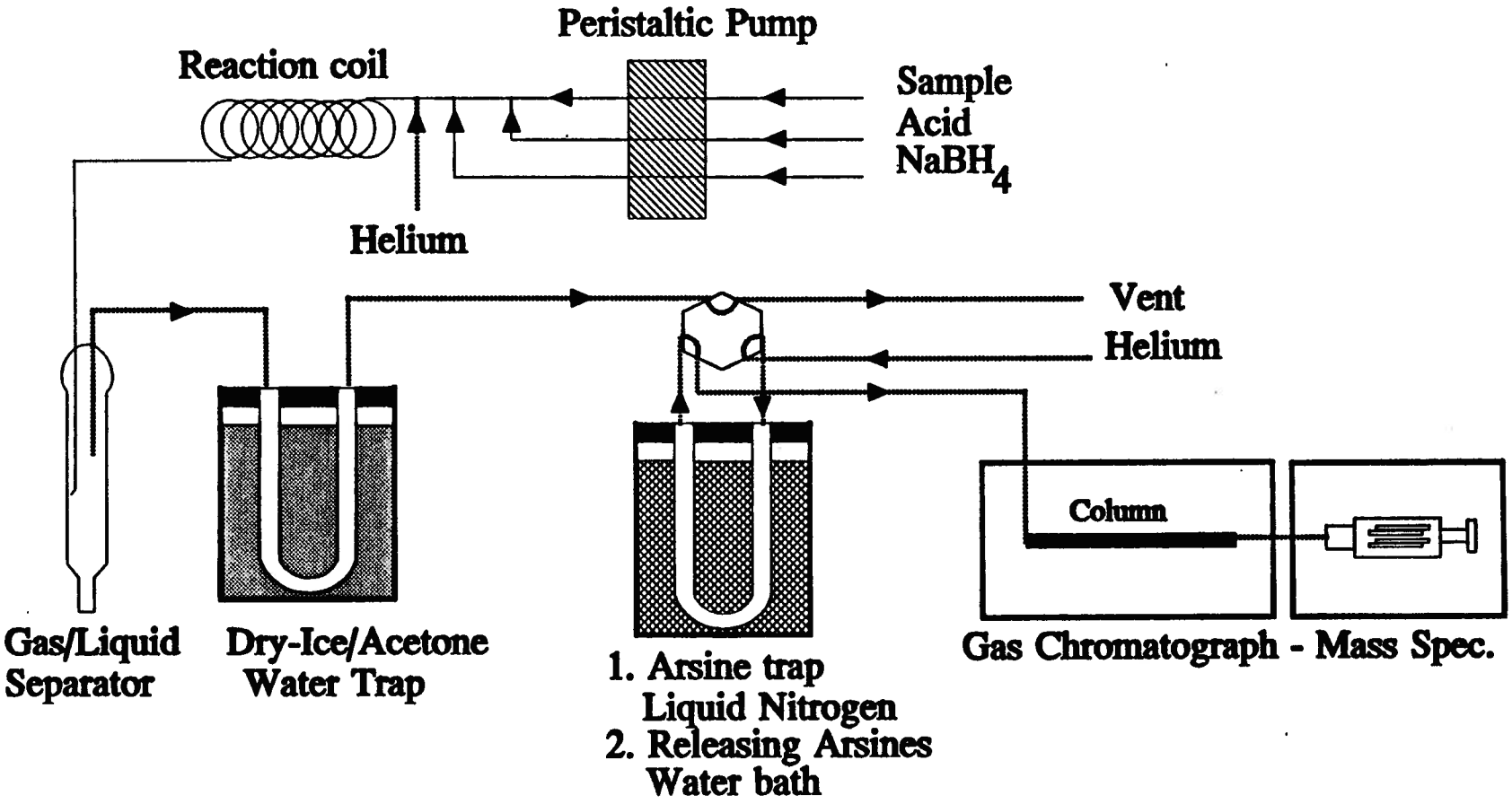


Figure 4.1 Hydride generation-gas chromatography-mass spectrometry experimental setup. Dotted lines represent gas lines.

Vector 2) interfaced to the mass spectrometer. The HG-GC-MS experimental conditions used are listed in Table 4.1. These conditions were arrived at after optimizing the hydride generation efficiency, the chromatographic resolution, and the mass spectrometric sensitivity for the arsines of interest.

Table 4.1 HG-GC-MS experimental parameters

Hydride Generation	
2.0% $NaBH_4$ solution, 4.0 M acetic acid, 3 mL sample	
Gas Chromatograph	
Initial temperature:	50°C
Ramp rate:	40°C. min^{-1}
Final temperature:	150°C for 2 min
Helium flow rate:	35 mL/min^{-1}
Mass Spectrometer	
Interface temperature:	140°C
Ion source temperature:	160°C
Scan range:	m/z 74-130
Scan time:	Scan every 0.1 s for 4 min

4.2.4 Experimental procedures

Aqueous solutions of the appropriate arsenical and L-methionine-methyl-d_3, filter sterilized (0.2 μm membrane) separately, were added to the autoclaved growth medium (see Table 3.1). The initial concentration of each of the arsenicals was 1 ppm (in arsenic) and L-methionine was 1.3 mM. A 10 mL aliquot of an actively growing culture of *A. humicola* was added to the growth medium, and the day of addition is referred to as day 0. A control experiment (without the addition of methionine) was also carried out. The incubation conditions, and the sampling of cultures were handled in the same manner as those previously described in Chapter 3. The transfer of the CD_3 group from L-methionine-methyl-d_3 to the arsenic substrates arsenate, arsenite, MMA, and DMA was investigated by examining the mass spectra of the arsines obtained by using HG-GC-MS.

4.3 RESULTS AND DISCUSSION

4.3.1 HG-GC-MS measurements

Hydride generation has proved to be an effective tool for detecting trace levels of arsenate, arsenite, MMA, DMA and TMAO in samples.[139-144] These arsenicals are precursors to the arsines that are ultimately detected by using atomic absorption spectrometry (AA),[139-144] and mass spectrometry (MS).[116,139,169] The reduction of these hydride-forming arsenicals to their corresponding arsines by sodium borohydride is a pH dependent process. Under pH < 1 conditions, all hydride forming arsenicals can be reduced to the arsines. At pH > 4 arsenite, but not arsenate, forms arsine.

The majority of previous studies reporting the use of a mass spectrometer as a

detector for arsines have used the selected-ion-monitoring (SIM) mode for characterizing arsine, methylarsine, dimethylarsine or trimethylarsine. In many cases the most abundant ions of each arsine were used for detection in the SIM mode, e.g. m/z 78 $[AsH_3]^+$ and 76 $[AsH]^+$ for arsine; m/z 92 $[CH_3AsH_2]^+$, 90 $[CH_3As]^+$ and 76 $[AsH]^+$ for methylarsine; m/z 106 $[(CH_3)_2AsH]^+$ and 90 $[CH_3As]^+$ for dimethylarsine; m/z 120 $[(CH_3)_3As]^+$, 105 $[(CH_3)_2As]^+$ and 103 $[(CH_2)_2As]^+$ for trimethylarsine. Odanaka *et al.*[169] were able to quantify arsines with detection limits between 10-20 ng of arsenic by using a SIM mode GC-MS system and an off-line hydride generation heptane cold trap procedure. Later, in a study involving the analysis of arsenic in NaOH digested tissue extracts of shellfish, fish, crustaceans, and seaweeds, Kaise *et al.*[139] collected the arsines, generated by $NaBH_4$ reduction, in a liquid-nitrogen trap coupled to a GC-MS system. The SIM mode gave detection limits of 0.3 ng arsenic. Norin *et al.*[116] reported the existence of TMAO in fish tissue based on analysis carried out on a HG-GC-MS system equipped with a liquid nitrogen trap for arsine collection.

In the present work , the mass spectrometer was set up to scan from m/z 74 to 130 at 1 scan per 0.1 s. Scans above m/z 130 were not made since the highest m/z for any anticipated volatile arsine is 129, corresponding to $(CD_3)_3As$. The gas chromatogram resulting from a mixture of arsines which were generated from a solution containing synthetic standards of arsenite, MMA, DMA, and TMAO, is shown in Figure 4.2. The mass spectra of each of the arsines AsH_3, CH_3AsH_2, $(CH_3)_2AsH$, and $(CH_3)_3As$ is shown in Figure 4.3. The wide-scan monitoring mode was used instead of the SIM mode because the former would allow for the observation of any unsuspected fragment ions resulting from the deuterium labeled arsines. This precaution was taken because the fragmentation patterns of deuterium labeled arsines apart from $(CD_3)_3As$, were unknown. Cullen *et al.*[33] demonstrated that the mass spectrum of $(CD_3)_3As$ has

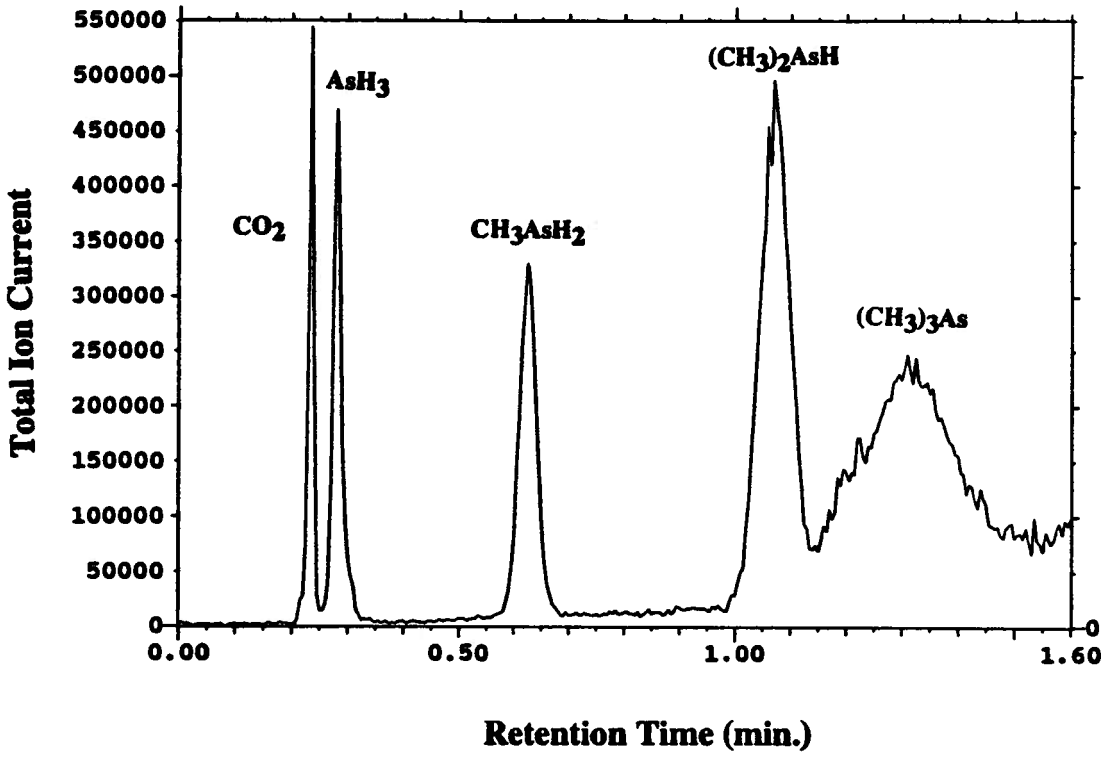


Figure 4.2 HG-GC-MS chromatogram of arsine, monomethylarsine, dimethylarsine, and trimethylarsine. Solutions of standard arsenite, MMA, DMA, and TMAO (100 ng of arsenic for each compound) were used.

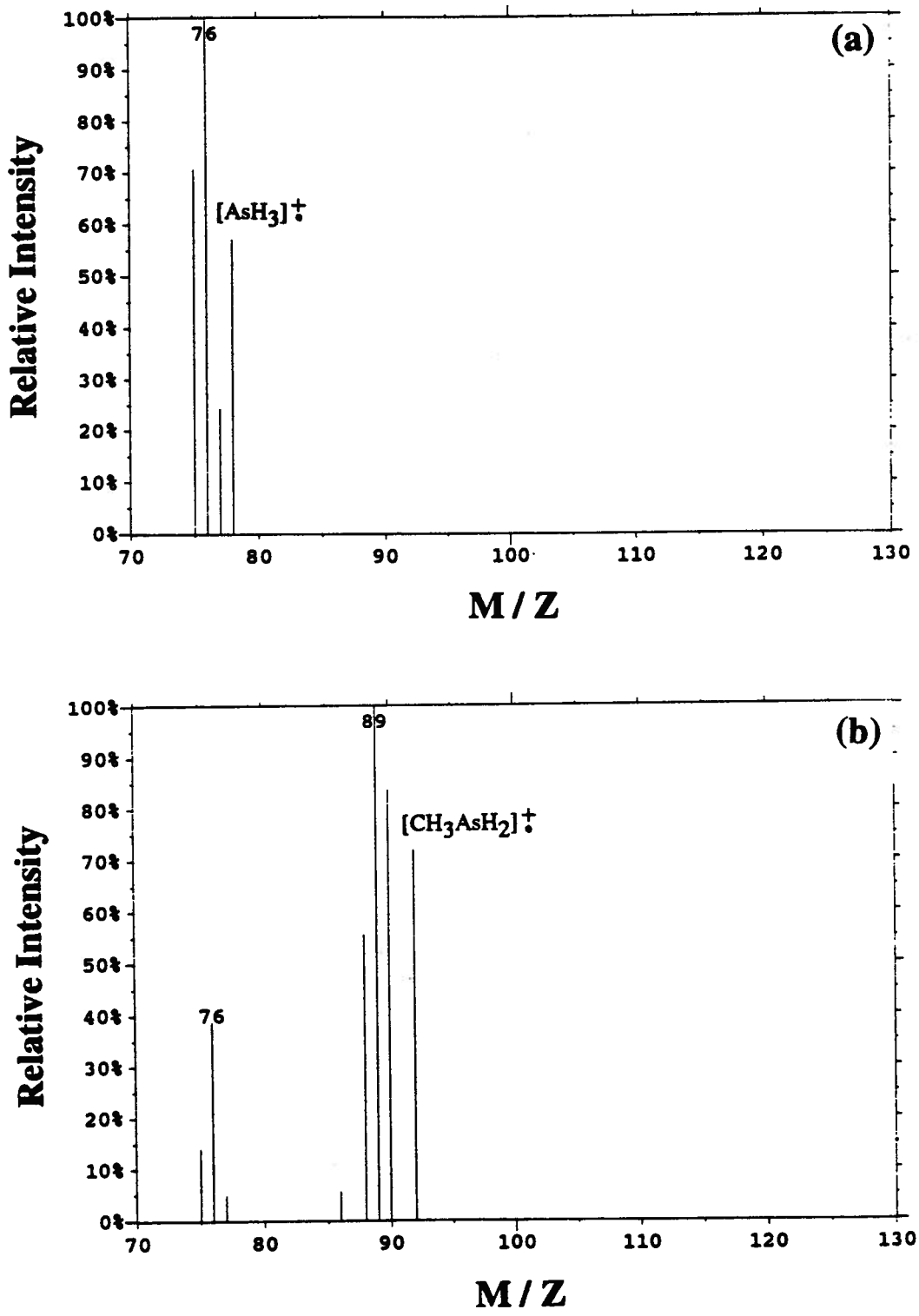
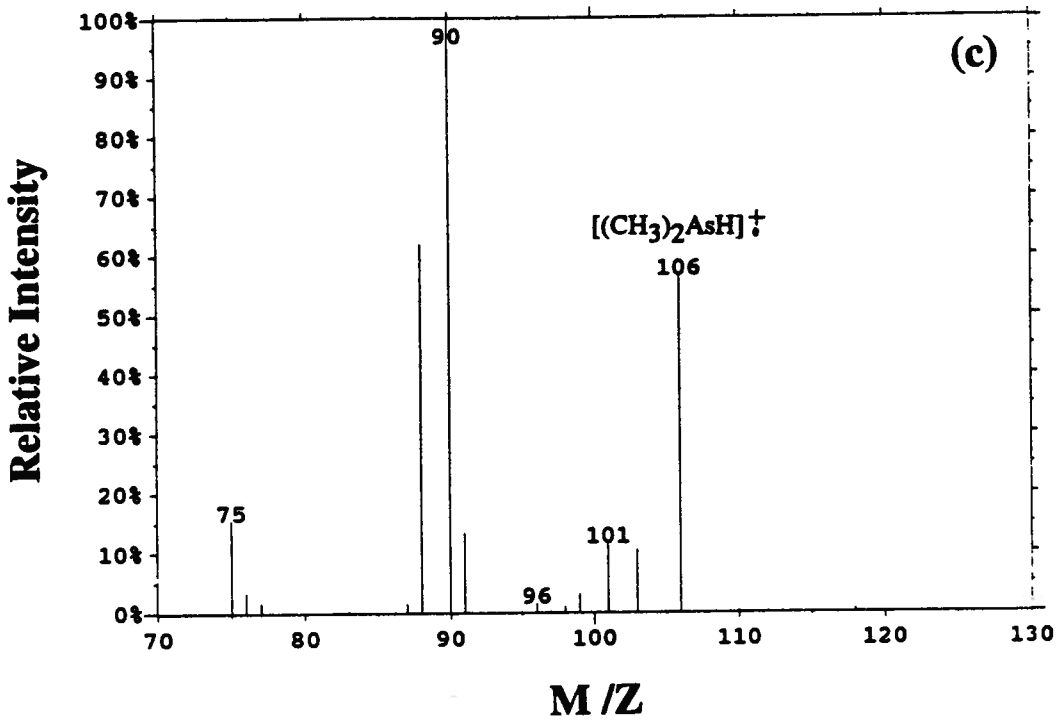
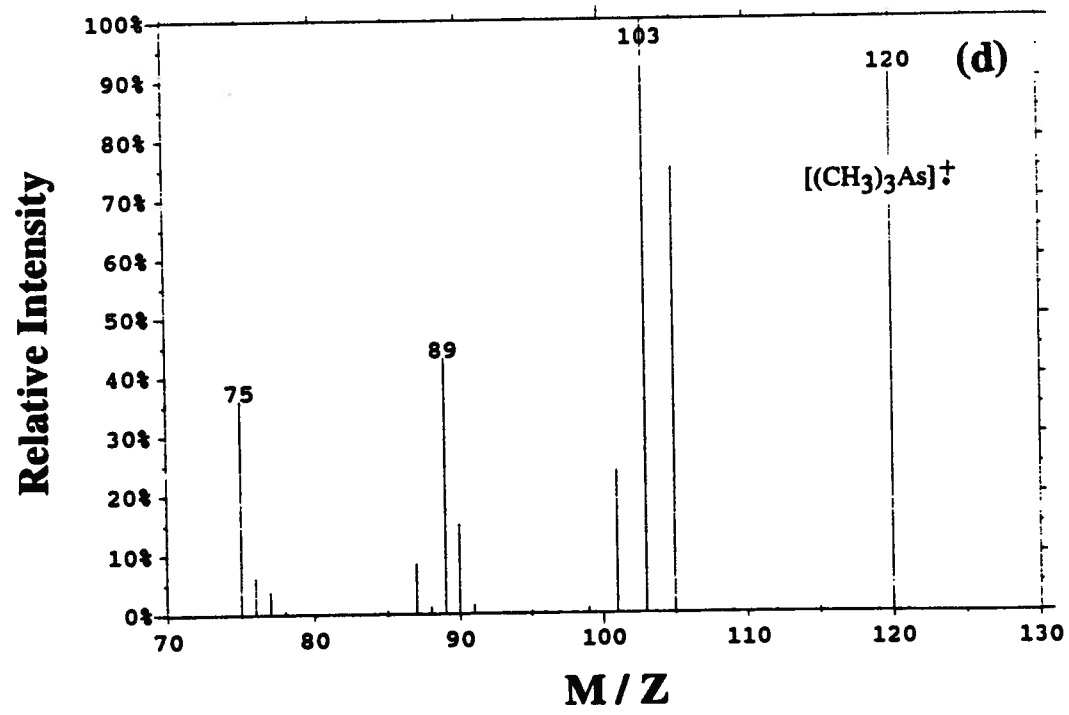


Figure 4.3 Mass spectra of arsines derived from standard solutions of arsenite, MMA, DMA, and TMAO. (a) arsine AsH_3, (b) monomethylarsine CH_3AsH_2, (c) dimethylarsine $(CH_3)_2AsH$, and (d) trimethylarsine $(CH_3)_3As$.


(c)
Relative Intensity
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
90
[(CH3)2AsH]+
106
75
101
96
70
80
90
100
110
120
130
M /Z


(d)
Relative Intensity
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
103
120
[(CH3)3As]+
89
75
70
80
90
100
110
120
130
M / Z

an identical fragmentation pattern to that of $(CH_3)_3As$ except that H is replaced by D. The main fragmentation path is as shown in Figure 4.4. Cullen *et al.*[33] also prepared $(CD_3)As(CH_3)_2$ for comparison purposes, and essentially the same fragmentation pattern is obtained. Fragment ions formed from the loss of both CD_3 and CH_3 from the parent ion are observed affording $[(CH_3)(CD_3)As]^+$ and $[(CH_3)_2As]^+$ in high relative abundance.[33] Although the mass spectra of standards CD_3AsH_2 and $(CD_3)_2AsH$ are not available, it is believed that their fragmentation patterns are similar to those of CH_3AsH_2, and $(CH_3)_2AsH$. The other reason for acquiring spectra in the wide-scan mode was to establish the identity of all hydride generated volatile compounds resulting from the cell culture samples because any interfering ions might lead to erroneous results in the SIM mode.

By using the HG-GC-MS methodology described above it was possible to detect down to 25 ng of arsenic. In the SIM mode the detection limit could be easily improved by at least an order of magnitude.

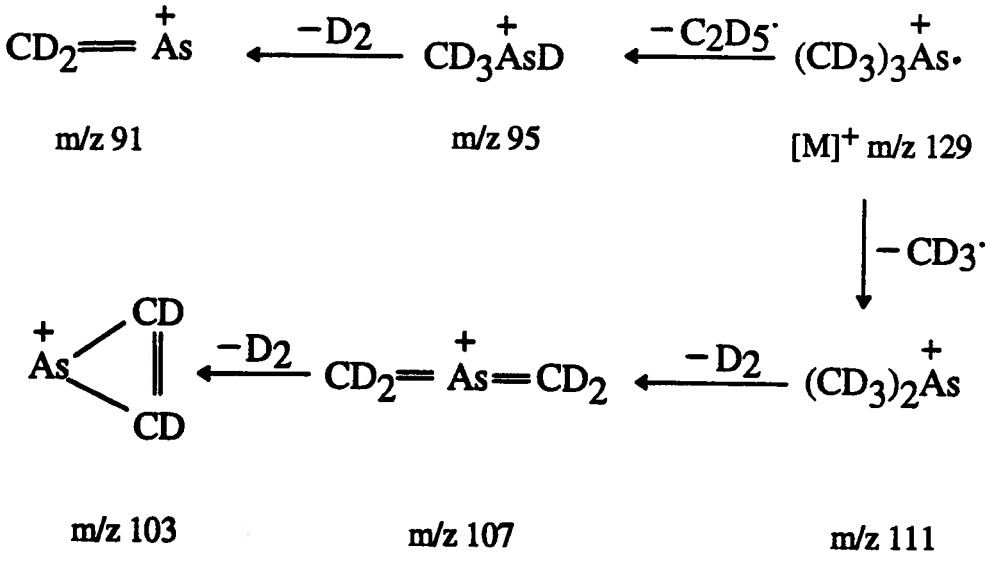


Figure 4.4 The predicted fragmentation pattern of $(CD_3)_3As$.[33]

4.3.2 Characterization of methylated arsenic intermediates in the growth medium of *A. humicola*

In order to verify that S-adenosylmethionine is the methyl donor of the extracellular arsenic metabolites produced by *A. humicola*, 1 ppm each of arsenate, arsenite, MMA, and DMA were added separately to the culture of *A. humicola* growing in the presence and absence of L-methionine-methyl-d_3. The arsenic metabolites in the growth medium were analyzed by using HG-GC-MS in the wide-scan mode.

4.3.2.1 Transformation of arsenate

When arsenate (1 ppm) was added to the growth medium in the absence of L-methionine-methyl-d_3, three arsenic compounds were found to be present in the growth medium after 5 days of incubation. Positive identification of the volatile arsenic compounds generated by borohydride treatment was made based on a comparison of their retention times and mass spectra with those exhibited by standards (Figures 4.2 and 4.3). Figure 4.5 shows the HG-GC-MS chromatogram of the sample collected on day 10. The first peak was identified as CO_2, which is produced probably during hydride generation. Its mass spectrum is shown in Figure 4.6. The second peak was identified as arsine AsH_3, derived from arsenite that was produced by *A. humicola* from the arsenate substrate as described in Chapter 3. The third peak was $(CH_3)_2AsH$ (Figure 4.7a) derived from DMA, and the fourth peak was $(CH_3)_3As$ (Figure 4.8a) derived from TMAO. The mass spectra of these arsines are identical with those of the arsines derived from standard arsenicals (Figure 4.3).

In the presence of L-methionine-methyl-d_3, dimethylarsenic and trimethylarsenic species were also detected in the growth medium. No apparent difference in the quantity

of these methylated arsenic intermediates was observed when L-methionine-methyl-d_3 was either present or absent from the medium. It has been proposed that the added methionine could increase the cell's internal concentration of SAM and thus might enhance the methylation process.[45] This assumption was based on some results that showed high methyl incorporation in arsenicals in the presence of methionine.[33] However, Cullen *et al.*[45] also reported that preculturing whole cell cultures of *A. humicola* or their cell-free extracts with methionine did not effect the amount of the methylated arsenic species produced from methylarsine oxide $(CH_3AsO)_n$.

The mass spectra of the dimethylarsenic (Figure 4.7b) and trimethylarsenic species (Figure 4.8b) which were produced by HG-GC-MS from cultures grown in the presence of L-methionine-methyl-d_3, exhibit additional ions, strongly indicating that a considerable amount of CD_3 was incorporated onto arsenic. The mass spectrum of the dimethylarsenic derivative contains additional ions at m/z 112 $[(CD_3)_2AsH]^+$, m/z 109 $[(CH_3)(CD_3)AsH]^+$, and m/z 93 $[CD_3As]^+$. The mass spectrum of the trimethylarsenic species contains additional ions at m/z 129 $[(CD_3)_3As]^+$, m/z 126 $[(CH_3)(CD_3)_2As]^+$, m/z 123 $[(CH_3)_2CD_3As]^+$, m/z 111 $[(CD_3)_2As]^+$, m/z 108 $[(CH_3)(CD_3)As]^+$, and m/z 93 $[CD_3As]^+$. These ions are absent from the HG-GC-MS traces resulting from the media containing no L-methionine-methyl-d_3 (Figure 4.7a, 4.8a).

The identification of deuterated arsenic metabolites in the growing cultures of *A. humicola* is noteworthy, as this is the first time that methionine has been demonstrated to be involved in the production of the non-volatile methylarsenic intermediates which have been proposed in Challenger's pathway (Figure 1.1). As described above, the labeled methyl group from methionine had been shown to be transferred to arsenate, arsenite, MMA, and DMA to form labeled volatile trimethylarsine.[33,48] Our work strongly reinforces the suggestion that S-adenosylmethionine or some related sulphonium

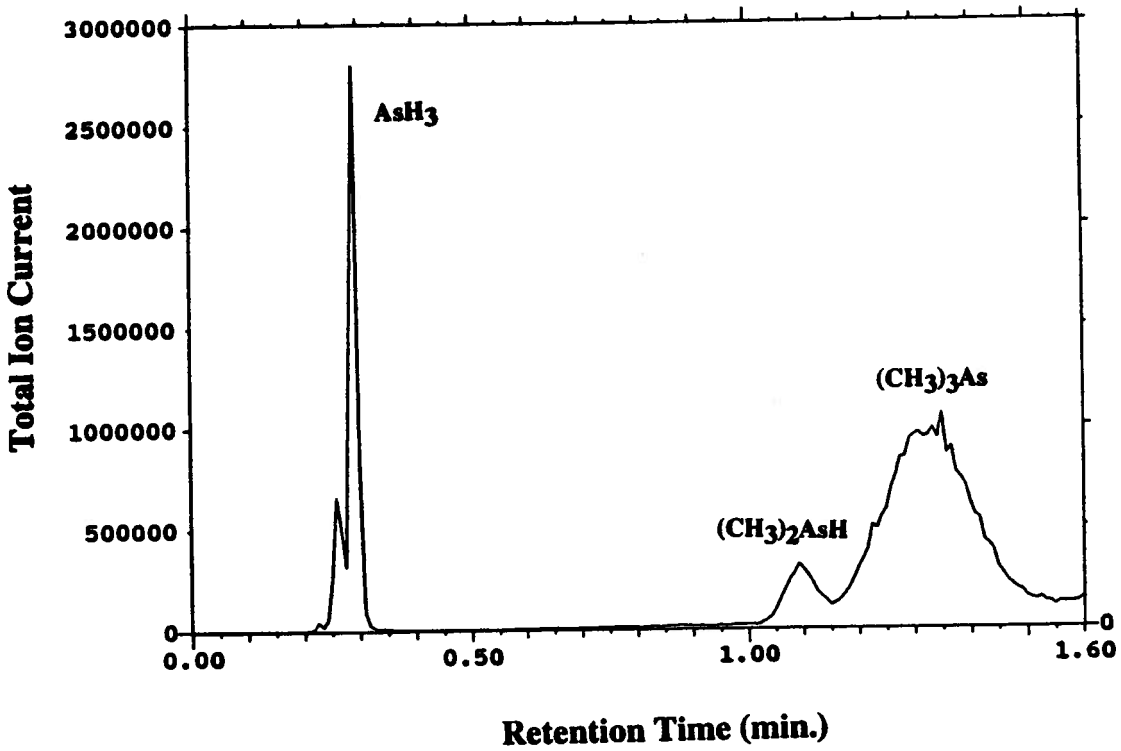


Figure 4.5 HG-GC-MS chromatogram of a 2 mL culture medium sample that was taken after 10 days of incubation. The medium was originally enriched with 1 ppm arsenate, but not with L-methionine-methyl-d_3.

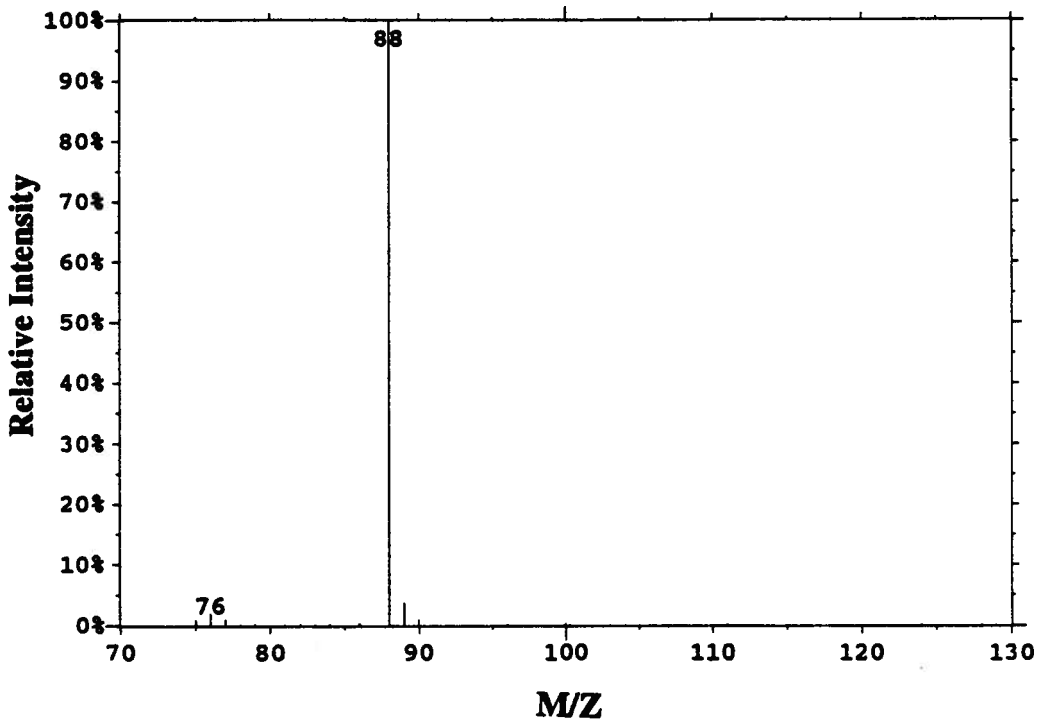


Figure 4.6 Mass spectrum of the first peak in Figure 4.5, identified as CO_2.

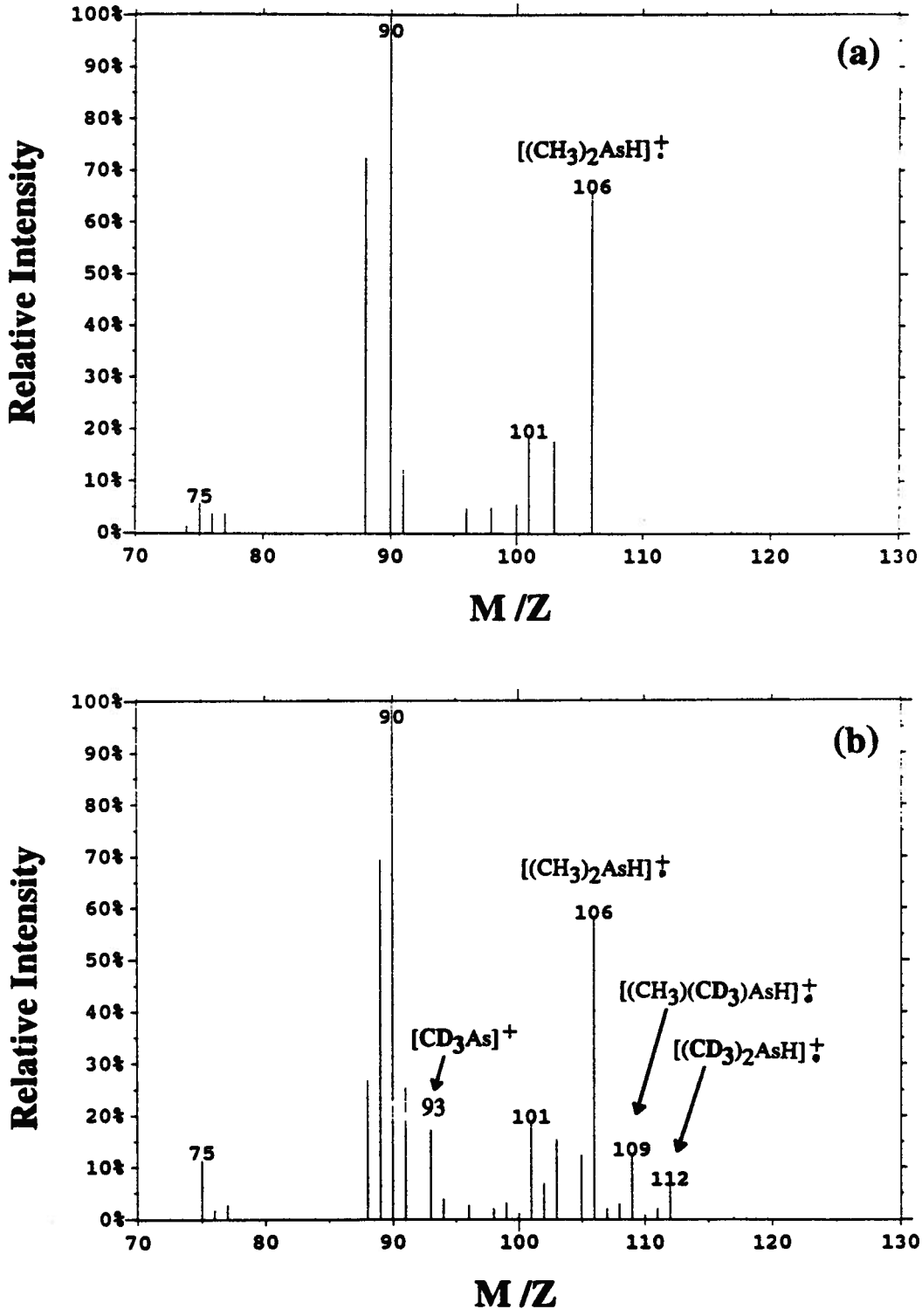


Figure 4.7 Mass spectra of the hydride derivative of the dimethylarsenic species present in the growing culture, enriched with arsenate, after 10 days of incubation. (a) in the absence of L-methionine-methyl-d_3, (b) in the presence L-methionine-methyl-d_3

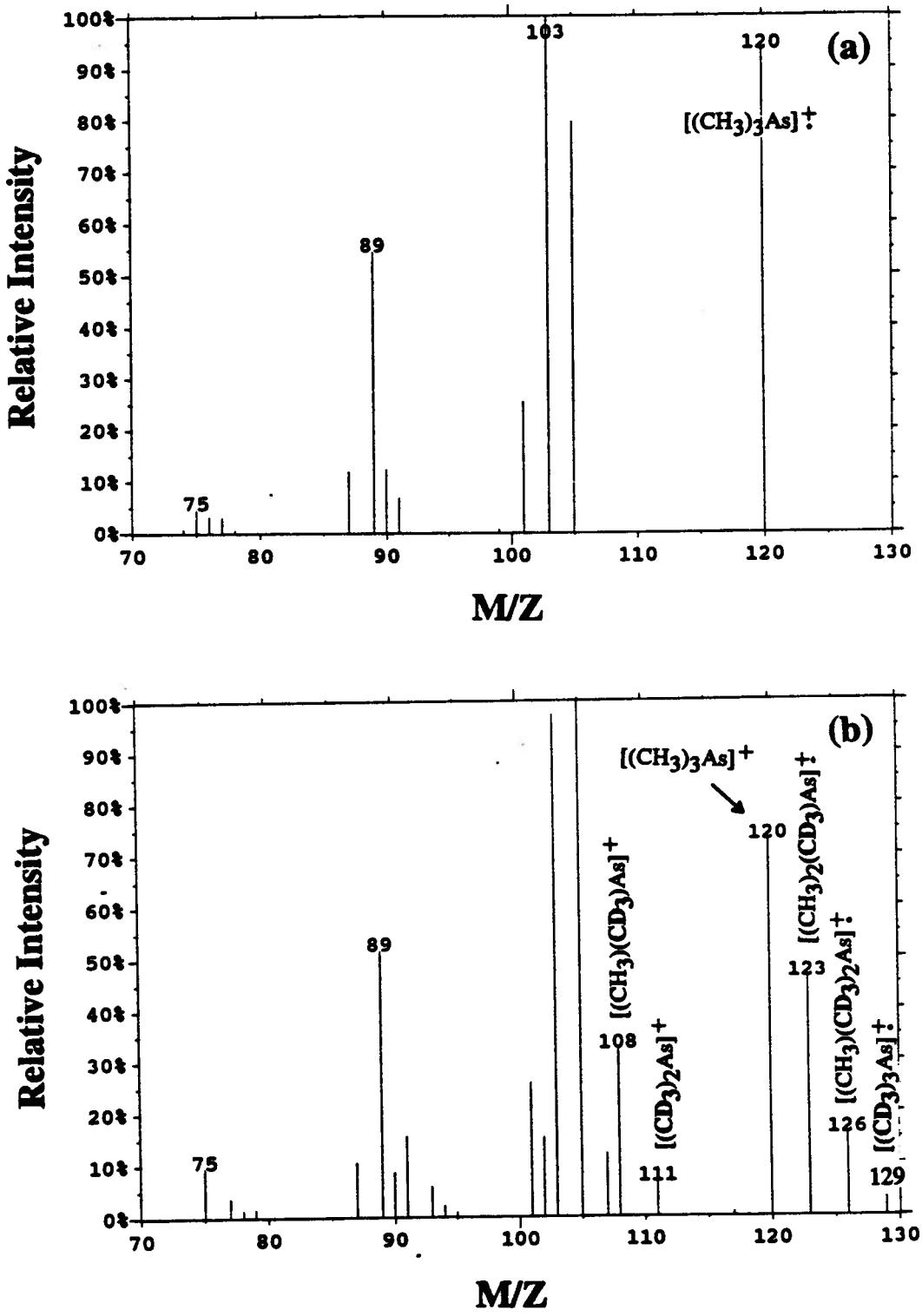


Figure 4.8 Mass spectra of the hydride derivative of the trimethylarsenic species present in the growing culture, enriched with arsenate, after 10 days of incubation: a) in the absence of L-methionine-methyl-d_3, b) in the presence L-methionine-methyl-d_3

compounds are the source of the $[CH_3]^+$ shown in Challenger's pathway.

The percentage of the CD_3 incorporation was determined by comparing the relative intensities of the parent ions, m/z 106 $[(CH_3)_2AsH]^+$, 109 $[(CH_3)(CD_3)AsH]^+$, and 112 $[(CD_3)_2AsH]^+$ for the dimethylarsenic species, and m/z 120 $[(CH_3)_3As]^+$, m/z 123 $[(CH_3)_2CD_3As]^+$, m/z 126 $[(CH_3)(CD_3)_2As]^+$, and m/z 129 $[(CD_3)_3As]^+$ for the trimethylarsenic species. The distribution change of the CD_3 label in the dimethyl and trimethylarsenic metabolites with incubation time is presented in Table 4.2. The relative standard deviations of the peak intensity of m/z 106, and m/z 120 for 4 determinations of standard dimethylarsinic acid and trimethylarsine oxide is 6%. For these calculations, we assumed that the responses of the deuterated arsines are identical to the responses of the undeuterated arsines; however, this assumption needs to be verified by using the appropriate deuterated methylarsenic standards. Consequently, the results presented in Table 4.2 should only be viewed as an indication of low or high incorporation.

4.3.2.2 Transformation of arsenite

Following the addition of arsenite to the growing culture of *A. humicola*, dimethylarsenic and trimethylarsenic species were detected in the growth medium after 5 days of incubation. The concentration of the arsenic metabolites was found to be independent of the presence or absence of L-methionine-methyl-d_3. In the absence of L-methionine-methyl-d_3, the HG-GC-MS traces of the dimethylarsenic and the trimethylarsenic species found in the medium were similar to those of the standard $(CH_3)_2AsH$ and $(CH_3)_3As$. While in the presence of L-methionine-methyl-d_3, the mass spectra of the two arsenic species showed the incorporation of the CD_3 group as indicated by the ions at m/z 112, 109, and 93 for the dimethylarsenic species, and m/z

Table 4.2 Percentage distribution of dimethylarsenic and trimethylarsenic compounds detected in the growth medium

	Incubation Time (days)			
Arsenic metabolites	day 5	day 10	day 15	day 20
	Arsenate (1 ppm) substrate			
$(CH_3)_2AsH$	30	47	56	
$(CH_3)(CD_3)AsH$	37	28	26	
$(CD_3)_2AsH$	33	25	18	
$(CH_3)_3As$	55	61	63	
$(CH_3)_2(CD_3)As$	28	28	27	
$(CH_3)(CD_3)_2As$	11	8	7	
$(CD_3)_3As$	6	3	3	
	Arsenite (1 ppm) substrate			
$(CH_3)_2AsH$	51	45	51	77
$(CH_3)(CD_3)AsH$	26	30	28	14
$(CD_3)_2AsH$	23	25	21	9
$(CH_3)_3As$	45	50	52	53
$(CH_3)_2(CD_3)As$	36	34	33	33
$(CH_3)(CD_3)_2As$	19	12	12	12
$(CD_3)_3As$	< L.O.D.	4	3	2
	MMA (1 ppm) substrate			
$(CH_3)_2AsH$	33	66	62	64
$(CH_3)(CD_3)AsH$	67	34	38	36
$(CH_3)_3As$	42	53	58	60
$(CH_3)_2(CD_3)As$	31	29	28	28
$(CH_3)(CD_3)_2As$	27	18	14	12
	DMA (1 ppm) substrate			
$(CH_3)_3As$	59	66	69	71
$(CH_3)_2(CD_3)As$	41	34	31	29

L.O.D.: limit of detection.

129, 126, 123, 111, 108, and 93 for the trimethylarsenic species. The distribution of the CD_3 label in the dimethyl and trimethylarsenic metabolites with incubation time is presented in Table 4.2.

4.3.2.3 Transformation of methylarsonic acid

Apiotrichum humicola transformed MMA into dimethylarsenic and trimethylarsenic species both in the presence and absence of L-methionine-methyl-d_3 in the growth medium. Again, the presence of methionine did not significantly affect the production of arsenic metabolites. Figure 4.9 shows that a large amount of DMA and TMAO is present in the growing culture collected on day 10 (in the absence of L-methionine-methyl-d_3). The mass spectra of their hydride derivatives are presented in Figure 4.10a and 4.11a, respectively.

When deuterated methionine was added to the growth medium, the CD_3 group from methionine was incorporated into MMA to form deuterated dimethyl and trimethylarsenic species. The mass spectra clearly show the presence of ions at m/z 109 $[(CH_3)(CD_3)AsH]^+$, and m/z 93 $[CD_3As]^+$ for the dimethylarsenic species (Figure 4.10b), and m/z 126 $[(CH_3)(CD_3)_2As]^+$, m/z 123 $[(CH_3)_2CD_3As]^+$, m/z 111 $[(CD_3)_2As]^+$, m/z 108 $[(CH_3)(CD_3)As]^+$, and m/z 93 $[CD_3As]^+$ for the trimethylarsenic species (Figure 4.11b). The absence of ions at m/z 112 $[(CD_3)_2AsH]^+$ for dimethylarsenic species and m/z 129 $[(CD_3)_3As]^+$ for trimethylarsenic species is expected since only one and two deuterated methyl groups can be incorporated into MMA to form dimethylarsenic and trimethylarsenic species, respectively. The mass spectrum of monomethylarsenic species does not show the presence of ion at m/z 95 $[CD_3AsH_2]^+$. The absence of ions at m/z 129, 112, and 95 indicates that the cleavage of

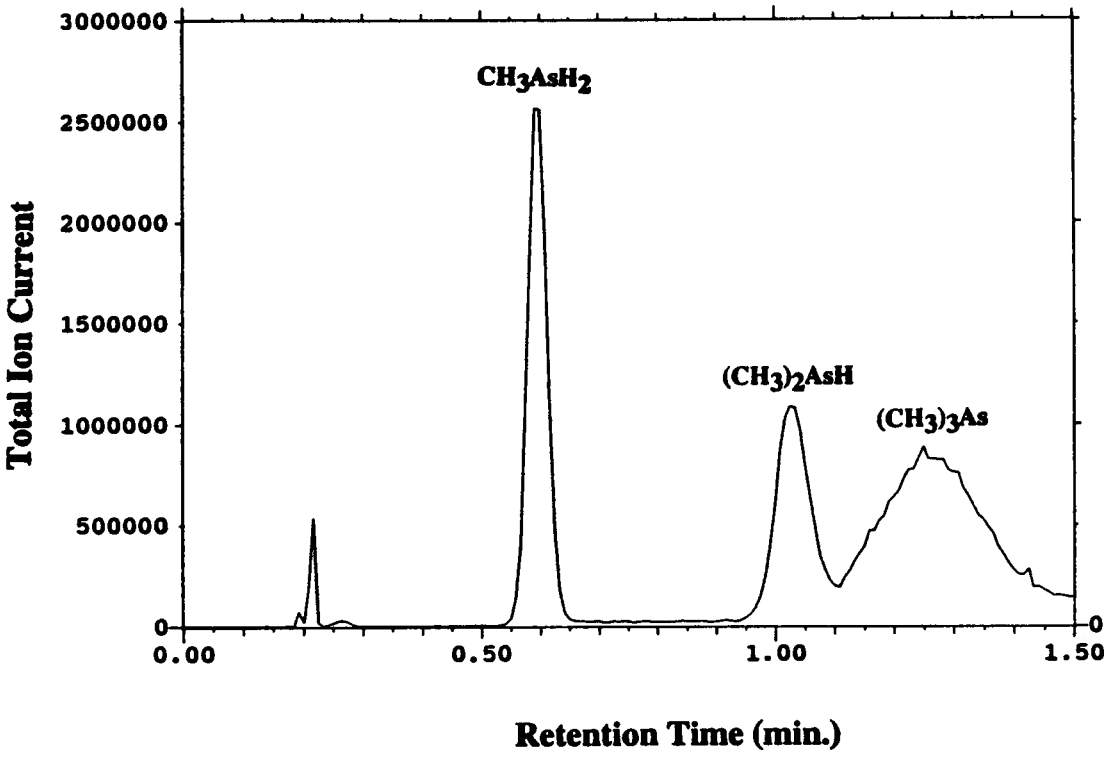


Figure 4.9 HG-GC-MS chromatogram of a 2 mL culture medium sample that was taken after 10 days of incubation. The medium was originally enriched with 1 ppm MMA, but not with L-methionine-methyl-d_3.

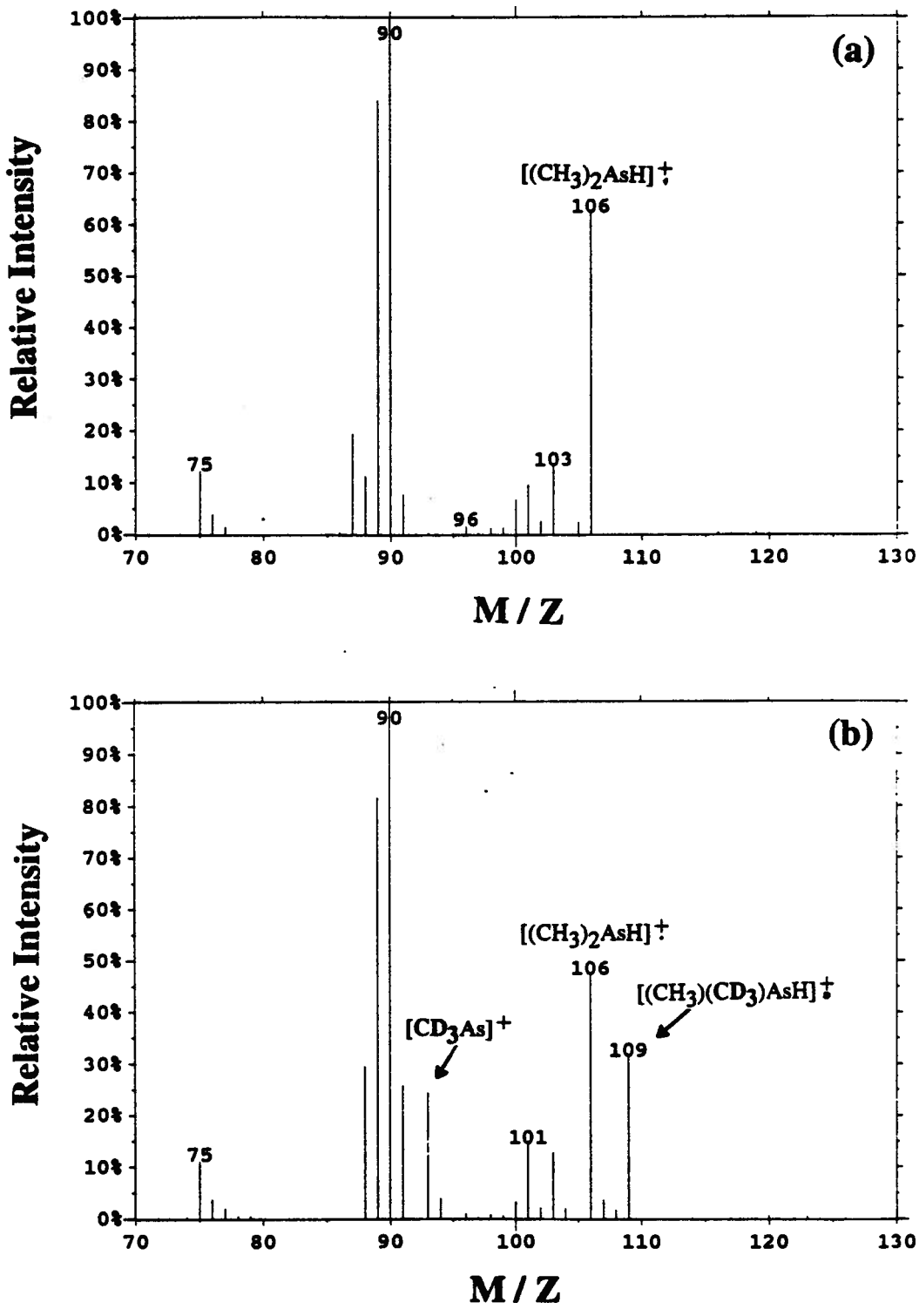


Figure 4.10 Mass spectra of the hydride derivative of the dimethylarsenic species present in the growing culture, enriched with MMA, after 10 days of incubation. (a) in the absence of L-methionine-methyl-d_3, (b) in the presence L-methionine-methyl-d_3

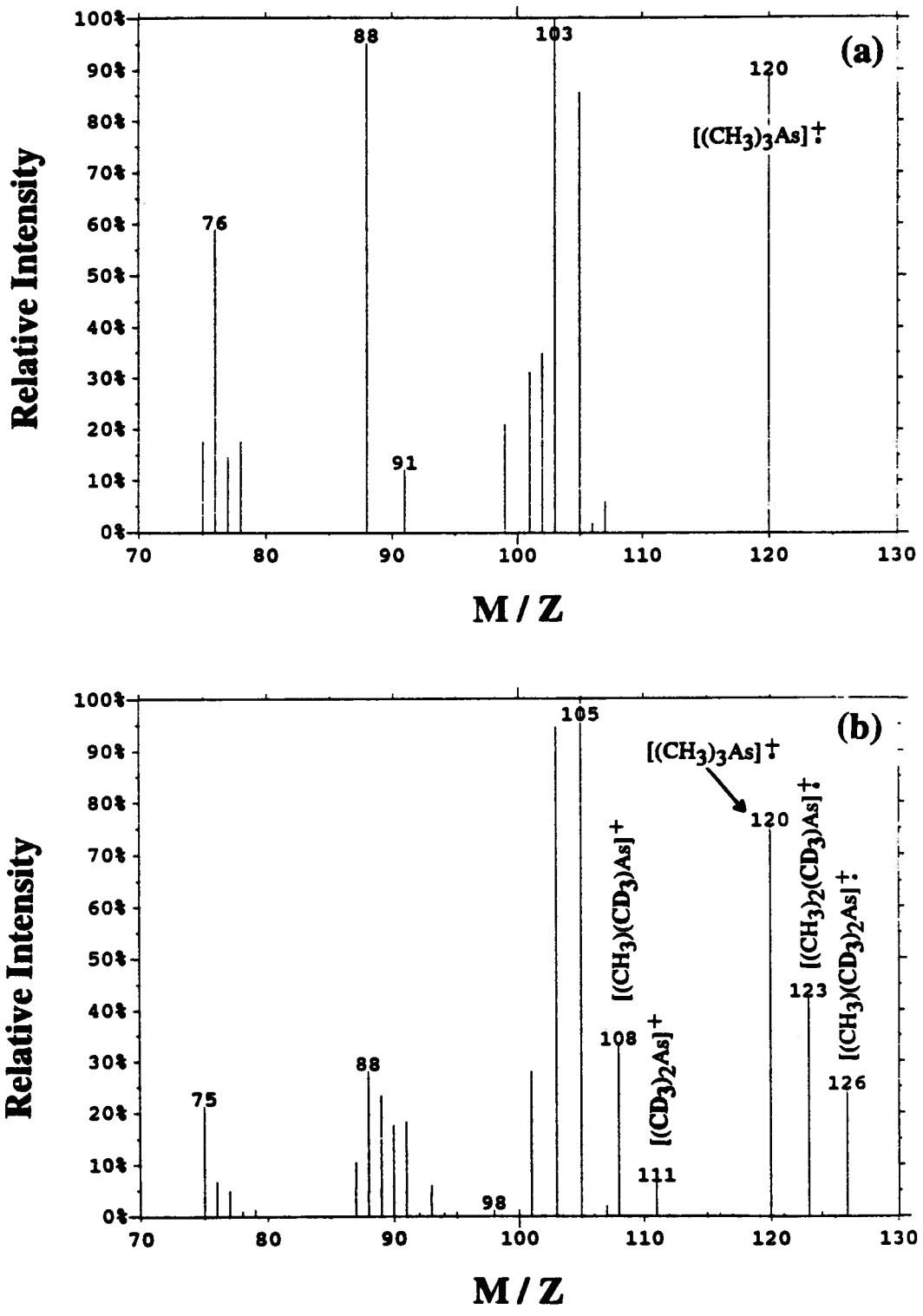


Figure 4.11 Mass spectra of the hydride derivative of the trimethylarsenic species present in the growing culture, enriched with MMA, after 10 days of incubation. (a) in the absence of L-methionine-methyl-d_3, (b) in the presence L-methionine-methyl-d_3

the H_3C-As bond is insignificant. The distribution of the CD_3 label in the arsenic metabolites is shown in Table 4.2.

4.3.2.4 Transformation of dimethylarsinic acid

The biotransformation of DMA to trimethylarsenic species in the absence of L-methionine-methyl-d_3 by the microorganism *A. humicola* is a slow process compared to the formation of trimethylarsenic species from arsenate, arsenite, and MMA. Most of the DMA substrate remained unchanged in the growing culture after 20 days of incubation (Figure 4.12). However, a small amount of a trimethylarsenic species was produced, and its HG-GC-MS spectrum is presented in Figure 4.13a. In the presence of L-methionine-methyl-d_3, the production of the trimethylarsenic species did not seem to be enhanced. The mass spectrum of this arsenic species (Figure 4.13b) exhibits ions at m/z 123 $[(CH_3)_2CD_3As]^+$, m/z 108 $[(CH_3)(CD_3)As]^+$, and m/z 93 $[CD_3As]^+$, indicating that the CD_3 group is incorporated into DMA. Not unexpectedly, the mass spectrum of the trimethylarsenic species does not show ions present at m/z 129 $[(CD_3)_3As]^+$, m/z 126 $[(CH_3)(CD_3)_2As]^+$, and 111 $[(CD_3)_2As]^+$, suggesting that H_3C-As bond cleavage is not significant. The distribution of the CD_3 label in the trimethylarsenic species is shown in Table 4.2.

In conclusion, the results obtained from this study strongly suggest that methionine, or SAM, is the source of the methyl groups in the biological methylation of arsenic in the microorganism *A. humicola*, and thus conform with the oxidation-reduction pathway involving carbonium ions suggested by Challenger.[30,43]

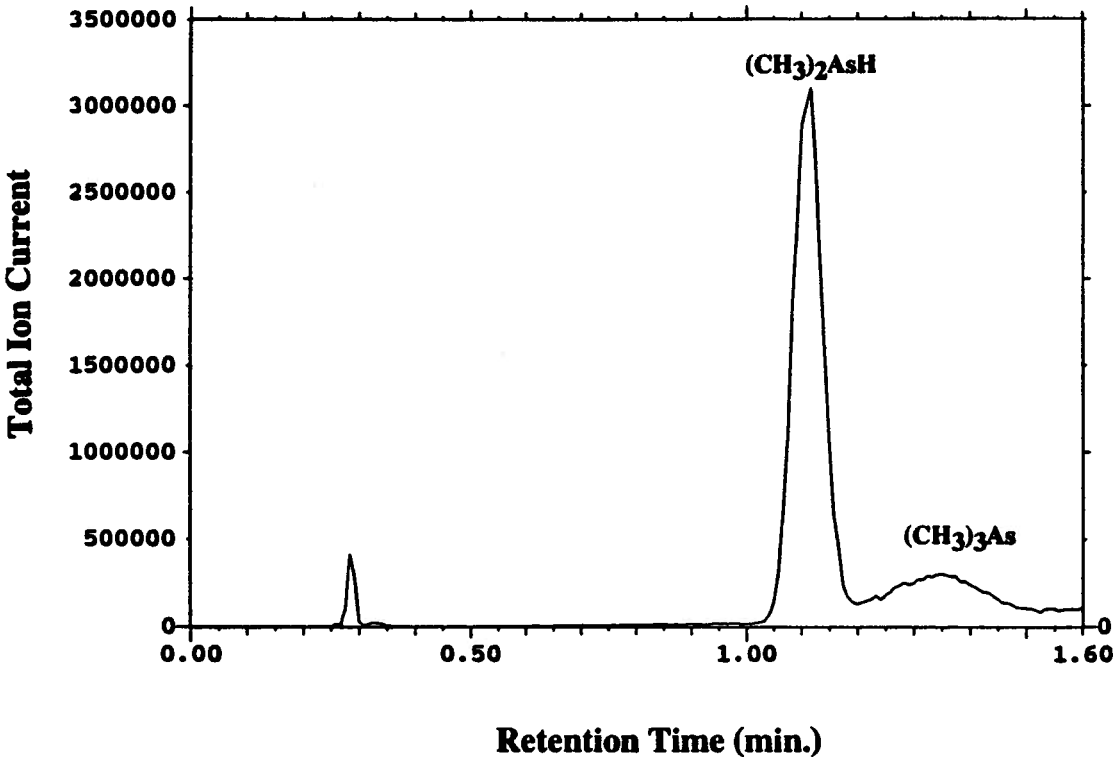


Figure 4.12 HG-GC-MS chromatogram of a 2 mL culture medium sample that was taken after 20 days of incubation. The medium was originally enriched with 1 ppm DMA, but not with L-methionine-methyl-d_3.

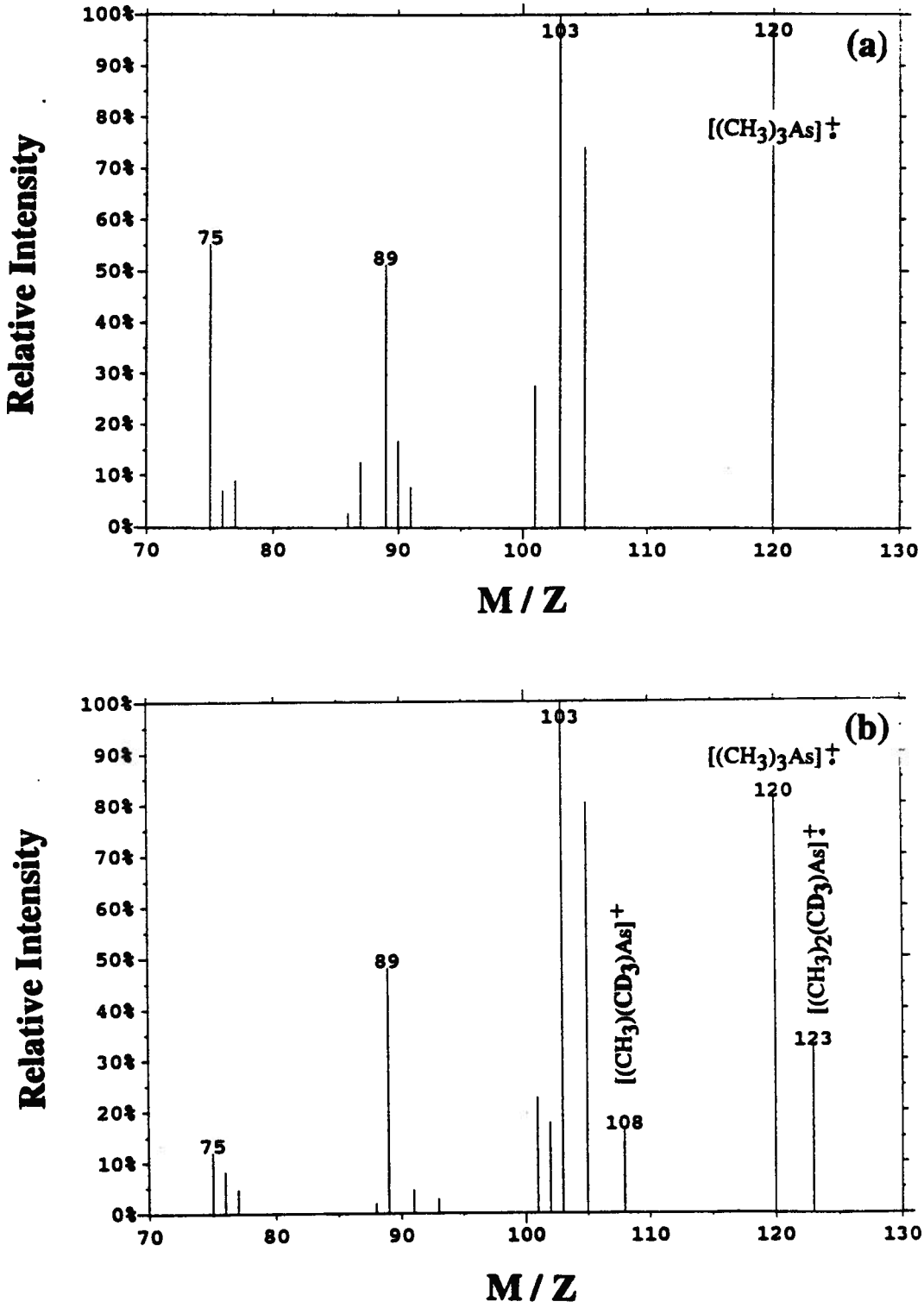


Figure 4.13 Mass spectra of the hydride derivative of the trimethylarsenic species present in the growing culture, enriched with DMA, after 20 days of incubation. (a) in the absence of L-methionine-methyl-d_3, (b) in the presence L-methionine-methyl-d_3

CHAPTER 5

THE BIOMETHYLATION AND BIOACCUMULATION OF ARSENICALS BY A MARINE ALGA *POLYPHYSA PENICULUS*

5.1 INTRODUCTION

Arsenic is probably one of the better known elements because of the toxic properties of some of its compounds. Against this background it is not surprising that reports, at the beginning of this century, of high levels of arsenic in marine organisms attracted much interest. Since then, concerns regarding the forms of arsenic in marine organisms, their toxicity, how they were accumulated, and what role they played in the biochemical functions of the organisms have been investigated. But even now, our knowledge of marine arsenic chemistry is incomplete.

Marine algae have been the subject of many arsenic metabolic studies because of their ecological and nutritional importance. Such studies of the interaction of marine algae with arsenicals are relevant because arsenic compounds produced by algae are generally believed to be the source of the arsenic compounds found in marine animals, although it is not well established how and when these transformations take place.

A number of investigators have reported that the total arsenic concentrations in a range of marine algae have much higher levels than seawater.[58-64] Arsenate, the predominant form of arsenic in seawater, is readily taken up by algae, possibly because of its similarity to the essential phosphate.[65-67] Once inside the algal cell, the arsenic is believed to be transformed by algae into a range of arsenic compounds, including MMA, DMA and other more complex aqueous-soluble or lipid-type arsenicals.[69-80] Several workers have been successfully isolated and characterized arsenosugar

derivatives from different seaweeds.[81-89] Many people now believe that the presence of arsenosugar derivatives in marine algae is ubiquitous.

Edmonds and Francesconi[80] have proposed a pathway for the biotransformation of arsenate by marine algae as shown in Figure 1.5. In order to provide support for this proposed mechanism a number of questions need to be addressed, such as: (1) whether this mechanism applies to all species of marine micro- and macroalgae; (2) whether the methylation of arsenic follows Challenger's proposed mechanism and involve the utilization of SAM as a donor of both methyl and adenosyl groups. We believe that experiments with controlled cultures may provide some of this information.

As mentioned in Chapter 1, there are only a limited number of reports that discuss the biotransformation of arsenicals by macroalgae in culture medium.[20,92,93] Complex arsenic compounds are reported to be produced by these algae, but they have not been positively identified as arsenosugars.

Studies on arsenic biotransformation by marine phytoplankton, mainly unicellular marine phytoplankton, only confirm the production of arsenite, MMA, and DMA from arsenate.[69,70,72,74,79] The presence of complex arsenic compounds is also reported although no positive identification of these compounds can be made.

We have chosen to study *Polyphysa peniculus*, a unicellular marine alga which has been cultivated in the laboratory of Dr. L. G. Harrison in artificial seawater under sterile conditions for more than two decades. Its cells are unusually large (4-5 cm in length, 0.4 mm in diameter), but, like many phytoplankton, it is a unicellular alga (Chlorophyta). In this chapter we report on the effect of adding arsenate, arsenite, methylarsonate and dimethylarsinic acid to artificial seawater containing *P. peniculus*. Arsenic accumulation, methylation and excretion by the alga were examined by using graphite furnace atomic absorption spectrometry (GFAA) and hydride generation-gas

chromatography-atomic absorption spectrometry (HG-GC-AA) methodology. The inability of *P. peniculus* to synthesize significant amounts of complex water and lipid-soluble arsenic compounds was also established by using flow injection-microwave digestion-hydride generation-atomic absorption spectrometry methodology.

We also report on the effect of adding L-methionine-methyl-d_3 and arsenate (1 ppm) to artificial seawater containing *P. peniculus*. The arsenic metabolites excreted by the alga in the growth medium, principally DMA, were identified by using hydride generation-gas chromatography-mass spectrometry (HG-GC-MS). These results provided conclusive evidence of CD_3 incorporation from L-methionine-d_3 in the dimethylarsenic species produced by *P. peniculus*, and supports the hypothesis that S-adenosylmethionine is the biological methyl donor.

5.2 EXPERIMENTAL

5.2.1 Reagents

Standard solutions of arsenic compounds were freshly prepared by serial dilution from stock solutions (1000 ppm of elemental arsenic) as described in Section 2.2.1. Solutions of 1.5% (w/v) potassium persulphate (BDH) in 0.1% (w/v) NaOH, 1.0 M HCl, 4.0 M acetic acid, and 2.0% (w/v) $NaBH_4$ in 0.1% (w/v) NaOH were freshly made daily. A solution of L-methionine-methyl-d_3, $CD_3SCH_2CH_2CH(NH_2)COOH$ (Aldrich) was prepared by dissolving the compound in deionized water.

5.2.2 Algal cultures

The alga used throughout the experiments was *Polyphysa peniculus* (Dasycladales, Chlorophyta), a marine alga closely related to the better known genus *Acetabularia*, and sometimes known as *A. cliftonii* and several other synonyms.[195] The *P. peniculus* culture used in this work has been maintained in sterile artificial seawater (Shephard's medium,[196] pH 7.9-8.3) for more than two decades, first in the laboratory of Dr. B. R. Green (Botany, this university) and then in the laboratory of Dr. L. G. Harrison. The composition of Shephard's medium is shown in Table 5.1. The culture had not been rendered anexic, but had been maintained and handled by using sterile techniques, and treated with antibiotics (a mixture of amphotericin, penicillin G, and kanamycin) if any bacterial infection arose.

5.2.3 Instrumentation

5.2.3.1 Graphite furnace atomic absorption spectrometry

Total amount of arsenic accumulated in cells of *P. peniculus* were measured by using graphite furnace atomic absorption spectrometry methodology as previously described in Chapter 2. The standard additions technique was used in these studies. The furnace operating parameters and the conditions for the standard additions method are found in Chapter 2 (Tables 2.1 and 2.2).

Table 5.1 Composition of Shephard's medium

Nutrients and Trace Metals in 1 L Medium			
NaCl	24 g	$NaNO_3$	0.04 g
$MgSO_4.7H_2O$	12 g	Na_2HPO_4	0.001 g
Tris(base)$(HOCH_2)_3CNH_2$	1 g	Micronutrients	10 mL
$CaCl_2.2H_2O$	1 g	$NaHCO_3$	0.1 g
KCl	0.75 g	Vitamins	0.2 g

Micronutrient Salts Stock Solution (1 L)			
Na_2EDTA	1.2 g	$MnCl_2.4H_2O$	0.02 g
$ZnSO_4$	0.2 g	$CoCl_2.6H_2O$	0.2 mg
$Na_2MoO_4.2H_2O$	0.1 g	$CuSO_4.5H_2O$	0.2 mg
$FeCl_3.6H_2O$	0.05 g	H_3BO_3	0.1 g

Vitamin Stock Solution (100 mL)			
Thiamine HCl	0.3 g	*p*-Aminobenzoate	20 mg
Vitamin B_{12}	4 mg	Calcium Pantothenate	10 mg

5.2.3.2 Hydride generation-gas chromatography-atomic absorption spectrometry

A hydride generation system was used for arsine production and collection as previously described in Chapter 2. After the sample introduction was completed, the arsines trapped in liquid nitrogen were volatilized when the hydride trap was warmed in a water bath (70°C). By using a Hewlett Packard Model 5830A gas chromatograph with a pre-set temperature program the arsines were then separated on a Porapak-PS column (80-100 mesh), atomized by using a hydrogen-air flame in a quartz cuvette, and detected by using a Jarrell-Ash Model 810 atomic absorption spectrometer equipped with a Varian Spectra arsenic hollow-cathode lamp set at 10 mA. The monochromator was set at 193.7 nm, and the slit width at 1 nm. Absorbance was recorded as peak area by using a Hewlett-Packard 3390A integrator.

5.2.3.3 Flow injection-microwave digestion-hydride generation-atomic absorption spectrometry

The flow injection-microwave digestion-HGAA system (Figure 5.1) described by Le *et al.*,[197,198] was used to determine non-hydride-forming, "hidden" arsenic compounds. The evolved arsines were carried into an open-ended T-shape quartz tube (11.5 cm long x 0.8 cm i.d.) which was mounted in the flame of a Varian Model AA-1275 atomic absorption spectrometer equipped with a standard Varian air-acetylene flame atomizer. The signals were recorded on a Hewlett-Packard 3390A integrator.

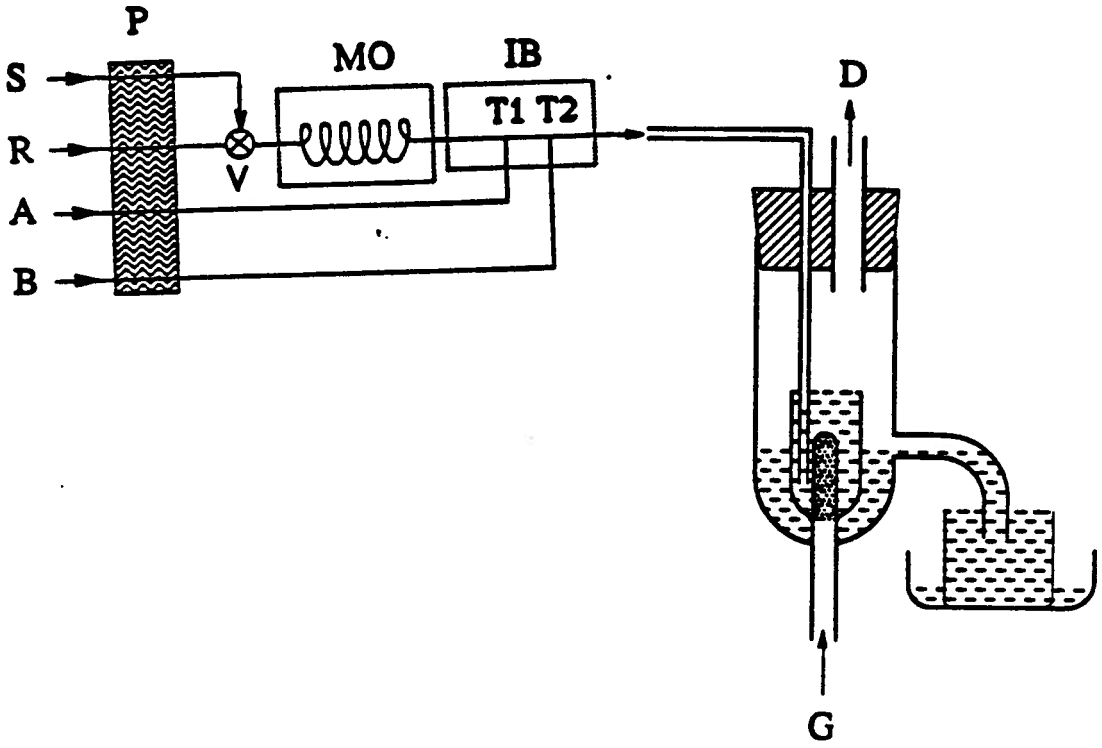


Figure 5.1 A schematic diagram of an on-line coupled flow injection-microwave oven decomposition-hydride generation system.

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

5.2.3.4 Hydride generation-gas chromatography-mass spectrometry

The hydride generation-gas chromatography-mass spectrometry system (HG-GC-MS) was used for characterization of the methylated arsenic species in the growth medium as previously described in Chapter 4. The experimental conditions are presented in Table 4.1. In this study, the mass spectrometer was scanned from m/z 74 to 115 at 1 scan per 0.1 second.

5.2.4 Experimental procedures

The alga (approximately 0.6 to 1 g dry weight) was added to sterile Erlenmeyer flasks (2 L) each containing 1 L of sterile Shephard's medium. Arsenicals were filter sterilized (0.2 μm membrane), and added to the medium separately. Two concentrations, 10 ppm and 0.9 ppm, were employed in our studies. During the growth period the cultures were maintained at 20°C. Fluorescent lamps that gave 3,200 lux intensity around the flasks were used as the light source, and the light:dark cycle was 16:8 hours. Once each day the culture was agitated and 10 mL aliquots of the medium were removed and frozen prior to analysis. The alga was harvested on day 7 and thoroughly rinsed with sterile Shephard's medium. A half portion of the alga was freeze-dried and stored in a freezer for future analysis. The rest of the alga was transferred to a 1 L sterile Erlenmeyer flask containing 500 mL fresh arsenic-free sterile Shephard's medium. The incubation conditions were not changed and the day of transfer is referred to as day 0 of the second cycle. The culture was handled in the same manner as in the first cycle, the medium was sampled each day, and the alga was again harvested after 7 days of incubation, rinsed, freeze-dried, weighed, and stored in a freezer. The growth of the cells in a 7 days period was limited as indicated by the length of the cells.

The possibility of non-metabolic interactions between arsenic and cells of *P. penicilus* was studied by exposure of heat-treated or 4.0% formalin-treated cells (4.0% formalin is an effective reagent for killing cells but causes only minimum damage to tissue integrity[92,93]) to arsenate enriched medium. The culture medium was collected once each day for 7 days, and frozen prior to analysis.

The total amount of arsenic in the cells was determined following acid digestion. Freeze-dried cells (50-100 mg) were dissolved in 1 mL of concentrated HNO_3 (69.0-71.0%), left overnight, then boiled with 1 mL of H_2O_2 for 5-10 minutes prior to analysis. The resultant pale-yellow transparent solution was neutralized with NaOH solution, diluted to an appropriate volume, and subjected to GFAA analysis. The amount of arsenic was quantified by using the standard additions technique. Palladium nitrate (100 ppm), prepared in citric acid (2.0% w/v), was used as a modifier.

For the arsenic speciation analysis, freeze-dried algal cells (0.1-0.2 g) were weighed and transferred into an Erlenmeyer flask (250 mL) containing 30 mL of mixed solvent, $CHCl_3$/MeOH/H_2O (1:1:1). The mixture was sonicated for 2 hours and then stoppered with a solid rubber plug and left on a mechanical shaker for 24 hours. It was then centrifuged and the residue was re-extracted with 10 mL of the mixed solvent for another 24 hours. The extracts were combined and centrifuged to separate the aqueous fraction with the organic fraction. The colorless aqueous layer was kept at -4°C prior to analysis. The organic extract and the residue were air dried, digested in 4 mL of 2.0 M NaOH in a water bath at 95°C for 3 hours, and finally neutralized with 6.0 M hydrochloric acid prior to analysis.

The HG-GC-AA system was used to analyze hydride-forming arsenicals in each of the three fractions of the cell extracts as well as in the growth medium. The flow injection-microwave digestion-HGAA technique was used to detect the existence of

"hidden" arsenic in the cells.

To investigate the utilization of L-methionine-methyl-d_3 in the biomethylation process of *P. peniculus*, arsenate and L-methionine-methyl-d_3, filter sterilized separately, were added to the growth medium. The initial concentration of arsenate and L-methionine-methyl-d_3 was 13 μM (1 ppm) and 130 μM, respectively. The day of arsenate addition is referred to as day 0. Four separate experiments were carried out. For the first experiment, only arsenate was added to the growth medium. In the second experiment L-methionine-methyl-d_3 and arsenate were inoculated in the medium at the same time. The third experiment was carried out by adding L-methionine-methyl-d_3 three days prior to the addition of arsenate and for the fourth experiment L-methionine-methyl-d_3 was not added until three days after the arsenate. Once each day, until day 11, the culture was agitated and a 10 mL of culture aliquot was removed and stored frozen prior to the HG-GC-MS analysis.

5.3 RESULTS

5.3.1 The accumulation of arsenicals in cells of *Polyphysa peniculus*

Two arsenic concentrations, 10 ppm and 0.9 ppm, were employed in this study. The total amount of arsenic accumulated in *P. peniculus* was determined by using GFAA. The results are presented in Table 5.2. With the exception of arsenate, alga exposed to 10 ppm arsenicals accumulated more arsenic in their cells than those exposed to 0.9 ppm arsenicals. In the presence of MMA, the arsenic accumulation in the cells was very low.

Table 5.2 Total amount of arsenic in cells of *P. peniculus* determined by using GFAA (μg/g, dry weight)

Arsenic inoculated	Cells treated with 10 ppm arsenicals		Cells treated with 0.9 ppm arsenicals	
	A[a]	B[b]	A[a]	B[b]
Arsenate	36.0±2.5[c]	11.5±0.7	43.3±2.4	7.7±0.5
Arsenite	52.7±4.2	5.6±0.5	17.4±1.2	3.5±0.3
MMA	18.3±1.1	4.4±0.3	3.1+0.2	trace
DMA	34.8±1.9	5.3±0.4	25.1±1.8	3.1±0.3

[a] 7 days after the cells were exposed to arsenicals
[b] 7 days after the cells were transferred to fresh media
[c] large fraction of dead cells was present within 2 days of incubation

Arsenate at high concentration (10 ppm) was more toxic to *P. peniculus* as indicated by the large percentage of dead cells found within 2 days of initiating the incubation. Certainly, high concentrations of arsenate affect the arsenic accumulating ability of the alga as shown in Table 5. 2.

Only small amounts of arsenic compounds were retained in the cells after the alga was transferred to an arsenic-free medium for 7 days (Table 5.2).

5.3.2 Arsenic speciation analysis in cells of *Polyphysa peniculus*

5.3.2.1 Water/methanol extracts

Most of the arsenic accumulated in cells of *P. peniculus* is present in the water/methanol fractions (> 90%) after the solvent extraction. The hydride-forming

arsenicals, arsenate, arsenite, MMA, and DMA in the aqueous extracts were detected as AsH_3, CH_3AsH_2 and $(CH_3)_2AsH$, respectively, by using HG-GC-AA. TMAO, which would have been detected as trimethylarsine, was not found in any of the samples. These arsenic speciation results are presented in Table 5.3. As expected the total amount of hydride-forming arsenicals in the alga is proportional to the external arsenic concentrations applied.

A large amount of inorganic arsenic, mainly arsenite (25.1 ppm), was detected in cells incubated with 10 ppm arsenate. Small amounts of DMA (1.9 ppm) were also found in the same sample. When *P. peniculus* was treated with 0.9 ppm arsenate, the amount of DMA found in the aqueous extract of the alga was as high as 21.7 ppm, and is about 45% of total hydride-forming arsenicals found in the aqueous extract. Substantial amounts of arsenite were also detected in both extracts. Neither MMA nor arsenate were found in cells incubated with 0.9 ppm arsenate. When the alga was transferred to an arsenic-free medium and incubated for 7 days, only small amounts of arsenate were retained.

Four arsenicals, principally arsenite, but also arsenate, MMA and DMA were found in the aqueous extracts after the alga was treated for 7 days with 10 ppm and 0.9 ppm arsenite. The percentage of MMA in both aqueous extracts is about 7%. The percentage of DMA in cells exposed to 0.9 ppm arsenite is 37% of total aqueous hydride-forming arsenicals. Little DMA was detected after the alga was treated with 10 ppm arsenite. The arsenical content of the alga was greatly reduced after the alga was transferred to an arsenic-free medium.

Similar amounts of MMA and DMA were found in cells incubated with 10 ppm and 0.9 ppm MMA. However, after the alga was transferred to an arsenic-free medium, small amounts of arsenicals remained in cells originally treated with 10 ppm MMA, but

Table 5.3 Arsenic distribution in aqueous extracts of the cells determined by using HG-GC-AA (μg/g, dry weight)

Arsenic inoculated	Arsenic species found in cells	Cells treated with 10 ppm arsenicals		Cells treated with 0.9 ppm arsenicals	
		A[a]	B[b]	A[a]	B[b]
Arsenate	Arsenate	12.8±0.8	9.7±0.7	0	5.1±0.3
	Arsenite	25.1±1.3	0	26.3±1.6	0
	MMA	0	0	0	0
	DMA	1.9±0.2	0	21.7±2.0	0
	Total	39.8	9.7	48.0	5.1
Arsenite	Arsenate	8.0±0.5	0	1.8±0.1	0
	Arsenite	37.7±2.5	2.3±0.2	6.5±0.4	1.2±0.1
	MMA	3.1±0.2	0.4±0.03	1.0±0.1	0
	DMA	0.8±0.1	0.8±0.08	5.4±0.5	0.8±0.1
	Total	49.6	3.5	14.7	2.0
MMA	Arsenate	0	0.3±0.03	0	0
	Arsenite	0	0.9±0.07	0	0
	MMA	5.3±0.3	0.8±0.06	1.4±0.1	0
	DMA	6.5±0.6	2.7±0.3	1.6±0.2	0
	Total	11.8	4.7	3.0	0
DMA	Arsenate	1.6±0.1	0	0	0
	Arsenite	0	0	0	0
	MMA	0.6±0.04	0.1±0.01	0.7±0.04	0
	DMA	28.1±2.5	3.6±0.3	20.4±1.8	2.5±0.3
	Total	30.3	3.7	21.1	2.5

[a] 7 days after the cells were exposed to arsenicals
[b] 7 days after the cells were transferred to fresh media

none were found in the cells exposed to 0.9 ppm MMA.

Arsenic speciation analysis of cells exposed to DMA shows that the accumulated arsenic exists mainly as DMA. Trace amounts of MMA were also present in the aqueous extracts. Most of the accumulated arsenic was discharged from the cells after the alga was transferred to an arsenic-free medium.

In order to determine if any "hidden" arsenic, possibly arsenosugars, existed in the aqueous extracts of the algal cells, a flow injection-microwave digestion-HGAA technique was applied. In this methodology, the "hidden" arsenic species are decomposed and oxidized by potassium persulphate to arsenate with the aid of microwave radiation.[197,198] The product, arsenate, can easily be reduced to arsine. Thus, by comparison of the arsine absorbance before and after microwave assisted digestion, the amount of total "hidden" arsenic in a sample can be calculated. The results are shown in Table 5.4: no significant differences are apparent in the amounts of arsenic detected before and after microwave assisted digestion. This suggests that very small amounts, if any, of "hidden" arsenic species such as arsenosugars, were produced and accumulated in the cells during the growth cycle.

Table 5.4 Arsenic distribution in aqueous extracts of the cells harvested from arsenical enriched media before and after microwave digestion (μg/g, dry weight)

Arsenic inoculated	Cells treated with 10 ppm arsenicals		Cells treated with 0.9 ppm arsenicals	
	before digestion	after digestion	before digestion	after digestion
Arsenate	37.3±2.6	38.7±2.7	45.4±3.2	47.1±3.8
Arsenite	48.7±2.9	50.6±4.1	15.9±0.8	14.7±0.9
MMA	12.7±0.8	11.5±1.1	3.8±0.3	3.4±0.3
DMA	32.2±2.1	33.1±2.2	20.4±1.2	20.0±1.8

5.3.2.2 Chloroform extracts

Chloroform fractions from the original $CHCl_3$/MeOH/H_2O cell extracts were air dried, digested with 2.0 M NaOH, neutralized with concentrated HCl, and then analyzed by using HG-GC-AA. Arsenosugars, if present, would be decomposed to DMA under these conditions and would be detected as dimethylarsine by using HG-GC-AA. No arsenicals were detected in these $CHCl_3$ extracts of cells exposed to arsenate and arsenite. Only trace amounts of dimethylarsenic compounds were detected in cells exposed to MMA and DMA. Arsenic compounds were not found in the $CHCl_3$ extracts following transfer of the cells to arsenic-free media.

The flow injection-microwave digestion-HGAA technique was used to detect if any "hidden" arsenic compounds, which may not have been hydrolyzed by NaOH, were present in the organic extracts. There was no significant difference in the amount of hydride-forming arsenicals present before and after microwave assisted digestion.

5.3.2.3 Insoluble residues

The residues were digested by using 2.0 M NaOH, and were subsequently analyzed by using HG-GC-AA. Trace amounts of monomethylarsenic and dimethylarsenic compounds were detected in cells exposed to arsenite, MMA and DMA. The flow injection-microwave digestion-HGAA technique did not show the presence of "hidden" arsenic species in these digested samples.

5.3.3 Arsenic speciation analysis in the growth media of *Polyphysa peniculus*

Arsenic speciation analysis of the growth media was carried out by using HG-GC-AA. The change in the chemical form of arsenic was very dramatic in the media enriched with arsenate. When the alga was exposed to 10 ppm arsenate, more than 70% of the substrate was reduced to arsenite after one day of incubation. The concentration of arsenate and arsenite remained relatively constant until the alga was harvested (Figure 5.2). Reduction of arsenate to arsenite was also observed in the growth medium of *P. peniculus* spiked with 0.9 ppm arsenate, but the reaction was slower (Figure 5.3). The concentration of arsenite reached a steady state after 2 days of incubation. In addition to arsenate and arsenite, DMA was detected in this culture medium on day 3. The concentration of DMA had increased slowly with time from 0 to about 0.15 ppm at the time when the experiment was terminated. No MMA was found in either of the experiments mentioned above. When the heat-treated or 4.0% formalin-treated cells were exposed to 0.9 ppm of arsenate, neither arsenite nor DMA was observed in the growth medium.

No MMA was detected in the growth medium when 10 ppm or 0.9 ppm arsenite was used as a substrate. When the alga was treated with 10 ppm arsenite, about 90% of the arsenite in the medium remained unchanged throughout the incubation period. Arsenate was detected in this medium after two days of incubation, but at a low level. After incubation of *P. peniculus* with 0.9 ppm arsenite for 1 day, small amounts of DMA were detected in the growth medium as well as arsenate (Figure 5.4). The concentration of DMA increased from 0 to 0.08 ppm after another five days of incubation.

When MMA or DMA was used as a substrate, the changes of arsenic species and arsenic concentration were minimal in the growth medium.

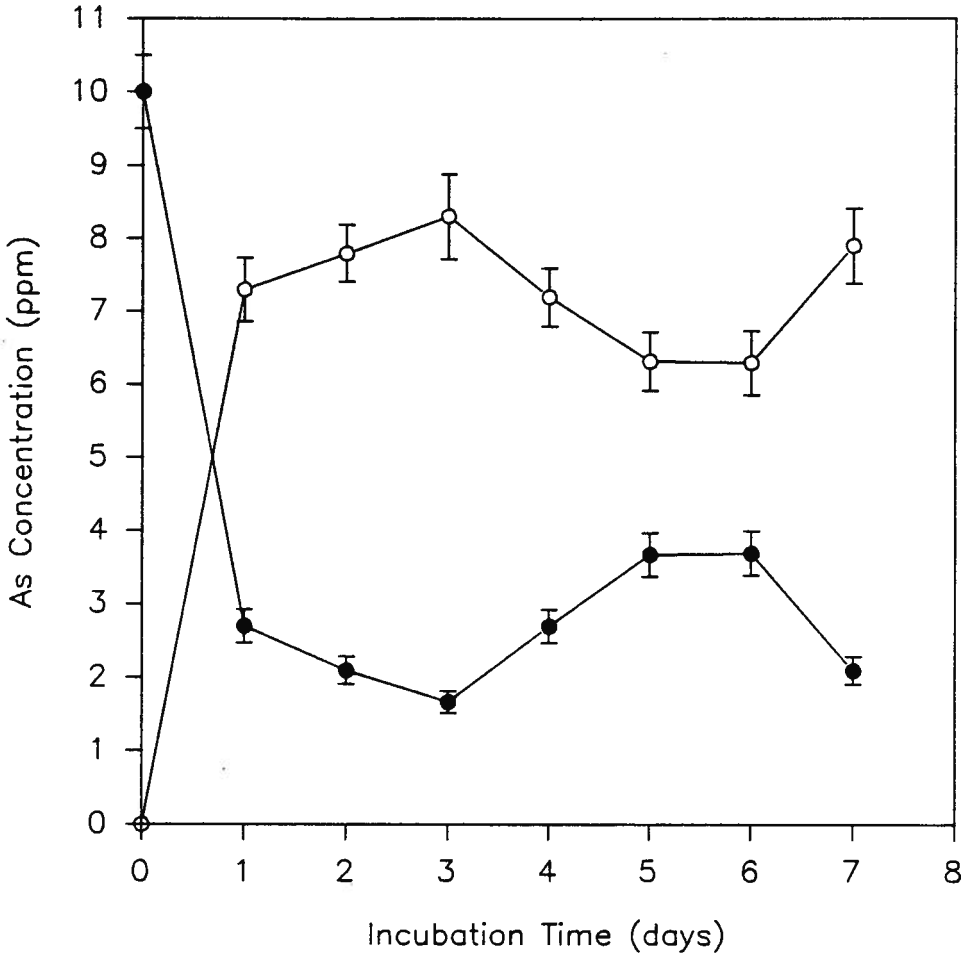


Figure 5.2 The change of arsenic species in the growth medium with incubation time. The growth medium was enriched with 10 ppm arsenate before incubation. (O) arsenite (●) arsenate

**The values are averages of four replicate determinations by HG-GC-AA of separate aliquots (0.02 mL) from the 6 mL sample taken at each time interval. The error bars represent 90% confidence intervals.

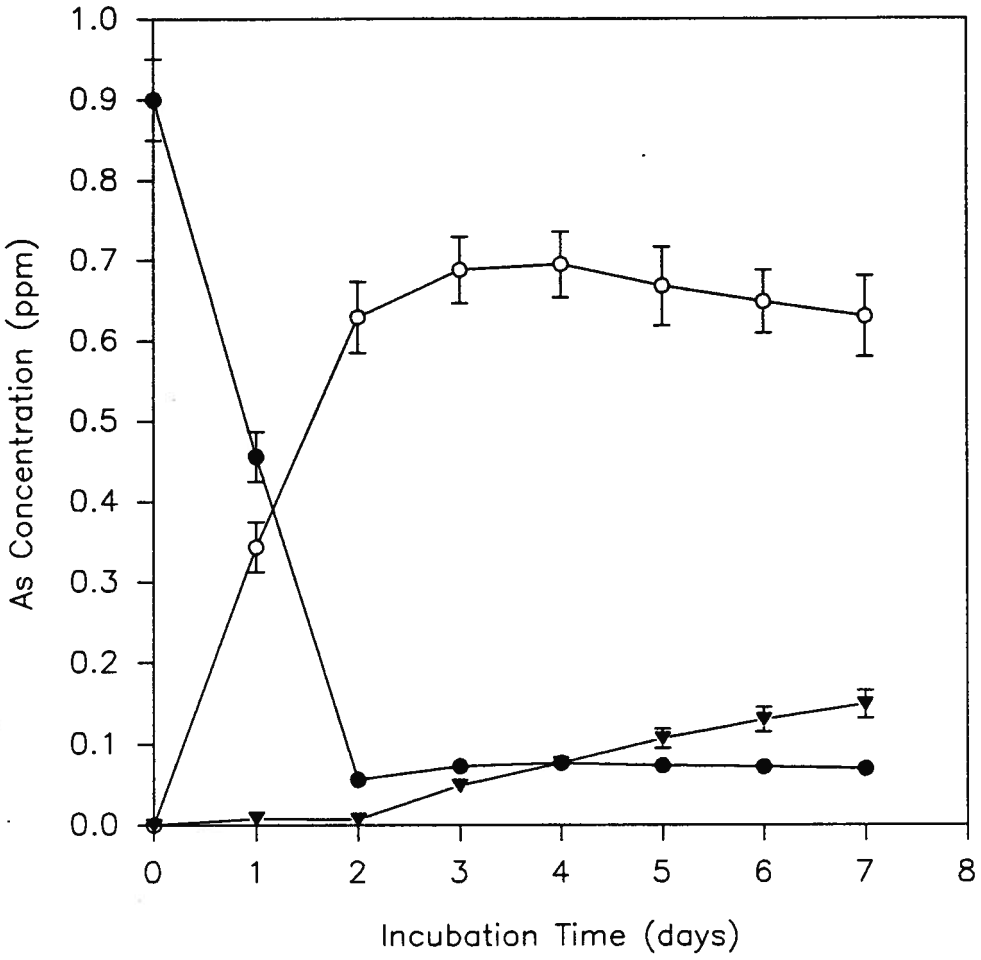


Figure 5.3 The change of arsenic species in the growth medium with incubation time. The growth medium was enriched with 0.9 ppm arsenate before incubation. (O) arsenite (●) arsenate (▼) DMA

****The values are averages of four replicate determinations by HG-GC-AA of separate aliquots (0.2 mL) from the 6 mL sample taken at each time interval. The error bars represent 90% confidence intervals, and are smaller than the size of the symbols when they are not evident on the graph.**

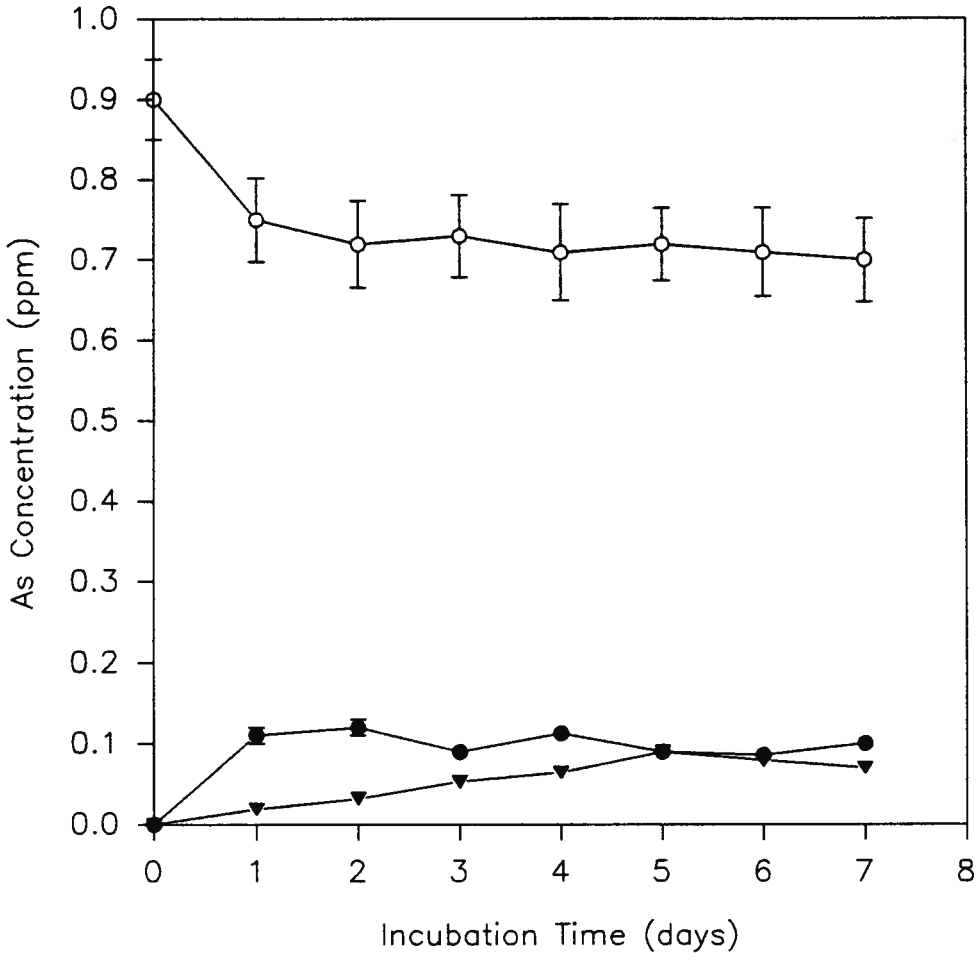


Figure 5.4 The change of arsenic species in the growth medium with incubation time. The growth medium was enriched with 0.9 ppm arsenite before incubation. (O) arsenite (●) arsenate (▼) DMA

****The values are averages of four replicate determinations by HG-GC-AA of separate aliquots (0.2 mL) from the 6 mL sample taken at each time interval. The error bars represent 90% confidence intervals, and are smaller than the size of the symbols when they are not evident on the graph.**

5.3.4 Arsenic efflux studies

After the cells were exposed to arsenicals for 7 days, they were washed and transferred to fresh arsenic-free media, and left for a further 7 days. Arsenic speciation analysis in this "fresh" media was carried out by using HG-GC-AA. Figure 5.5 shows that the accumulated arsenicals were rapidly excreted to the media by the cells, usually within 1-2 days. The difference in the amounts of hydride-forming arsenicals in the cells after the first 7 days and after 14 days was compared with the amounts of hydride-forming arsenicals released in the medium during the second 7 days period. The results are, in general, in agreement, indicating that "hidden" arsenic species were not produced during this period. This conclusion is also reinforced by the results obtained by using the flow injection-microwave digestion-HGAA technique.

The amount of DMA found in the medium after 14 days from cells treated with either 10 ppm arsenate or arsenite was higher than that detected in the cells before the transfer. Consequently, the amount of inorganic arsenicals, mainly arsenite, was greatly decreased. In contrast, in the 0.9 ppm arsenate or arsenite experiments, a decrease in the amount of DMA and an increase in the amount of inorganic arsenicals was observed, indicating that a demethylation process took place. After the cells which had been exposed to MMA were transferred to an arsenic-free medium, the endocellular MMA and DMA were excreted to the medium. An increase in the concentration of DMA and a decrease in the concentration of MMA was also observed after 1-2 days.

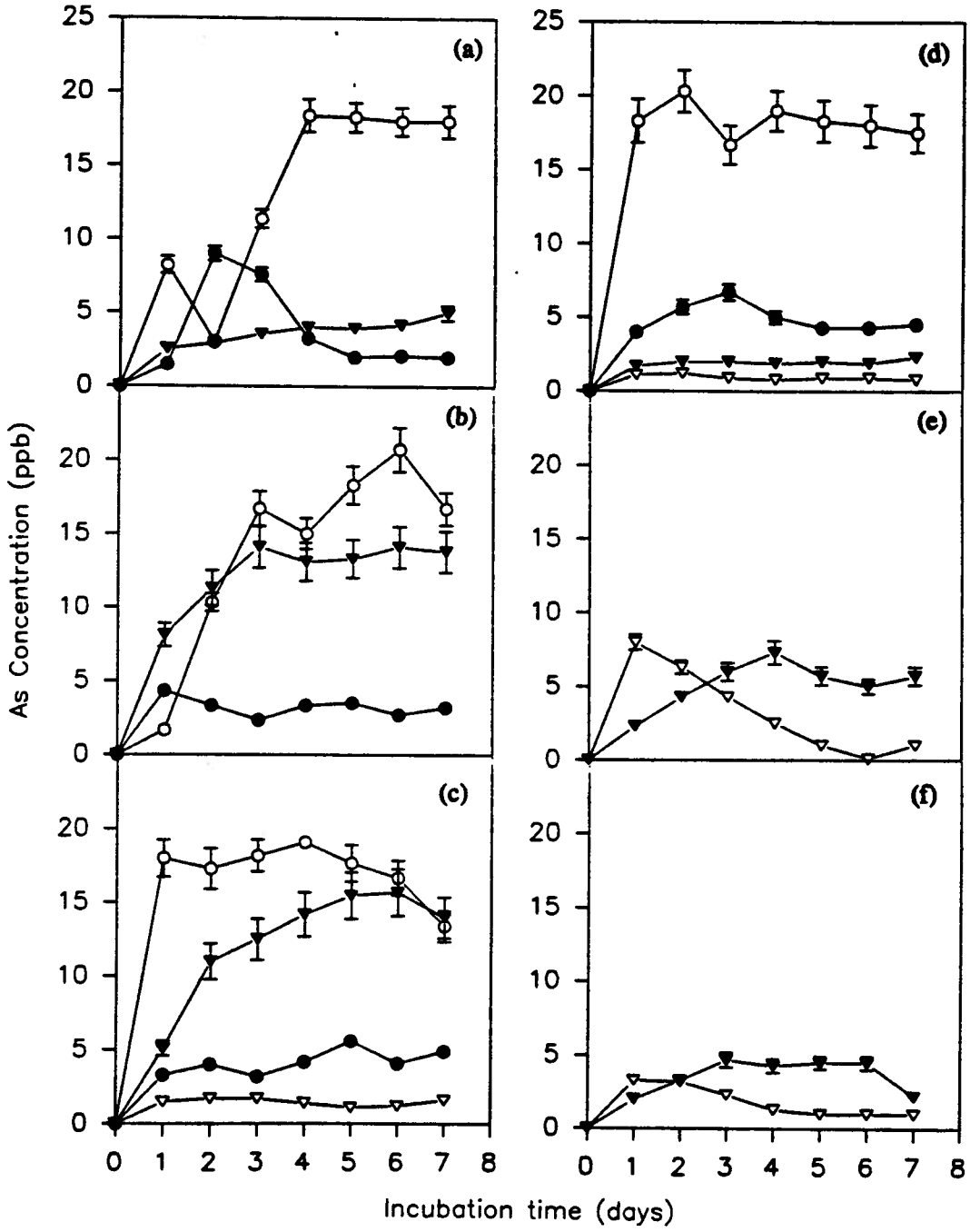


Figure 5.5 The change of arsenic species in media with incubation time after the transfer of cells that had been previously grown in (a) 10 ppm arsenate (b) 0.9 ppm arsenate (c) 10 ppm arsenite (d) 0.9 ppm arsenite (e) 10 ppm MMA (f) 0.9 ppm MMA. (O) arsenite (●) arsenate (∇) MMA (▼) DMA

**The values are averages of four replicate determinations by HG-GC-AA from the sample taken at each time interval. The error bars represent 90% confidence intervals, and are smaller than the size of the symbols when they are not evident on the graph.

5.3.5 Characterization of dimethylarsenic derivative in growth medium by using HG-GC-MS

The HG-GC-MS methodology can be used for isolation and characterization of arsines derived from hydride-forming arsenicals. In this study, the mass spectrometer was scanned from m/z 74 to 115 at 1 scan per 0.1 s by using a wide scan mode. Scans above m/z 115 were not made since no trimethylarsenicals were produced as mentioned previously. The present study mainly concerned the detection of dimethylarsine. The chromatogram and mass spectra of arsine, methylarsine and dimethylarsine which were produced from standard arsenate, MMA, and DMA, obtained by using wide-scan monitoring mode are shown in Figure 5.6; the spectra are similar to those shown in Chapter 4.

In order to investigate the arsenic methylation pathway in *P. peniculus*, we added L-methionine-methyl-d_3 and arsenate (1 ppm) to the growing culture of the alga and analyzed the aqueous arsenic metabolites in the medium by using HG-GC-MS. In the first experiment, only arsenate was added to the growth medium. After four days of incubation, a dimethylarsenic derivative was detected in the medium, as can be seen from the gas chromatogram obtained by using HG-GC-MS (Figure 5.7). This result is similar to those obtained by using HG-GC-AA. The first peak in the gas chromatogram was identified as arsine [AsH_3] (Figure 5.8), and the second peak as dimethylarsine [$(CH_3)_2AsH$] (Figure 5.9). This assignment was made based on a comparison of their retention times and mass spectra with those produced by standards (Figure 5.6).

In the second experiment arsenate and L-methionine-methyl-d_3 were added to the growing culture at the same time. The HG-GC-MS chromatogram obtained from samples collected on day 4 (the day of the addition of arsenate is referred to as day 0) by using HG-GC-MS is similar to that shown in Figure 5.7. The mass spectrum for the

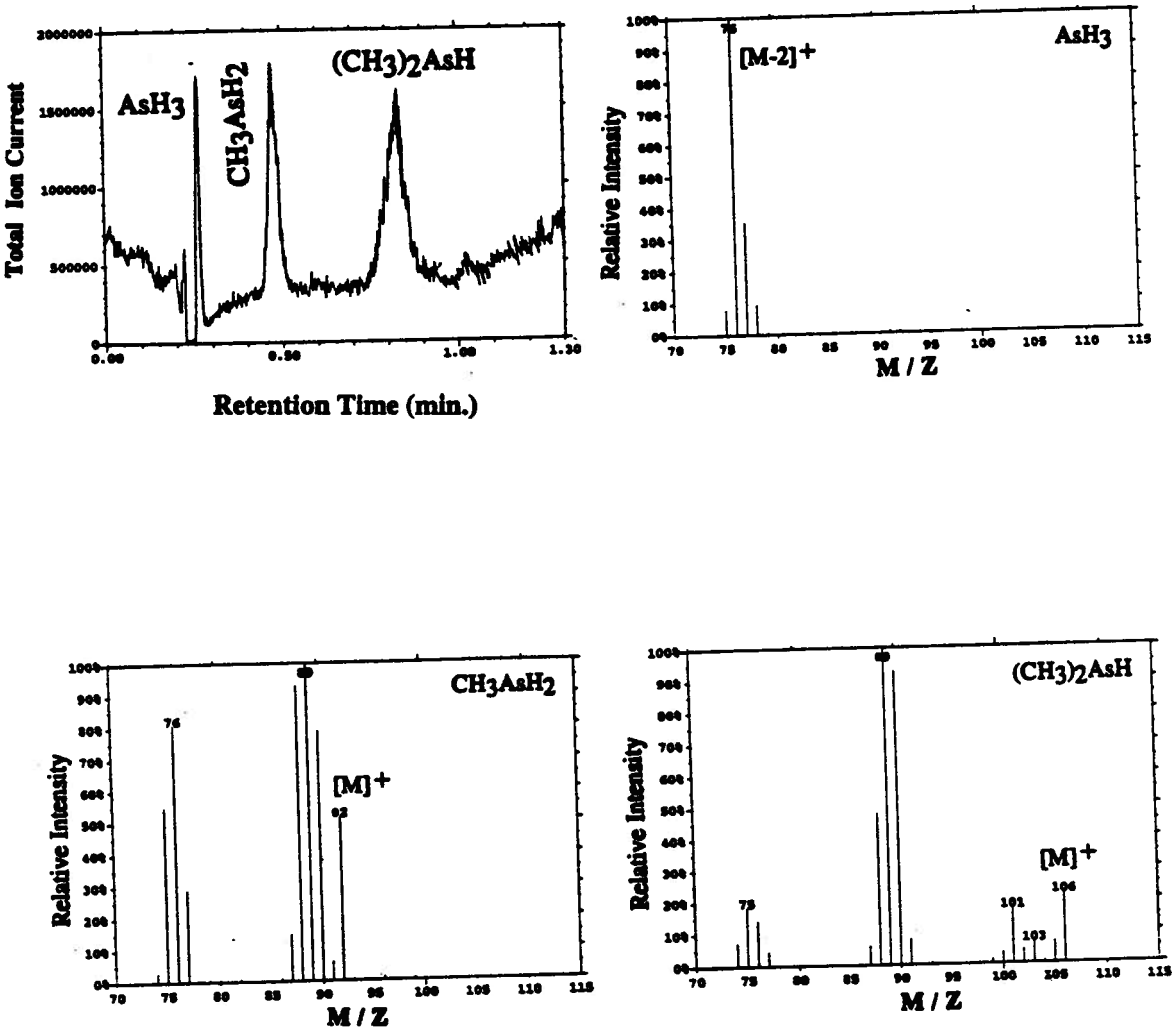


Figure 5.6 HG-GC-MS chromatogram and mass spectra of arsine, monomethylarsine, and dimethylarsine. Solutions of standard arsenite, MMA, and DMA (100 ng of arsenic for each compound) were used.

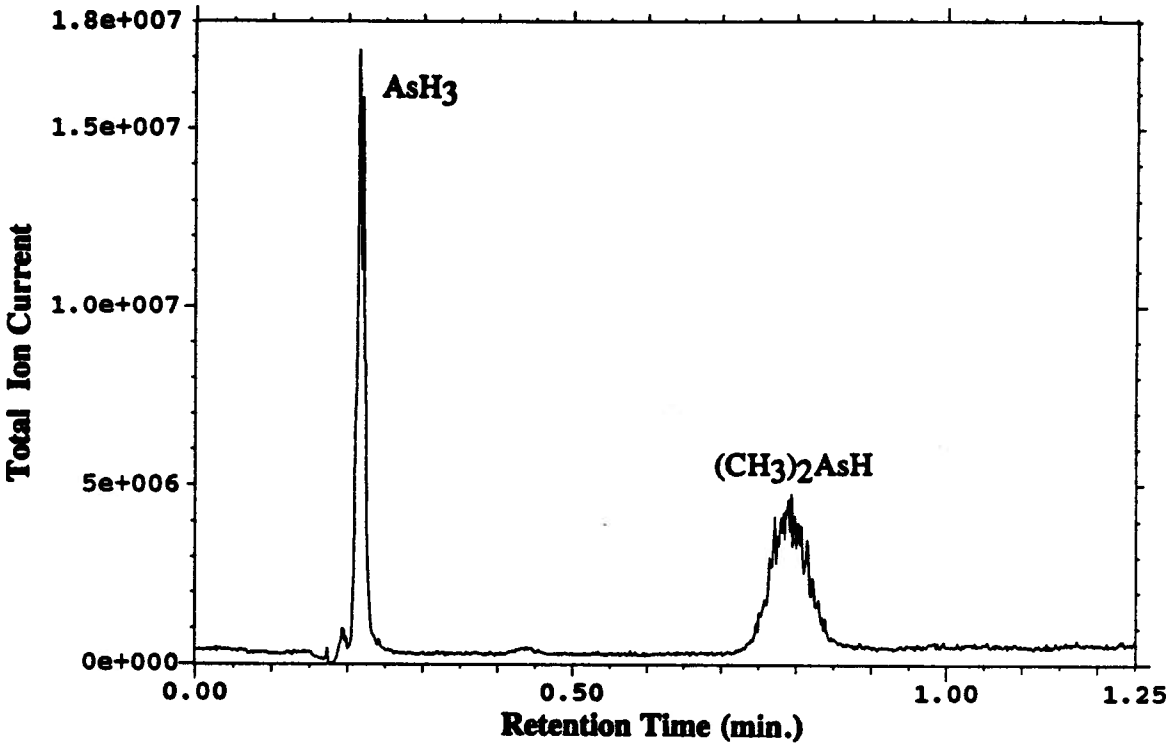


Figure 5.7 HG-GC-MS chromatogram of a 3 mL culture medium inoculated with arsenate (no methionine present), after 4 days of growth.

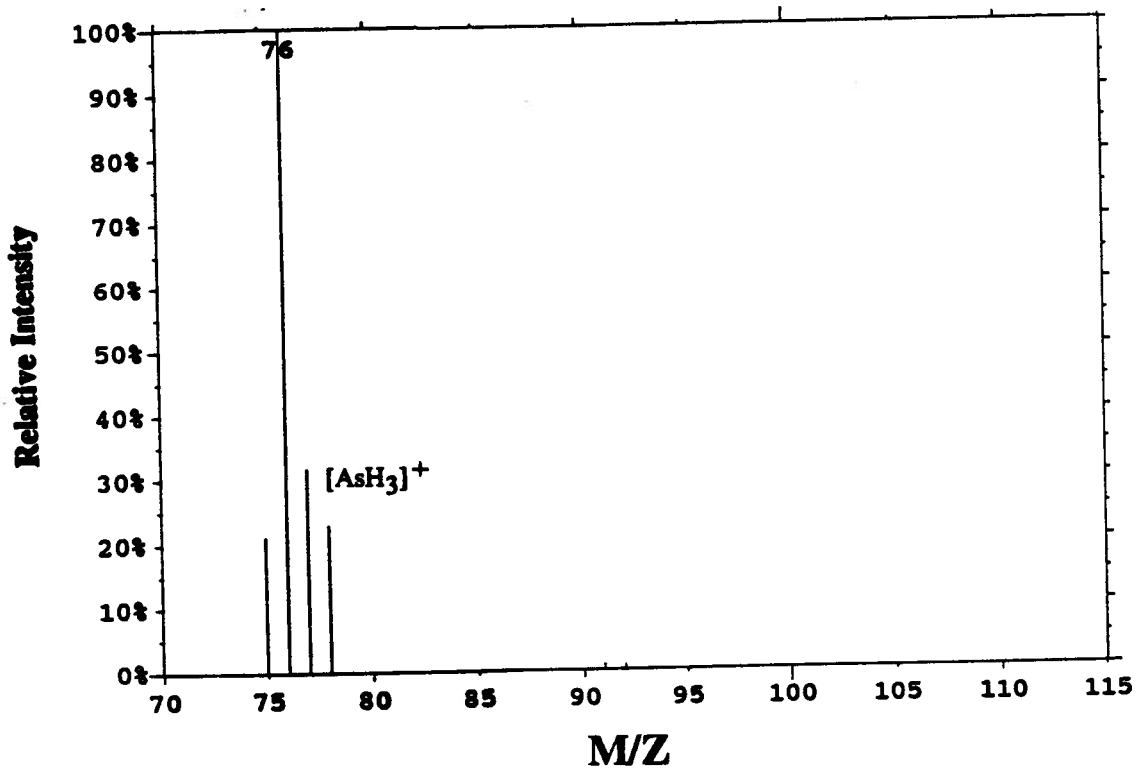


Figure 5.8 Mass spectrum of AsH_3 produced from a 3 mL culture medium inoculated with arsenate (no methionine present), after 4 days of growth.

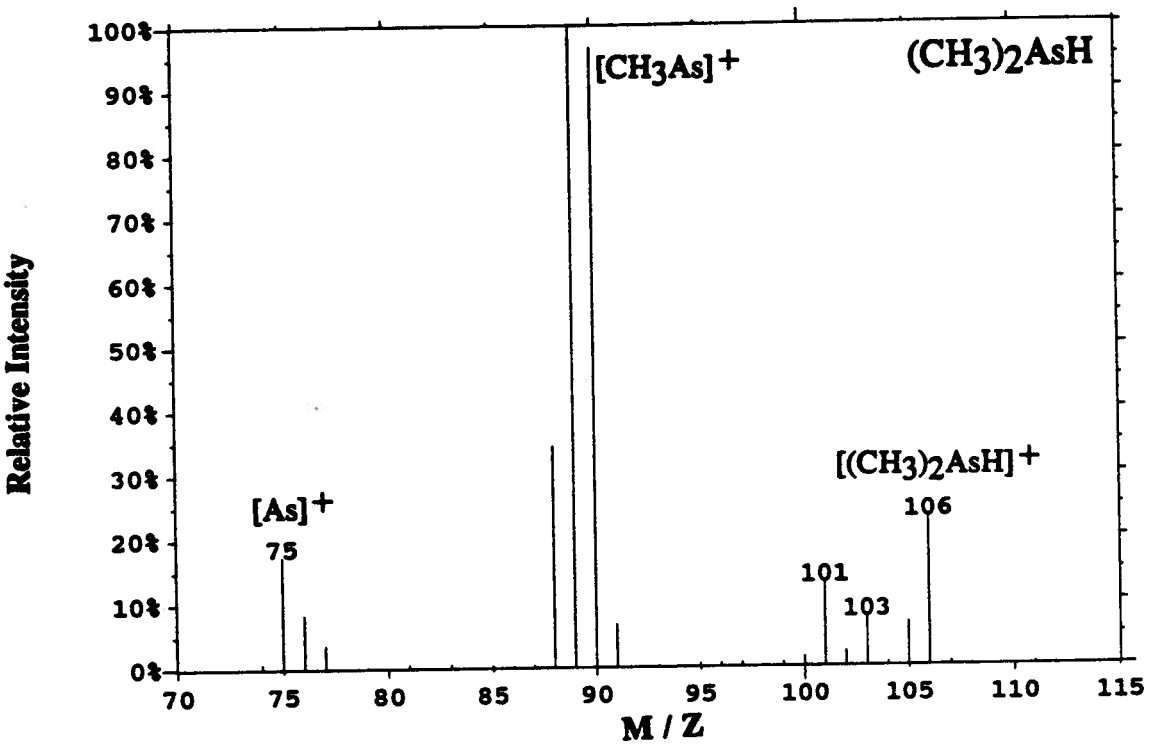


Figure 5.9 Mass spectrum of $(CH_3)_2AsH$ produced from a 3 mL culture medium inoculated with arsenate (no methionine present), after 4 days of growth.

second peak indicates that the CD_3 incorporation in the dimethylarsenic species is significant as indicated by ions at m/z 112 $[(CD_3)_2AsH]^+$, m/z 109 $[(CH_3)(CD_3)AsH]^+$, m/z 94 $[CD_3AsH]^+$ and m/z 93 $[CD_3As]^+$ (Figure 5.10). These ions are absent from the mass spectra resulting from the medium containing no L-methionine-methyl-d_3 (Figure 5.9). Further evidence of the incorporation of the CD_3 in the dimethylarsenic species is provided by the single ion chromatograms of m/z 93, 109, and 112 shown in Figure 5.11. It can be seen that their retention times are identical to the retention time of dimethylarsine shown in Figure 5.8. These peaks do not exist in the absence of L-methionine-methyl-d_3. The amount of DMA detected in the growth medium had increased slightly with time during the 11 days incubation. This result is similar to those obtained by using HG-GC-AA.

In the third experiment, L-methionine-methyl-d_3 was inoculated into the medium three days prior to the addition of arsenate. Two peaks are shown in the gas chromatogram from samples collected on day 4 (the day of the addition of arsenate is referred to as day 0). The mass spectrum of the second peak also confirms the incorporation of the deuterated CD_3 group in the dimethylarsenic species as shown by the presence of ion m/z 112, 109, and 93. The extent of CD_3 incorporation is similar to the second experiment.

When L-methionine-methyl-d_3 was added to the culture medium three days after the incubation of arsenate with the alga, the mass spectrum of the dimethylarsenic compound (Figure 5.12) shows that the incorporation of CD_3 is less than that found in the experiments described above.

The distribution of the CD_3 label in the dimethylarsenic metabolites with incubation time is presented in Table 5.5. The percentage of CD_3 incorporation was determined by comparing the relative peak intensities of the parent ions, m/z 106

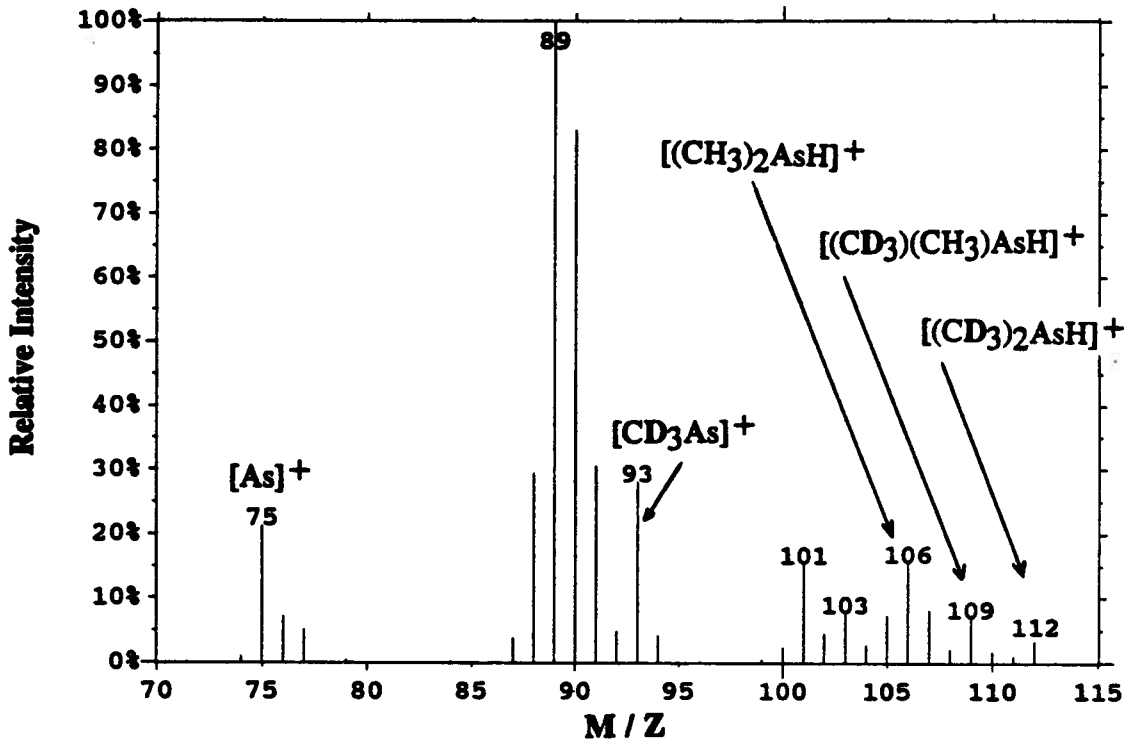


Figure 5.10 Mass spectrum obtained from the culture medium inoculated with L-methionine-methyl-d_3 and arsenate simultaneously, after 4 days of growth.

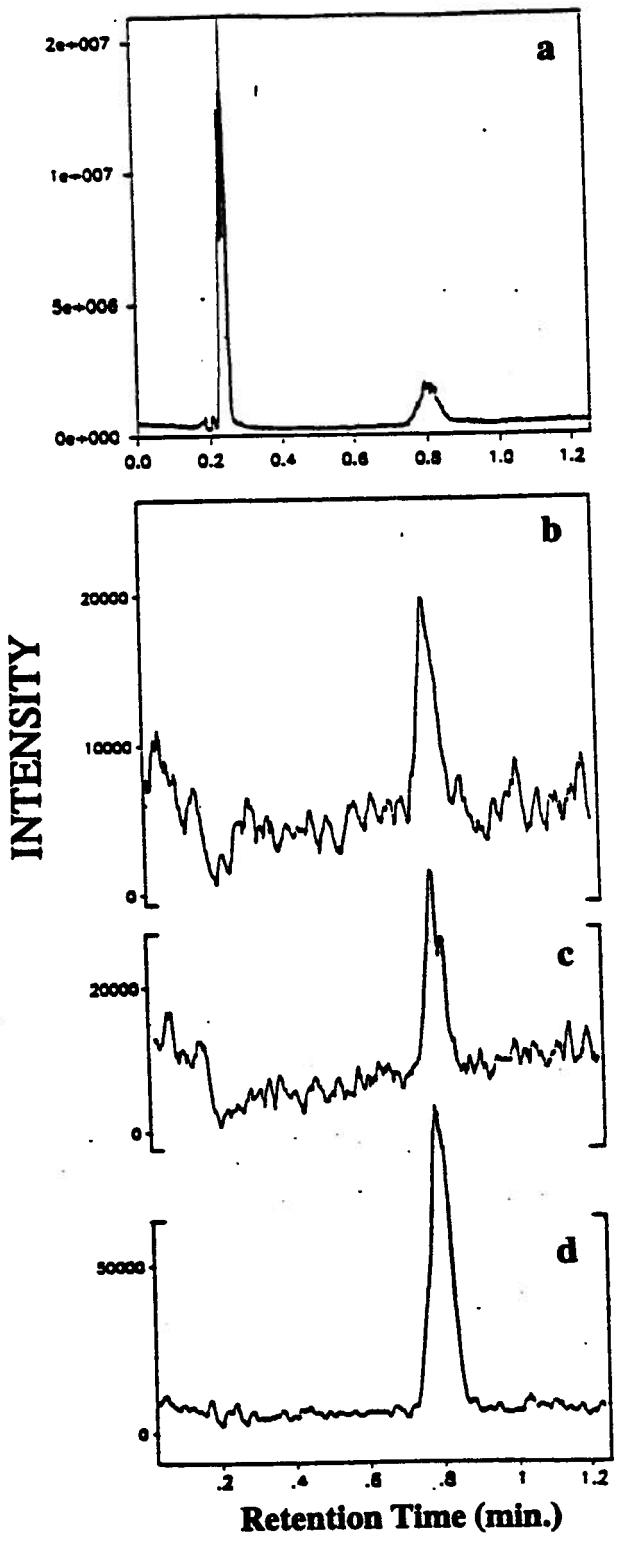


Figure 5.11 Wide scan and Selected Ion Chromatograms (SIC) obtained from the analysis of culture medium inoculated with both L-methionine-d3 and arsenate. a) Wide scan m/z 74-115 b) SIC of m/z 112 c) SIC of m/z 109 d) SIC of m/z 93

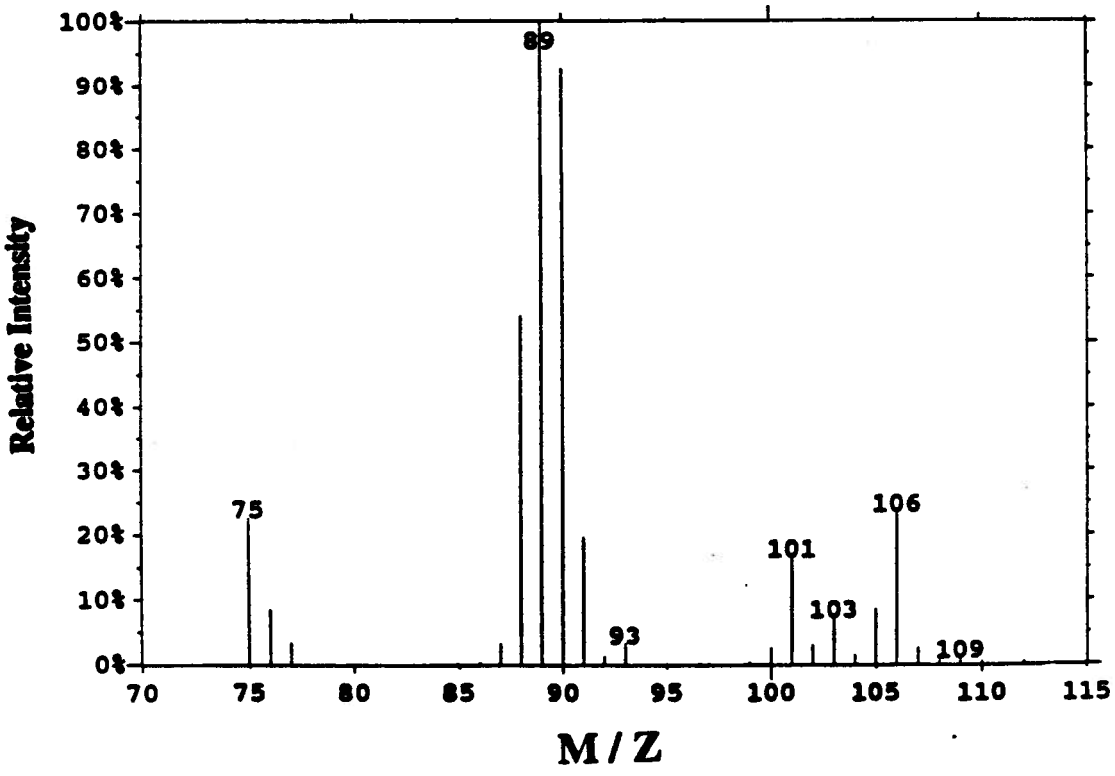


Figure 5.12 Mass spectrum obtained from culture medium inoculated with L-methionine-methyl-d_3 and arsenate (4 days after the addition of methionine). The arsenate was added 3 days prior to the addition of methionine.

$[(CH_3)_2AsH]^+$, 109 $[(CH_3)(CD_3)AsH]^+$, and 112 $[(CD_3)_2AsH]^+$. The relative standard deviation of the peak intensity of m/z 106 for 4 determinations of standard dimethylarsinic acid was 6%. For lack of a standard, the standard deviation of the peak intensity of m/z 112 from deuterated dimethylarsine was not determined.

Table 5.5 Percentage distribution of dimethylarsenic compounds detected in the medium

Experiment Number:	1	2			3		4		
Experimental condition:	Arsenate[a] added only	Arsenate and methionine added on the same day			Arsenate added 3 days after methionine inoculation		Arsenate added 3 days prior to methionine inoculation		
Day[b]	5	4	7	11	4	7	4	7	11
Species detected:									
$(CH_3)_2AsH$	100	73	62	67	60	69	92	91	86
$(CD_3)(CH_3)AsH$	0	20	29	20	27	21	4	4	9
$(CD_3)_2AsH$	0	7	9	13	13	10	4	5	5

[a] The medium in all experiments contained 1 ppm arsenate.
[b] The day of arsenate addition is referred to as Day 0.

5.4 DISCUSSION

As mentioned in the introduction, Edmonds and Francesconi[80] have proposed the biotransformation pathway (Figure 1.5) to explain the production of arsenosugars found in marine macroalgae. In the present work, we demonstrate that when arsenate, arsenite and MMA are used as substrates for the unicellular alga *P. peniculus* the principal methylation product is DMA. No trimethylarsenical species are found in the cells or in the growth medium. Exposure of *P. peniculus* to arsenate yields arsenite (in

cells, and in the media) and DMA (in cells and in the medium spiked with 0.9 ppm arsenate). When the alga is treated with arsenite, MMA and DMA are detected in the cells; the metabolite DMA can also be found in the growth medium spiked with 0.9 ppm arsenite. The substrate MMA is biotransformed by *P. peniculus* to produce DMA in the cells. When DMA is used as a substrate, trace amounts of the demethylation product MMA are detected in the cells. When *P. peniculus* is transferred from arsenic enriched media to arsenic free media, the accumulated arsenicals in the algal cells are excreted into the "fresh" media. Biotransformation of arsenic, including methylation and demethylation, also takes place in this media.

The most significant result from these studies is that no complex arsenic compounds, such as arsenosugars, are produced by *P. peniculus*. When the concentration of arsenicals varies from as high as 10 ppm to as low as 20 ppb (in the second cycle), there are no "hidden" arsenicals in either the cells or the media as judged by the flow injection-microwave digestion-HGAA methodology which has been shown to be very effective in decomposing and converting complex arsenicals to hydride-forming species.[197,198] Results obtained by using flow injection-microwave digestion-HGAA are also in agreement with those obtained by using HG-GC-AA for speciation. It seems that the alga *P. peniculus* follows the microbial biomethylation pathway proposed by Challenger for microbial processes (Figure 1.1), and in the case of *P. peniculus*, DMA is the probable end product of this biomethylation.

The reduction of arsenate to arsenite is proposed to be the first step towards methylation,[30,43] and our results are in agreement: most arsenate in the medium is reduced to arsenite by *P. peniculus* prior to the detection of DMA in the medium. Arsenate reduction to arsenite proceeds rapidly and most of the arsenate is reduced to arsenite within 1-2 days. It is possible that arsenate, being chemically similar to

phosphate, is readily taken up by algae[65-67] and then reduced by thiols or dithiols as a detoxification process.[2] This reduction could be enzymatic or could be a chemical reaction resulting from the interaction of arsenate with an enzymatically produced reducing agent.[36] Regardless of which mechanism is correct, the results show that it is necessary to have a biologically intact organism capable of generating the appropriate reducing conditions, because nonmetabolizing, enzymatically inactive cells do not reduce arsenate to arsenite in the growth medium.

Compared to cells exposed to 0.9 ppm arsenate, the accumulation of arsenic in cells is much lower when the cells are exposed to the same concentration of arsenite, MMA, or DMA; entry of MMA and DMA into the cell probably occurs by means of passive diffusion.[2] In particular, the uptake of MMA seems to be the least efficient. This result agrees with that reported by Cullen and Nelson[191-193] that MMA has a much lower diffusion coefficient than DMA. The diffusion coefficient was determined when liposomes were employed as model membranes and showed that the diffusion coefficient of MMA was shown to be 10 times lower than DMA.

Arsenate at high concentration (10 ppm) is more toxic to *P. peniculus,* and affects the arsenic accumulating ability of the alga as shown in Table 5.2. It is possible that high concentrations of arsenate inactivate the phosphate transport system and interfere with oxidative phosphorylation.[199-201]

A high concentration (10 ppm) of arsenate or arsenite also seems to inhibit the biomethylating ability of *P. peniculus*. As shown in Table 5.2, only a small amount of methylated arsenicals is found in the cells after 7 days growth. In a lower arsenate or arsenite concentration (0.9 ppm), *P. peniculus* can efficiently methylate inorganic arsenic to DMA which can either be excreted into the medium or kept in the cells. When the alga is transferred from a hostile environment, such as medium containing 10 ppm

arsenate or arsenite, to a fresh arsenic-free environment, the biomethylating ability of *P. peniculus* seems to be restored. This was indicated by an increase in the amount of DMA in the new medium compared to the amount of DMA in the cells harvested at the end of the first cycle.

It is not surprising to see that little or no MMA is detected when the alga is treated with arsenate and arsenite. Cullen *et al.*[51] reported that only traces of MMA were produced from arsenate by broken cell homogenates of *Apiotrichum humicola* and that MMA is the least transformed arsenical substrate. They tentatively concluded that MMA is not a free intermediate in Challenger's arsenate-to-trimethylarsine pathway. The results obtained from whole cell cultures of *A. humicola* as previously described in Chapter 3 also show that only limited amounts of MMA are found as an extracellular arsenic metabolite in the growth medium. As mentioned previously, MMA has a very low diffusion coefficient, and the cells may prefer to metabolize the endocellular MMA to DMA than to wait for MMA to diffuse into the growth medium. The DMA thus produced has a higher permeability coefficient, and can be excreted by the cells to the growth medium. This surmise seems to be consistent with what we have observed. Work on arsenic speciation in seawater shows that arsenite and DMA are the main arsenical products of natural phytoplankton blooms; MMA has not been detected in high concentrations.[202-204]

It has been shown that a variety of marine phytoplankton take up arsenate from seawater and produce arsenite, MMA and DMA,[20,60,70,72,74,79] and release them into the surrounding media.[20,70,79] The efflux studies of *P. peniculus* demonstrate that the excretion of arsenicals is a rapid process. No "hidden" arsenic species are detected in the cells and in the medium, indicating that *P. peniculus* does not produce arsenosugars when it is exposed to low concentrations of arsenic species. We suggest that the fast

excretion of the reduced and /or methylated arsenic compounds to the media reduces the requirement for further detoxification process. It seems that the cells interact not only with endocellular arsenicals but also with the excreted arsenicals as indicated by the differences in the amount of individual arsenical species before and after the transfer. The biotransformation, the excretion, and the re-uptake of the excreted arsenicals may take place simultaneously. These interactions seem to reach a steady states after 2-3 days in the "fresh" media.

Both HG-GC-AA and HG-GC-MS technique have proved to be very useful tools for the determination of hydride-forming arsenic compounds.[116,139,140,162,169,170] The HG-GC-AA system is more widely used because it is cheaper and easier to access than the latter. However, HG-GC-MS system is a very useful tool for characterizing an unknown volatile compound because both the retention time and its mass spectrum are thus available. The results from the culture samples obtained by using the mass spectrometer in the wide scan monitoring mode clearly shows that only a dimethylarsenic species is produced, and excreted to the growth medium by the alga.

The most significant result is that when L-methionine-methyl-d_3 is added to the growth medium, the CD_3 group is incorporated to a considerable extent by the cells to form deuterated dimethylarsenic species. This is revealed by the ions at m/z 112, 109, and 93. The mass spectrum of the volatile dimethylarsenic compound indicates that a mixture of deuterated and non-deuterated DMA is present in the growth medium of alga *P. peniculus*.

The confirmation of CD_3 incorporation in the dimethylarsenic compound is important, as it is the first time that methionine, or S-adenosylmethionine, has been shown to be the source of the methyl groups in the biological alkylation of arsenic in marine algae. This result confirms that *P. peniculus* also follows the oxidation-reduction

pathway involving carbonium ions originally suggested by Challenger for the alkylation of arsenic by microorganisms.[30,43] As previously mentioned, DMA is probably the end product of this methylation process in *P. peniculus*.

In addition to systematic and random errors, the errors such as the assumption that the ionization efficiencies, and thus the responses, of the deuterated arsines are identical to the responses of the undeuterated arsines, may affect the calculated incorporation percentages of CD_3. A previous study by Cullen *et al.*[33] demonstrated that the deuterated trimethylarsine shows slightly lower sensitivity than the non-deuterated trimethylarsine under mass spectrometry conditions. Thus, the results presented in Table 5.5 should only be viewed as an indication of low or high incorporation without too much reliance being placed on the actual values. Nevertheless, it can be clearly seen that a high incorporation of the CD_3 label in the dimethylarsenic species does occur.

The added methionine is expected to be incorporated into the cell's pool of SAM, and could enhance any methylation process. This expectation is based on previous result obtained by Cullen and coworkers.[33] In their experiment different concentrations of methionine were applied to the growing culture of microorganisms for production of trimethylarsine, and it was found that the methyl incorporation in arsenicals is proportional to the concentration of methionine. Later, Cullen *et al.*[45] reported that pre-incubation of whole cell cultures of *A. humicola* or of their cell-free extracts with methionine did not effect the amount of the methylated arsenic species produced from methylarsine oxide. In our experiments, no apparent increase or decrease in the quantity of the dimethylarsenic metabolite was observed when L-methionine-methyl-d_3 was either present or absent in the medium. The data of Table 5.5 show that the quantity of CD_3 label incorporattion in the dimethylarsenic species in experiments 2 and 3 is quite

similar, about 27-40%. Pre-incubation of methionine with the cells neither affects the amount of DMA produced nor increases the percentage of deuterated DMA in the culture medium. In the fourth experiment L-methionine-methyl-d_3 was not added until 3 days after the incubation of arsenate with *P. peniculus*. The deuterated dimethylarsenic derivative was observed one day after the addition of methionine but in lower amounts than those observed in experiments 2 and 3. In addition, no significant increase is observed in the total amount of dimethylarsenic species and in the amount of deuterated arsenic species present after 11 days of incubation. This suggests that most of the arsenate had been incorporated and metabolized by the cells prior to the addition of L-methionine-methyl-d_3. After the methylated dimethylarsenic species is excreted into the growth medium, the methylation process slows down and reaches a steady state.

Based on these results we now propose a model for the methylation of arsenate by cells of *P. peniculus*. The basic steps are outlined in Figure 5.13. First, algal cells take up arsenate from the medium via the phosphate transport system, reduce the arsenate to arsenite inside the cells by using thiols and/or dithiols, and excrete most of the arsenite into the growth medium by means of an active transport system. This process is reasonably fast. Second, arsenite in the cells is methylated to MMA by using SAM; however, due to the low passive diffusion coefficient, the endocellular MMA is not excreted to the growth medium. As a consequence, the MMA remains in the cell where it is more likely to be reduced and further methylated to DMA. This arsenical which has a greater diffusion coefficient can diffuse into the growth medium by means of passive diffusion. The methylation of MMA could be a fast process. In Chapter 3, we reported that MMA is metabolized to DMA by whole cell cultures of *A. humicola* and is then excreted rapidly to the growth medium. After the excretion of DMA by the algal cells, the methylation process slows down as indicated by the absence of any change in

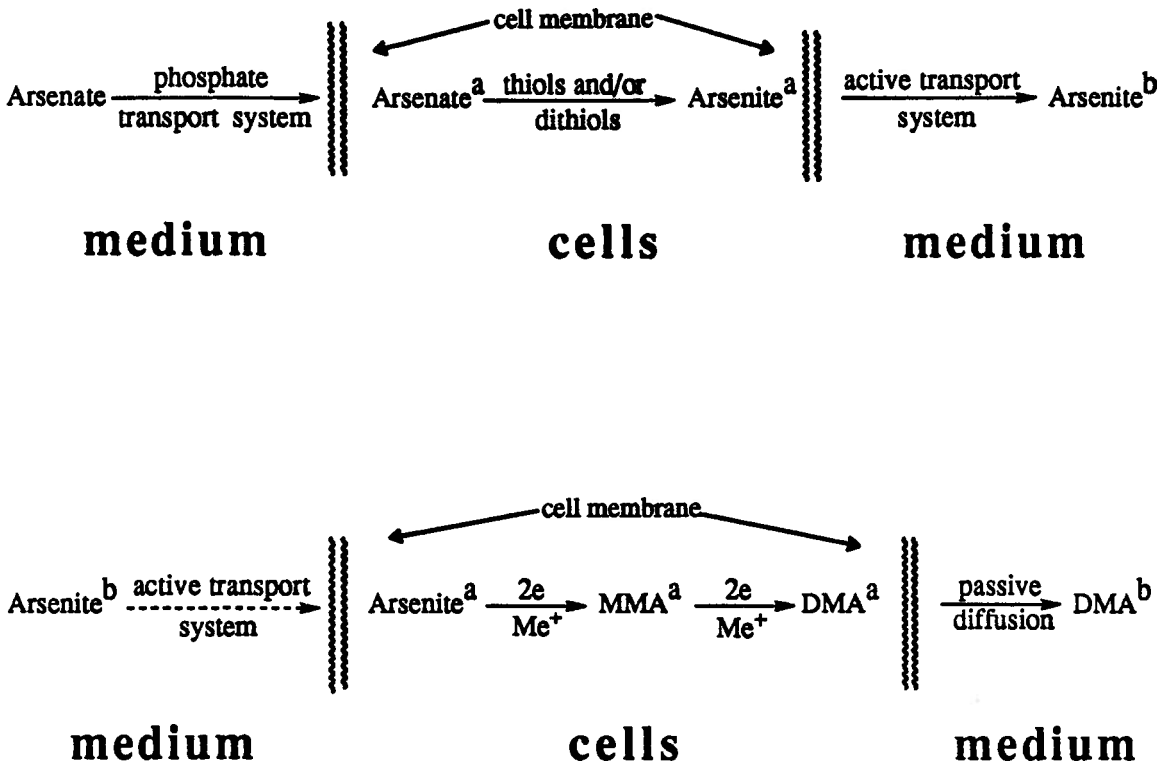


Figure 5.13 Proposed model for biomethylation of arsenate in marine algae *P. peniculus.* [a] endocellular arsenicals [b] extracellular arsenicals

the concentrations of DMA in the growth medium and by the results obtained in Experiment 4 where the addition of the deuterated methionine 3 days prior to the addition of arsenate does not increase the extent of CD_3 incorporation in the DMA. This model explains many of the results obtained in the present investigations, but in reality the biotransformation process is probably much more complex. For example, an uptake/excretion equilibrium between endocellular and extracellular arsenicals, and the cleavage of As-C bonds may also be involved in the metabolic process.

In conclusion, the results obtained from this study strongly suggest that methionine via S-adenosylmethionine, is the source of the methyl groups in the biological alkylation of arsenic in marine algae. The final arsenical biotransformation product of *P. peniculus* is a dimethylarsenic compound, probably DMA.

CHAPTER 6

THE IDENTIFICATION AND CHARACTERIZATION OF ANTIMONY(III), ANTIMONY(V), AND METHYLANTIMONY SPECIES BY USING HG-GC-MS

6.1 INTRODUCTION

Antimony is present in the aquatic environment as a result of the weathering of rocks, from soil runoff, and through effluents from mining and smelting.[205] It is now recognized that the toxicity and physiological behavior of antimony depend on its oxidation state.[206] Typical concentrations of antimony in unpolluted waters are less than 1 ppb.[207,208]

Hydride generation-atomic absorption spectrometry (HGAA) has been by far the most frequently used technique for determination of antimony in aqueous solutions.[26,205,209-212] The hydride forming antimony compounds are reduced to stibines by sodium borohydride solution ($NaBH_4$), and detected by an atomic absorption spectrometer.

Using a batch type hydride generator, Andreae *et al.*[26,205,211] identified the methylantimony compounds in natural water samples by using HG-GC-AA where the hydrides cochromatographed with those produced from supposedly authentic samples. On this basis the organoantimony species found in the samples were said to be methylstibonic acid $MeSbO(OH)_2$ and dimethylstibinic acid $Me_2SbO(OH)$.

However, Dodd *et al.*[212] reported that molecular rearrangement of stibine produced from pure methylantimony compounds $Me_3Sb(OH)_2$, Me_3SbCl_2, and $Me_2SbCl(OH)_2$ occurred when a batch type hydride generator was used, giving a mixture of antimony hydrides, possibly SbH_3, $MeSbH_2$, Me_2SbH, and Me_3Sb from all

three antimony(V) compounds. These results cast some doubt on the ability of the hydride generation technique to clearly distinguish different alkylated compounds of antimony. As a consequence, the results of earlier studies of antimony speciation in the aquatic environment[26,205,211] need to be regarded with caution.

In the present work, we use a continuous type hydride generation system coupled with GC-MS to identify the antimony hydrides produced from the trimethylantimony compounds $Me_3Sb(OH)_2$ and Me_3SbCl_2. The possible cause of the molecular rearrangement noted above was investigated. The HG-GC-MS system was also used to analyze the extracts of plant samples collected in Kam Lake and Keg Lake (Yellowknife). The results provided conclusive evidence of the presence of methylantimony compounds in these samples.

6.2 EXPERIMENTAL

6.2.1 Reagents

All chemicals used were of reagent grade. Distilled water was used for all dilution. Glass and plasticware were cleaned by soaking overnight in 2.0% Extran solution, followed by a water rinse, a soak in dilute hydrochloric acid, and finally a water rinse.

A solution of antimony potassium tartrate containing 1000 ppm of Sb was used to prepare the standards for the determination of antimony(III). The antimony(V) standard (1000 ppm of Sb) was prepared from potassium antimonate. The standards for $Me_3Sb(OH)_2$ and Me_3SbCl_2 were supplied by Dr. M. Dodd (Royal Roads Military College, Victoria, B.C., Canada). They were dissolved in distilled water, and diluted to

make a stock solution containing 100 ppm of Sb. The standard solutions were diluted to the appropriate concentrations daily for analytical use. Solutions of 2.0% (w/v) $NaBH_4$ prepared in 0.1% (w/v) NaOH, and 4.0 M acetic acid were freshly made daily.

6.2.2 Hydride generation-gas chromatography-mass spectrometry

The hydride generation-gas chromatography-mass spectrometry system (HG-GC-MS) used was previously described in Chapter 4. The experimental conditions are presented in Table 4.1. In this study, the mass spectrometer was scanned from m/z 120 to 170 at 1 scan per 0.3 second. The highest m/z value for any anticipated volatile stibine was 168, corresponding to $(CH_3)_3{}^{123}Sb$.

6.2.3 Experimental procedures

A Gilson Minipuls 2 four-channel peristaltic pump was used to deliver and mix sample, acid and sodium borohydride solution. The standard solution of either antimony(III), antimony(V), or the trimethylantimony standards was first mixed with 4.0 M acetic acid, and then 2.0% (w/v) $NaBH_4$. The hydrides were produced in an 18-turn glass reaction coil, carried by a helium flow to a glass liquid/gas separator where the spent solution constantly drained out, and then collected in a Teflon U-tube trap immersed in liquid nitrogen. The liquid nitrogen bath was then removed and the U-tube trap immersed in a 80°C water bath. The released stibines were separated by using GC on a silanized Porapak-PS column, and characterized by using the mass spectrometer. When the plant extracts were analyzed, 2 mL of 10% (w/v) EDTA was added to the sample (5 mL) prior to analysis.

6.3 RESULTS AND DISCUSSION

The HG-GC-MS technique has proved to be an effective tool for arsenic determination.[116,139,169] However, there has been no published report regarding the use of a HG-GC-MS system for the determination of antimony compounds. Most of the reported work concerning the analysis of Sb(III), Sb(V), and methylantimony species used a batch-type hydride generator coupled with a GC-AA system. The hydrides produced were usually concentrated in a cold trap, separated by using temperature programmed GC, and detected by using an AA.[26,205,211,212] Since the methylated antimony species can be separated from inorganic antimony on the basis of their chromatographic behavior, only Sb(III) and Sb(V) need to be differentiate by using the hydride generation step. The efficiency of the hydride generation process for Sb(III) and Sb(V) is related to their pK_a values, and depends strongly on the pH of the reaction medium.[205] At pH > 4, Sb(III) (pK_{a1} 11.0) is reduced to SbH_3 by $NaBH_4$; and at pH = 1, both Sb(III) and Sb(V) (pK_{a1} 2.7) form SbH_3.

The continuous type HG-GC-MS system described in Chapters 4 and 5 was used successfully to characterize the arsenic metabolites produced by a microorganism and by a marine alga. We showed that 4.0 M acetic acid could be used successfully for the analysis of arsenite, MMA, DMA, and TMAO. This approach was also applied to the analysis of Sb(III) and methylantimony species. Andreae *et al.*[205] suggested that slightly higher values of pK_{a1} are to be expected for the methylantimony species, compared to methylarsenic species, due to the larger ionic radius of antimony. Therefore samples acidified with 4.0 M acetic acid could be analyzed successfully for methylantimony species. Indeed trimethylstibine is easily produced from $(CH_3)_3Sb(OH)_2$ and $(CH_3)_3SbCl_2$ under these conditions as will be shown later. The principle aim of the present work was to identify the methylated antimony compounds

present in the samples, so further optimization of the hydride generation process was not attempted.

When a solution of Sb(III) was mixed with 4.0 M acetic acid and 2.0% (w/v) $NaBH_4$, the production of stibine SbH_3 was confirmed by the mass spectrometer. The HG-GC-MS chromatogram and the mass spectrum of SbH_3 are shown in Figure 6.1 and Figure 6.2, respectively. Because antimony has two major isotopes, ^{121}Sb (57%) and ^{123}Sb(43%), and arsenic has only one major isotope ^{75}As, the mass spectrum of stibine SbH_3 is more complex than that of arsine AsH_3. The ions at m/z 126 and 125 are from $[^{123}SbH_3]^+$ and $[^{123}SbH_2]^+$, and the ions at m/z 122 and 121 are from $[^{121}SbH]^+$ and $[^{121}Sb]^+$, respectively.

When a trimethylantimony compound $Me_3Sb(OH)_2$ was examined by using HG-GC-MS, the chromatogram (Figure 6.3) shows the presence of three peaks. The mass spectra of the first two peaks (Peak A and Peak B) are shown in Figures 6.4 and 6.5, and can not be assigned to either SbH_3, $MeSbH_2$, or Me_2SbH and do not appear to be Sb containing compounds as judged by the isotope pattern in the mass spectra. The mass spectra of the third peak in Figure 6.3 clearly shows the presence of $(CH_3)_3Sb$ as indicated by the ions at m/z 168 $[(CH_3)_3{}^{123}Sb]^+$, m/z 166 $[(CH_3)_3{}^{121}Sb]^+$, m/z 153 $[(CH_3)_2{}^{123}Sb]^+$, m/z 138 $[CH_3{}^{123}Sb]^+$, and m/z 121 $[^{121}Sb]^+$ (Figure 6.6). The HG-GC-MS chromatogram (Figure 6.7) obtained from a blank solution shows the presence of two peaks corresponding to Peak A and Peak B, indicating that these compounds are probably not from the original standard solution. The HG-GC-MS chromatogram and mass spectrum of the hydride produced from Me_3SbCl_2 are similar to that from $Me_3Sb(OH)_2$.

As mentioned above Dodd *et al.*[212] reported that the trimethylantimony compounds $Me_3Sb(OH)_2$ and Me_3SbCl_2 formed four hydrides SbH_3, $MeSbH_2$,

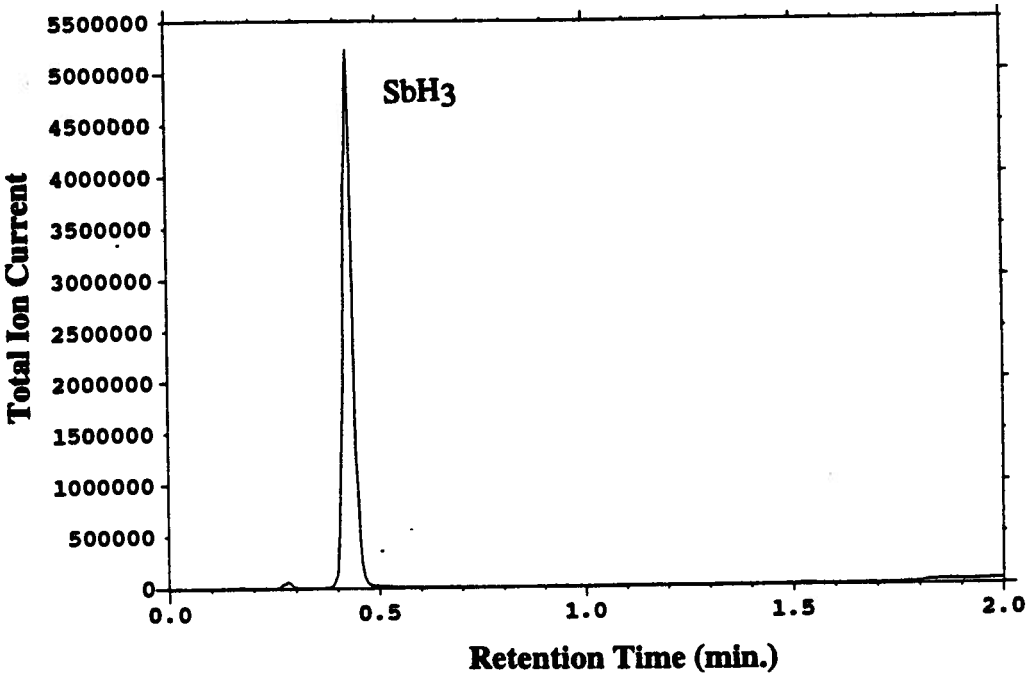


Figure 6.1 HG-GC-MS chromatogram of stibine SbH_3. A standard solution of Sb(III) (300 ng of antimony) was used.

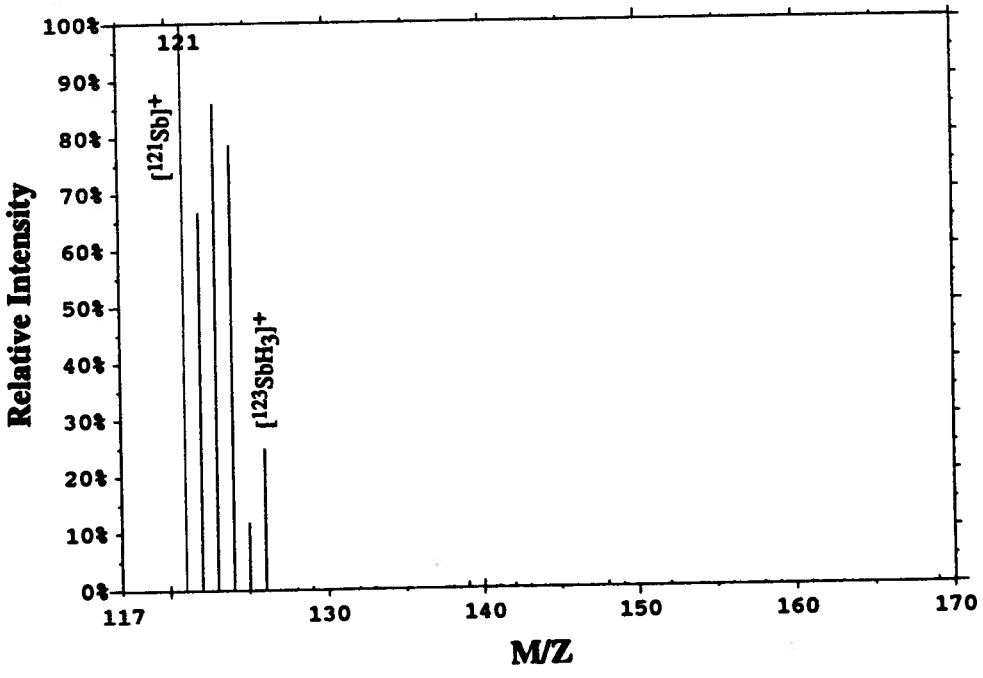


Figure 6.2 Mass spectrum of stibine SbH_3 derived from a standard solution of Sb(III).

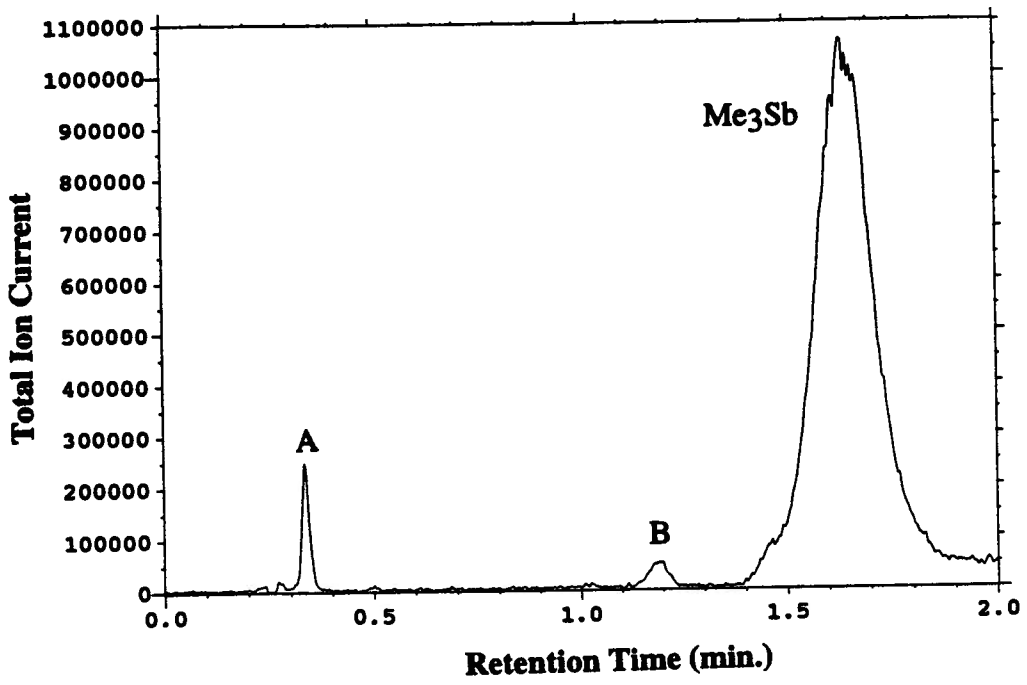


Figure 6.3 HG-GC-MS chromatogram of trimethylstibine Me_3Sb. A standard solution of $Me_3Sb(OH)_2$ (200 ng of antimony) was used.

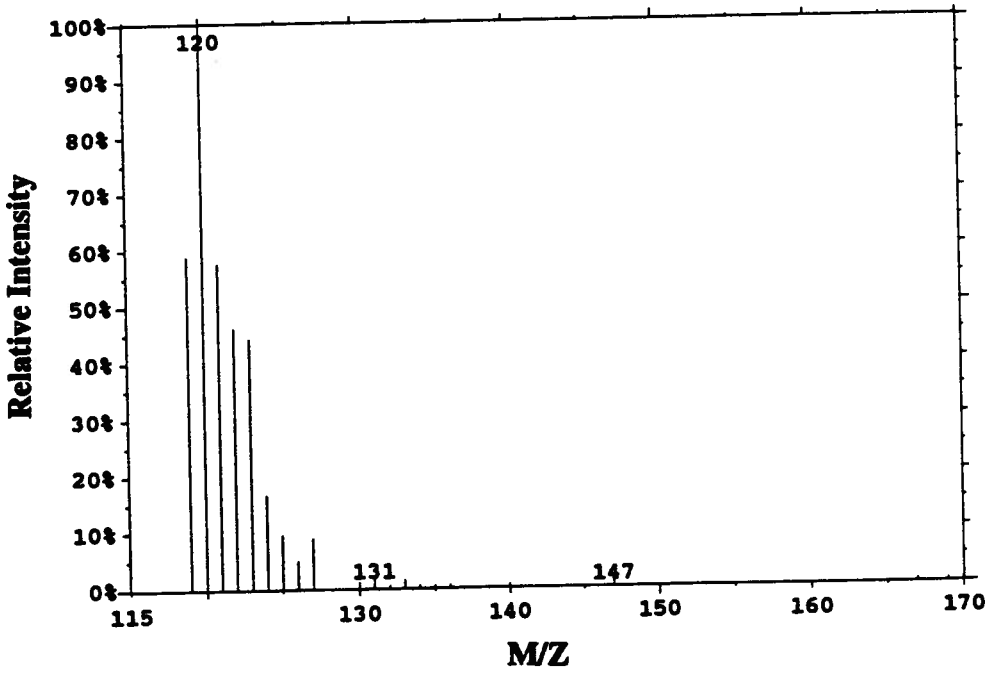


Figure 6.4 Mass spectrum of Peak A shown in figure 6.3.

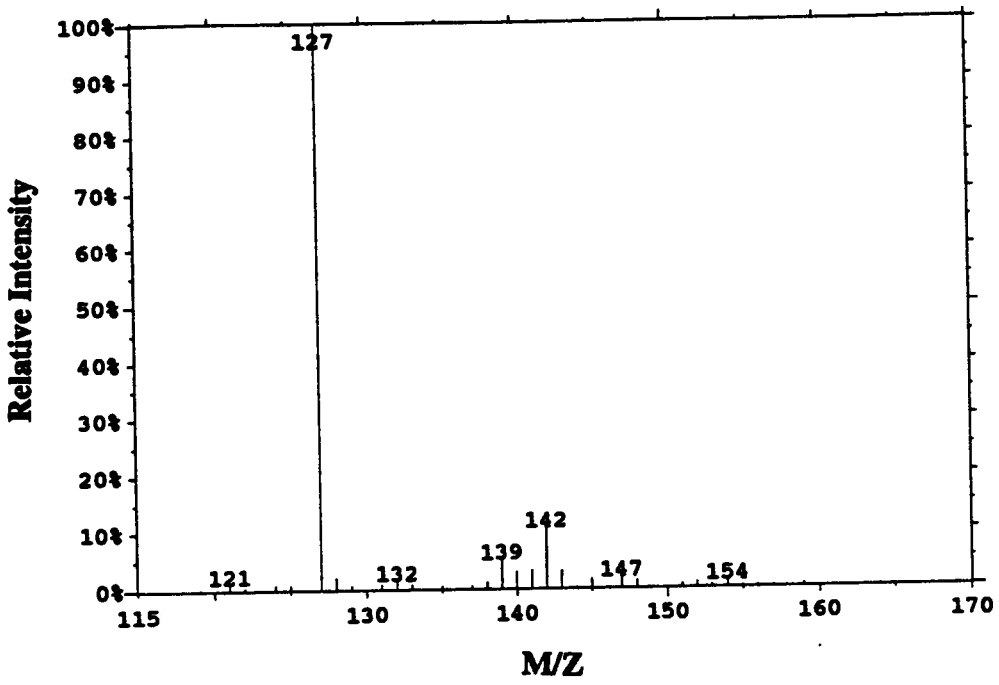


Figure 6.5 Mass spectrum of Peak B shown in figure 6.3.

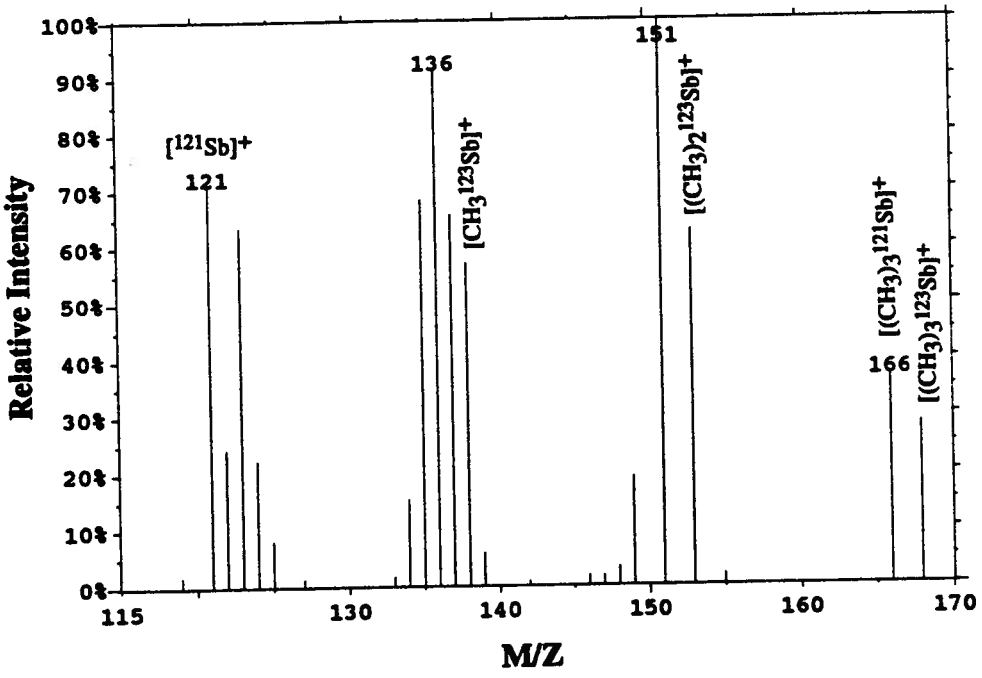


Figure 6.6 Mass spectrum of trimethylstibine Me_3Sb derived from a standard solution of $Me_3Sb(OH)_2$.

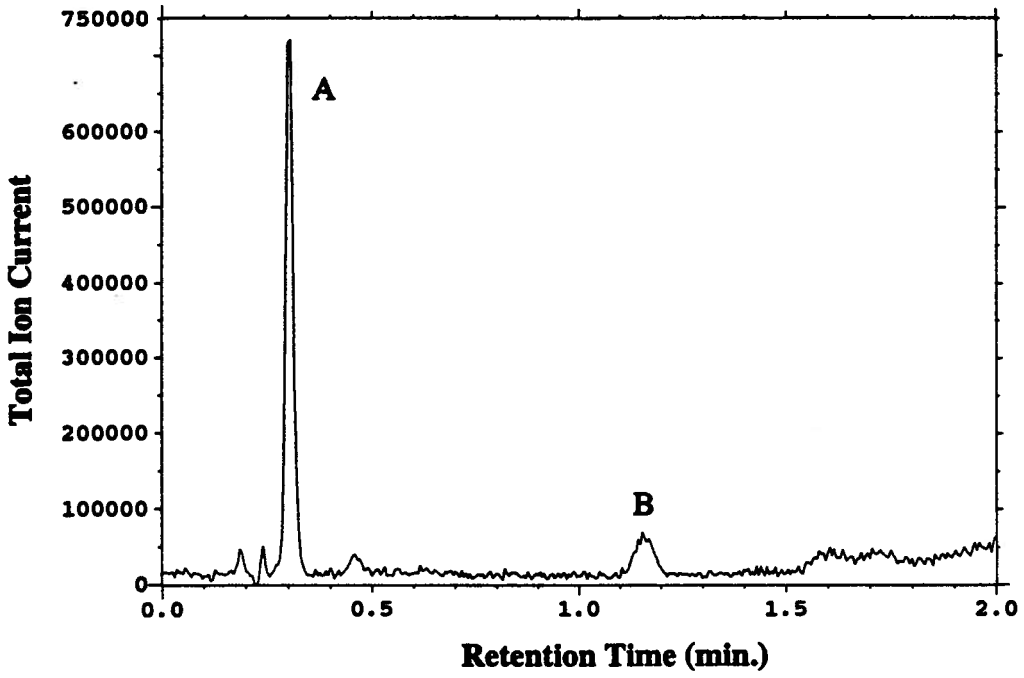


Figure 6.7 HG-GC-MS chromatogram of a blank solution. Solutions of 4.0 M acetic acid and 2.0% (w/v) $NaBH_4$ were used for the reaction.

Me_2SbH, and Me_3Sb when a batch type hydride generator was used for hydride generation, indicating that the molecular rearrangement had occurred during the hydride generation. When the same trimethylantimony compounds (from the same source) were analyzed by using the semi-continuous mode hydride generation system described in Chapter 2, the only stibine produced is Me_3Sb as can be seen from Figure 6.3. However, the molecular rearrangement of the trimethylstibine can take place if certain precautions are not taken. This usually happens when the hydride generation system including the probes (for the introduction of acid, $NaBH_4$, and sample), the reaction coil, and the liquid/gas separator are rinsed with distilled water and then used immediately for sample analysis. The HG-GC-MS chromatogram (Figure 6.8) obtained from a sample of $(CH_3)_3Sb(OH)_2$ (with the molecular rearrangement) shows the presence of four

stibines. The first antimony containing peak was identified as SbH_3 whose mass spectrum (Figure 6.9) is similar to that seen in Figure 6.2. The second antimony containing peak was characterized as $MeSbH_2$ (Figure 6.10) on the basis of ions at m/z 140 $[CH_3{}^{123}SbH_2]^+$, m/z 139 $[CH_3{}^{123}SbH]^+$, and m/z 121 $[{}^{121}Sb]^+$. The mass spectrum of the third antimony containing peak is shown in Figure 6.11. The ions at m/z 154 $[(CH_3)_2{}^{123}SbH]^+$, m/z 152 $[(CH_3)_2{}^{121}SbH]^+$, m/z 139 $[CH_3{}^{123}SbH]^+$, m/z 138 $[CH_3{}^{123}Sb]^+$, and m/z 121 $[{}^{121}Sb]^+$ confirm the presence of Me_2SbH. The fourth antimony containing peak is due to Me_3Sb on the basis of the mass spectrum shown in Figure 6.12 (cf. Figure 6.6).

The above results are noteworthy as they confirm the identity of the molecular rearrangement products. These spectra can also be used as qualitative standards for the analysis of environmental samples.

The possible causes of the rearrangement were investigated. At first it was suspected that the rearrangement could be due to active sites on the inner glass surface of the hydride generation system. However, the use of a Teflon reaction coil and liquid/gas separator did not eliminate the rearrangement. Only when acetic acid, $NaBH_4$, and distilled water were introduced by using a peristaltic pump and continuously mixed in the reaction coil with the aid of a stream of helium for a few minutes (usually more than 3 minutes) prior to sample analysis was the rearrangement prevented (Figure 6.3). At the present time the cause of the rearrangement remains unknown. After the system was conditioned by analyzing the blank solution at beginning of a routine analysis session, no more conditioning was needed.

In the light of these results it is not surprising that Dodd *et al.*[212] encountered the rearrangement of trimethylstibine when using the batch type hydride generator. Their apparatus was cleaned with deionized water after each analysis, and was never

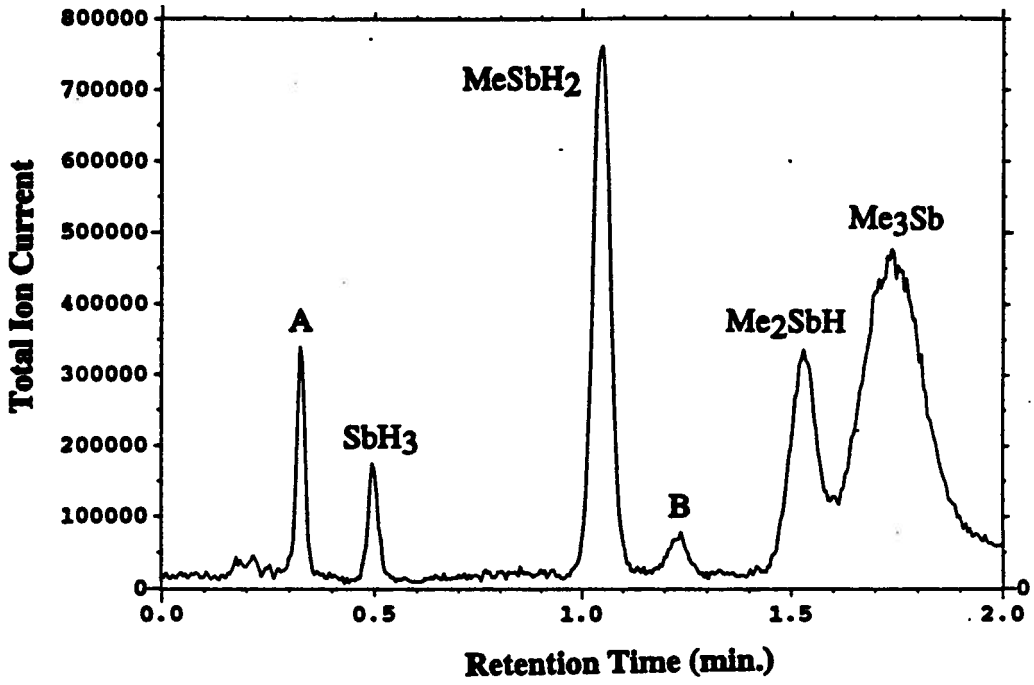


Figure 6.8 HG-GC-MS chromatogram of the hydrides derived from a solution of standard $Me_3Sb(OH)_2$ (200 ng of antimony) exhibiting molecular rearrangement. The sample analysis was performed immediately after the hydride generation system was rinsed with distilled water.

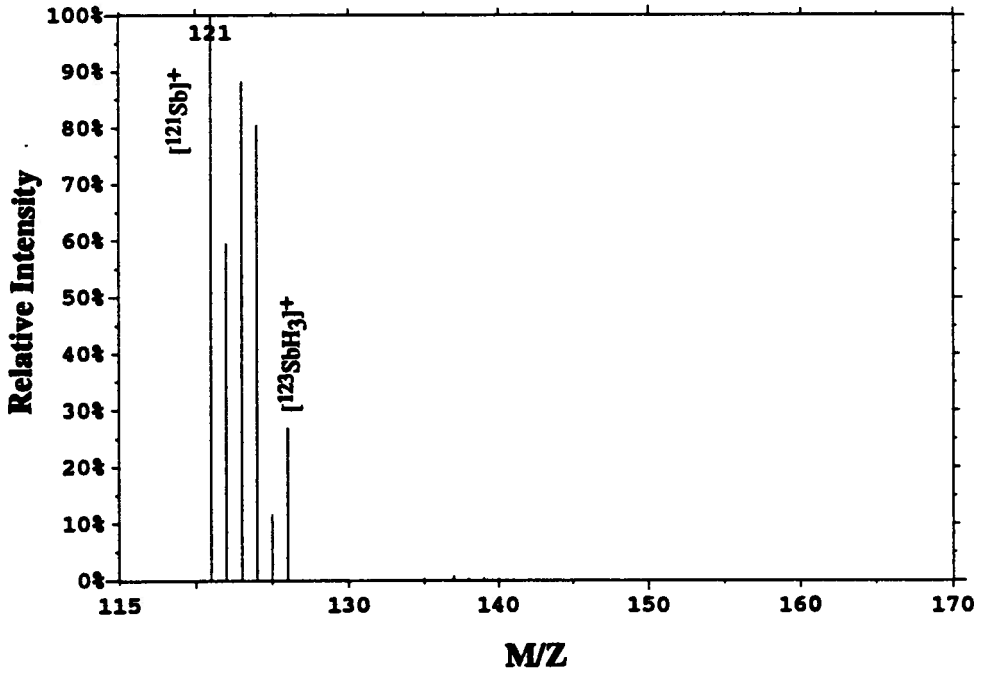


Figure 6.9 Mass spectrum of the first antimony containing peak in Figure 6.8, identified as SbH_3.

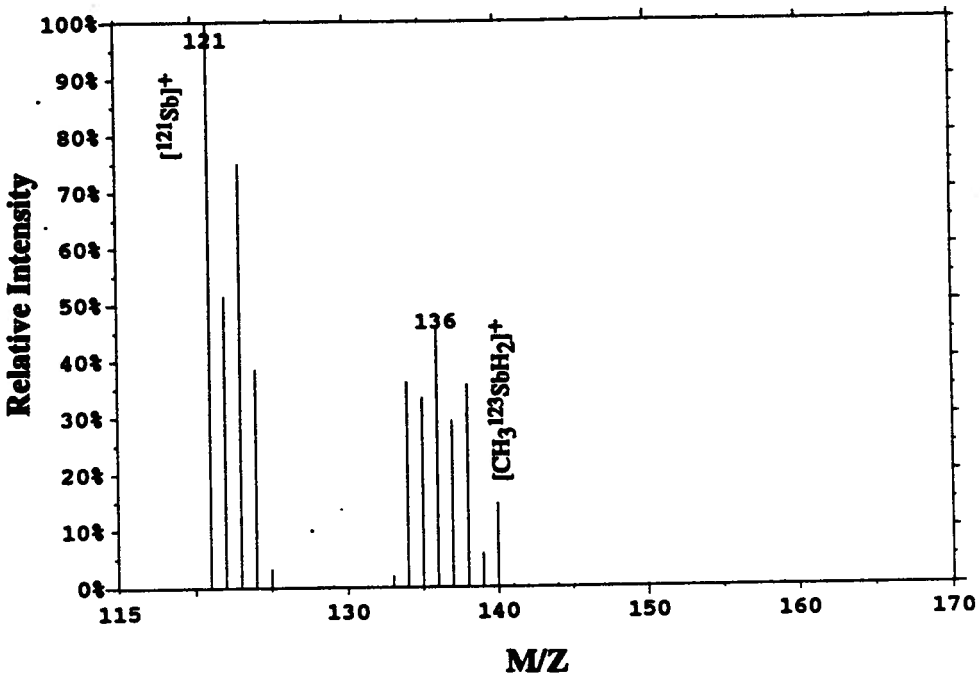


Figure 6.10 Mass spectrum of the second antimony containing peak in Figure 6.8, identified as $MeSbH_2$.

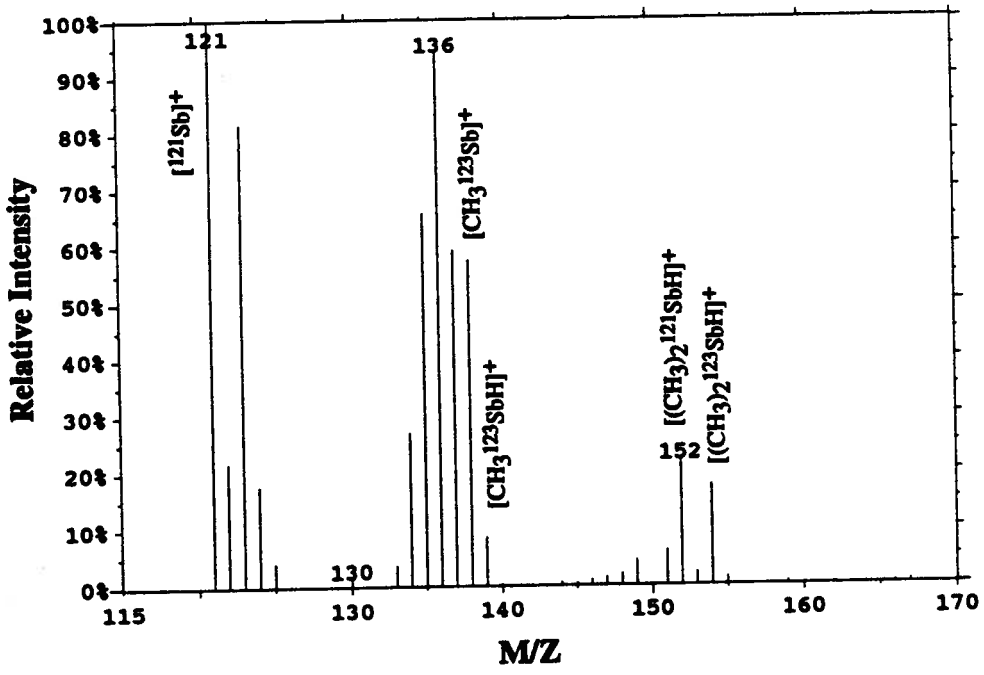


Figure 6.11 Mass spectrum of the third antimony containing peak in Figure 6.8, identified as Me_2SbH.

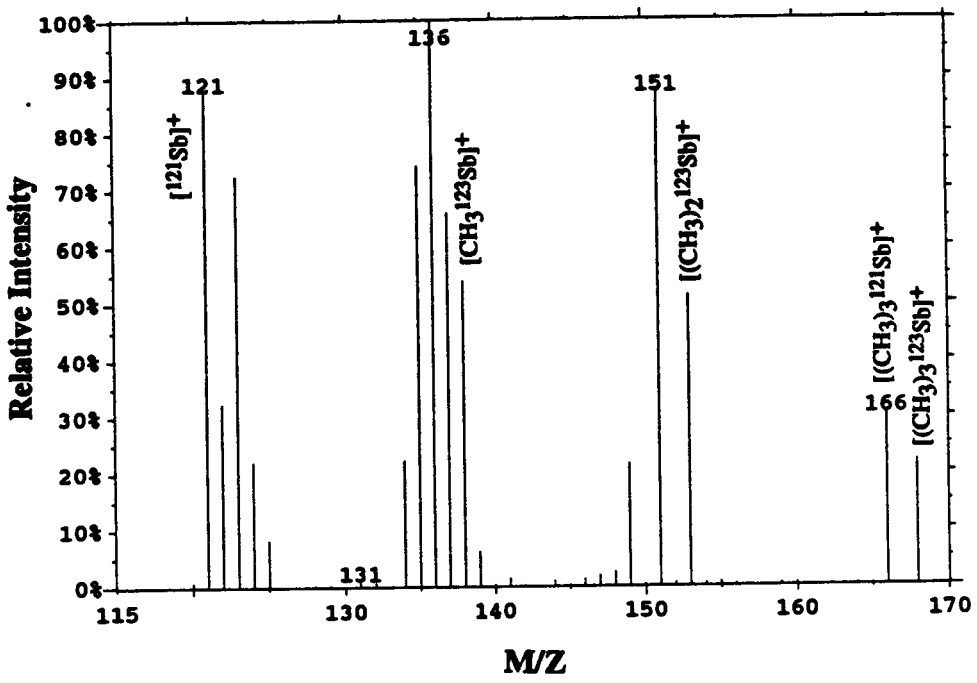


Figure 6.12 Mass spectrum of the fourth antimony containing peak in Figure 6.8, identified as Me_3Sb.

conditioned with the mixture described in the above paragraph. Andreae *et al.*[26,205,211] also used a batch type hydride generator for the analysis of Sb(III), Sb(V), and methylantimony species. They reported that the methylantimony standards had been stored for several years, and thus the solutions prepared from them contained some demethylation products: methylstibonic acid contained inorganic Sb; and the dimethylstibinic acid contained inorganic Sb and methylstibonic acid as judged by using HG-GC-AA. In light of the present work, it is possible that these demethylated compounds were produced in the hydride generation process rather than in the storage period. The results reported by Andreae *et al.*[26,205,211] that Sb(III), methylstibonic acid, and dimethylstibinic acid were found in natural water samples must be interpreted with caution because they used a batch type HG-GC-AA system.

In the present work, the HG-GC-MS system was used to analyze the extracts of plant samples collected in Yellowknife. Identification of the antimony species produced from these samples was achieved based on the comparison of their retention times and mass spectra with those shown in Figures 6.8, 6.9, 6.10, 6.11, and 6.12. The HG-GC-MS chromatogram (Figure 6.13) of the extract of plant samples collected in Kam Lake shows three major peaks. The first two peaks are from the same unknown compounds (Peak A and Peak B) described previously. The mass spectrum of the third peak indicates that this is a trimethylantimony compound (Figure 6.14). In the extract of plant samples collected in Keg Lake, four antimony containing peaks were found (Figure 6.15). Peak 1 was identified as SbH_3 (Figure 6.16) produced from Sb(III), Peak 2 as monomethylstibine $MeSbH_2$ (Figure 6.17), Peak 3 as dimethylstibine Me_2SbH (Figure 6.18), and Peak 4 as Me_3Sb (Figure 6.19).

These results are significant as it is the first time that convincing evidence is provided for the existence of methylantimony compounds in the environment. These compounds are likely to be biological metabolites since the anthropogenic sources of methylantimony compounds are not known.

Sb(V) must be determined under hydrochloric acid conditions since Sb(V) can not form stibine when 4.0 M acetic acid is used. In the presence of 1.0 M hydrochloric acid, both Sb(V) and Sb(III) are reduced to SbH_3 by sodium borohydride. Because of the limited time available and the lack of standard compounds of methylstibonic acid and dimethylstibinic acid, we did not quantify any of these antimony compounds in the two samples.

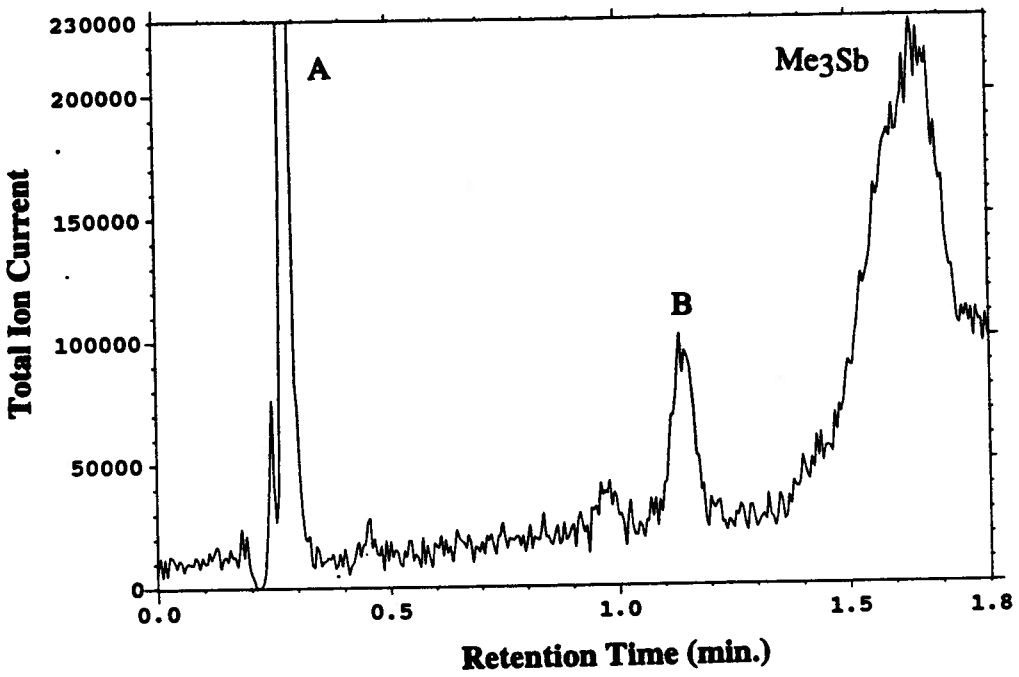


Figure 6.13 HG-GC-MS chromatogram of a 5 mL extract of plant samples collected in Kam Lake.

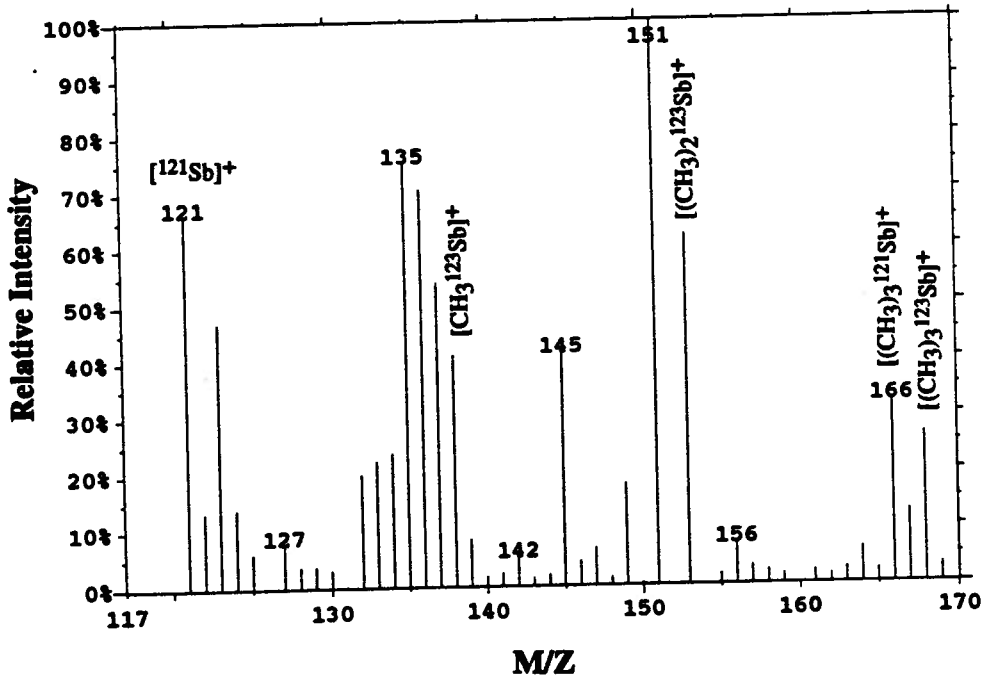


Figure 6.14 Mass spectrum of Me_3Sb derived from a 5 mL extract of plant samples collected in Kam Lake.

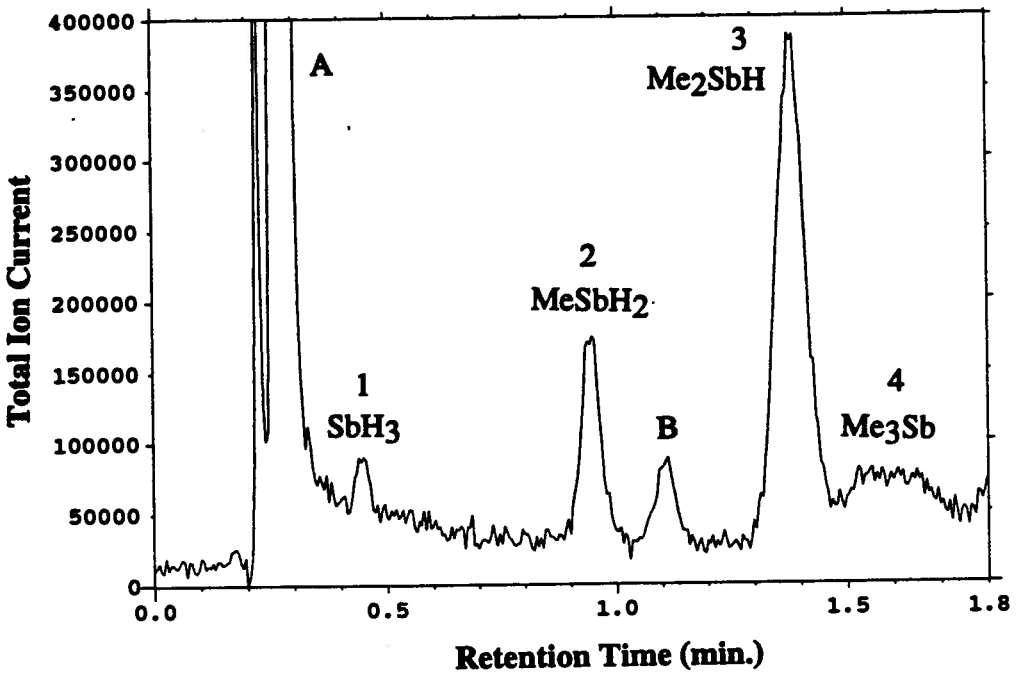


Figure 6.15 HG-GC-MS chromatogram of a 5 mL extract of plant samples collected in Keg Lake.

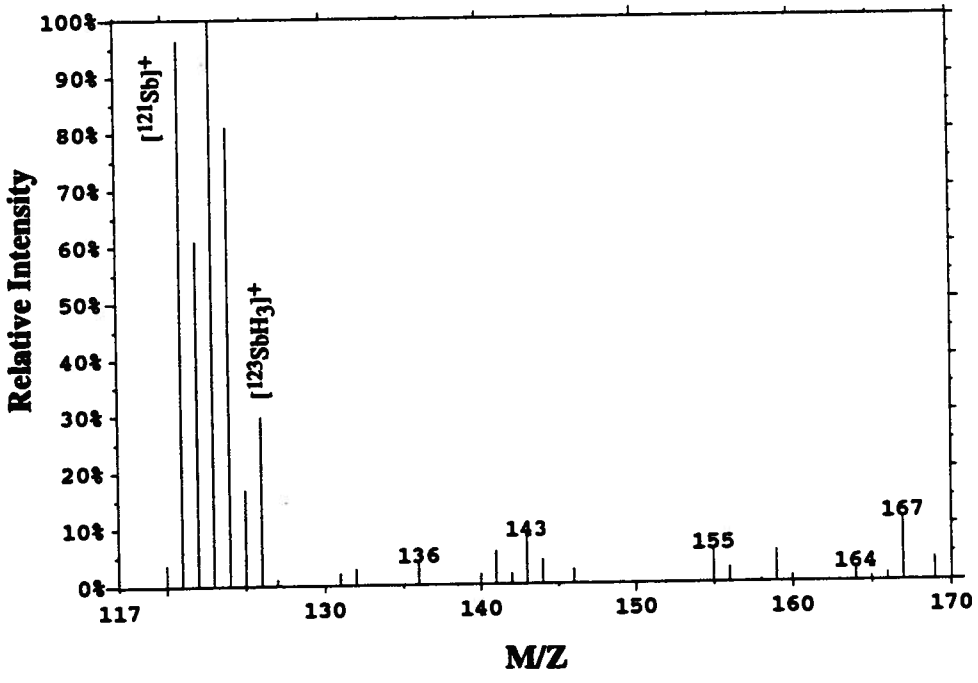


Figure 6.16 Mass spectrum of Peak 1 in Figure 6.15, identified as SbH_3.

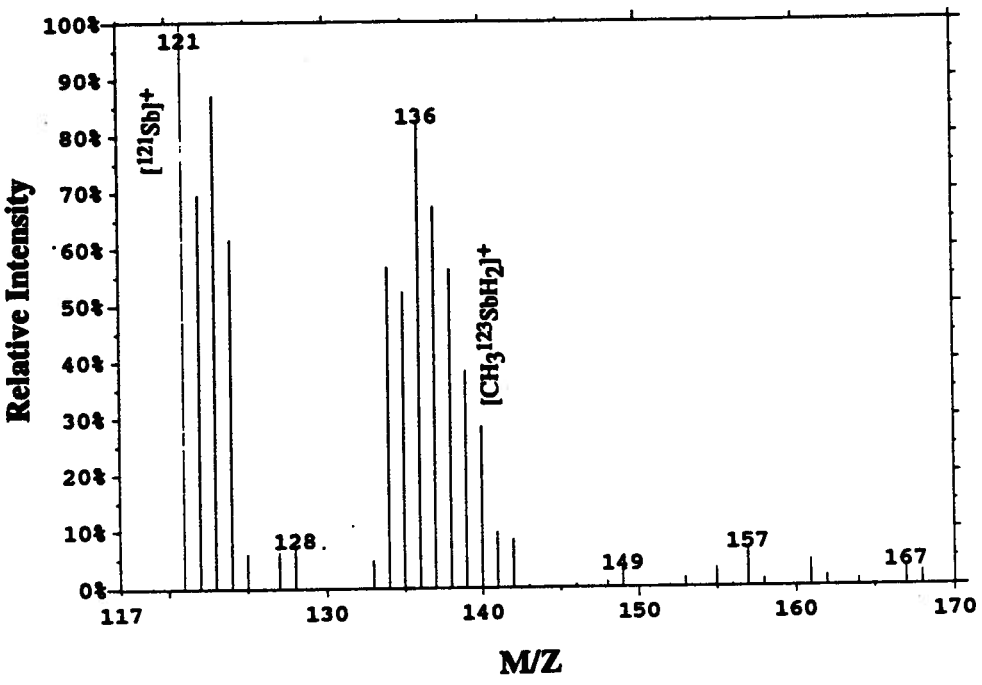


Figure 6.17 Mass spectrum of Peak 2 in Figure 6.15, identified as $MeSbH_2$.

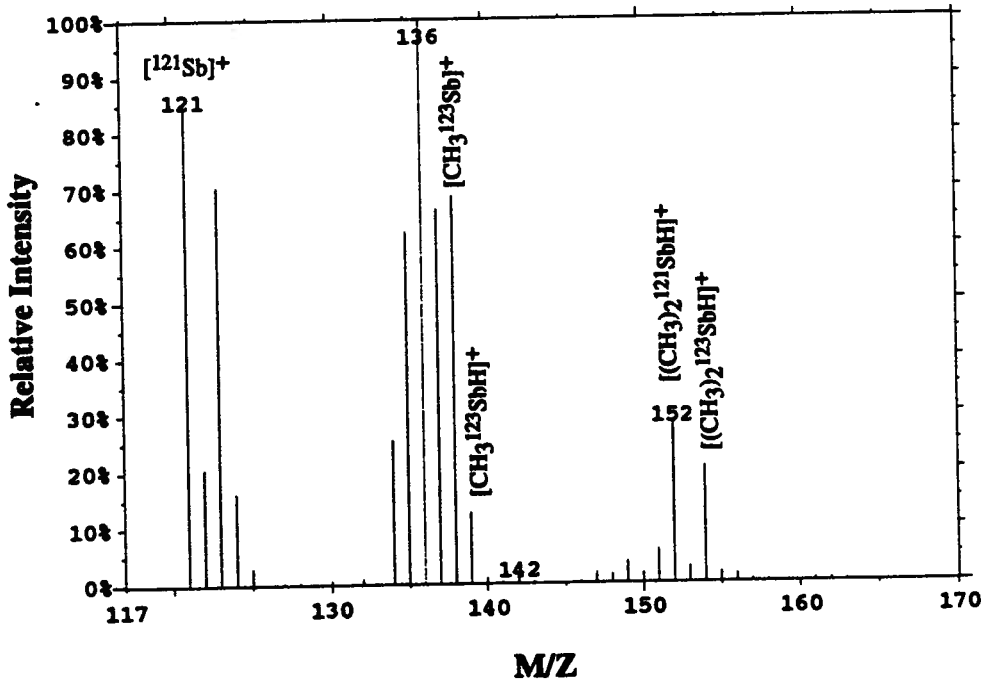


Figure 6.18 Mass spectrum of Peak 3 in Figure 6.15, identified as Me_2SbH.

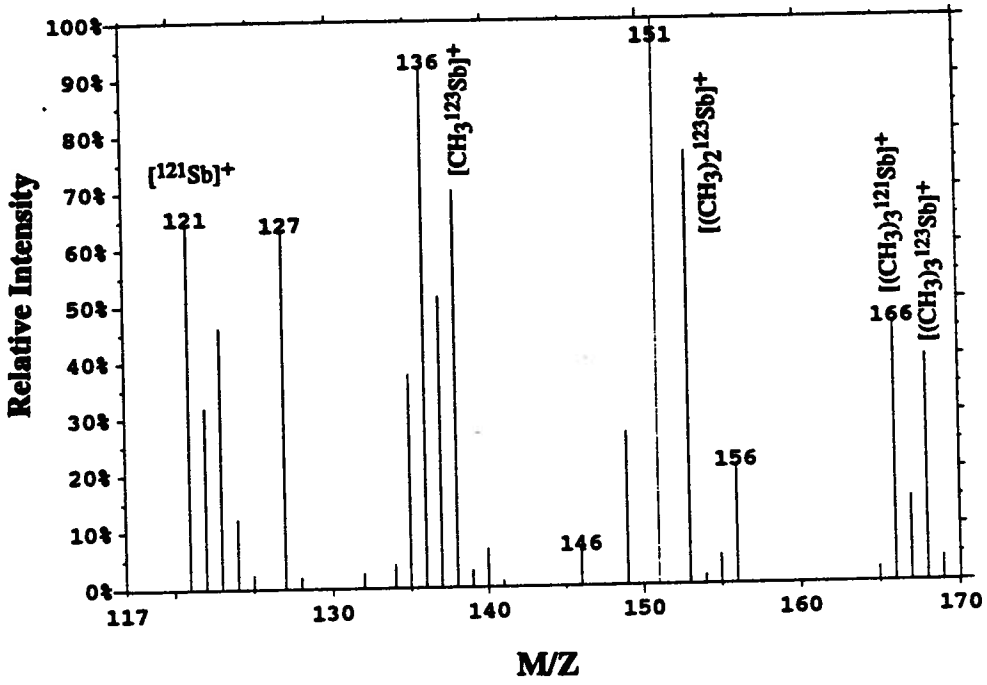


Figure 6.19 Mass spectrum of Peak 4 in Figure 6.15, identified as Me_3Sb.

CHAPTER 7

SUMMARY

The hydride generation technique is a very useful tool for the determination of hydride-forming arsenic species such as arsenite, arsenate, MMA, DMA and TMAO. The semi-continuous flow HG-GC-AA system developed in the course of this project is easy to construct and simple to operate. The operational procedures are simplified compared to those of the batch type hydride generation systems, and the time for one sample analysis can be reduced to less than 7 minutes. The system has proved to be efficient and gives reproducible results in analyzing a large number of samples (> 40 samples per day).

The biotransformation processes for low levels of arsenic (1 ppm) in the microorganisms *A. humicola* and *S. brevicaulis* were investigated.

Exposure of *A. humicola* to arsenate yields arsenite prior to the detection of any methylated arsenicals MMA, DMA and TMAO in the growth medium. Most of the arsenate substrate is transformed into TMAO, and the production of MMA and DMA is limited. The substrate arsenite is metabolized by *A. humicola* to produce MMA (in trace amounts), DMA (in small amounts), and TMAO (the major metabolite). The oxidation of arsenite to arsenate by the microorganism is insignificant. When MMA is used as a substrate, DMA and TMAO are the major metabolites, and the demethylation of MMA to arsenate and/or arsenite by *A. humicola* is insignificant. The transformation of the substrate DMA to the metabolite TMAO is a slow process, and only small amounts of TMAO are present in the growth medium at the end of the experiment.

The microorganism *S. brevicaulis* also transforms the arsenic substrates into a variety of metabolites including arsenite from arsenate, DMA and TMAO from arsenate,

arsenite and MMA, and TMAO from DMA. However, the yields of the methylated arsenic metabolites are low due probably to the growth medium used in the experiments since this medium was optimized for the production of trimethylarsine by *A. humicola.*

Trimethylarsine is not produced by either of the microorganisms under the experimental conditions used in the work. It now seems likely that low concentrations of TMAO (< 1 ppm) do not greatly affect the living fungal system. Therefore, further detoxification by reducing TMAO to trimethylarsine is not necessary.

The identification of these arsenicals is noteworthy as it is the first time that any of the non-volatile arsenic intermediates have been identified in the growth medium of a pure culture of microorganisms spiked with either arsenate, arsenite, MMA or DMA. The production of the anticipated methylated intermediates from the substrates, the absence of oxidation of arsenite to arsenate, and the lack of demethylation products strongly support the metabolic sequence proposed by Challenger.

On the basis of the results obtained from the experiments, an extended model is proposed to supplement Challenger's proposed pathway, which explains our experimental observations in terms of the uptake, the biotransformation, and the excretion of the arsenicals by the cells of microorganisms.

The effect of adding L-methionine-methyl-d_3 and the arsenic substrates (1 ppm) to the growing culture of *A. humicola* was studied by using a specially developed HG-GC-MS methodology. When either arsenate or arsenite is added to the growth medium of *A. humicola* in the presence of L-methionine-methyl-d_3, the mass spectra of the hydride derivative of the dimethylarsenic metabolite exhibit ions at m/z 112 $[(CD_3)_2AsH]^+$, and m/z 109 $[(CH_3)(CD_3)AsH]^+$ indicating incorporation of the CD_3 moiety. Similarly, the mass spectra of the produced trimethylarsenic species contains ions at m/z 129 $[(CD_3)_3As]^+$, m/z 126 $[(CH_3)(CD_3)_2As]^+$, and m/z 123

$[(CH_3)_2(CD_3)As]^+$. When MMA is used as the substrate in the presence of L-methionine-methyl-d_3, the mass spectra of the produced dimethylarsenic and trimethylarsenic species show the ions at m/z 109 $[(CH_3)(CD_3)AsH]^+$, m/z 126 $[(CH_3)(CD_3)_2As]^+$ and m/z 123 $[(CH_3)_2(CD_3)As]^+$. The absence of ions at m/z 112 $[(CD_3)_2AsH]^+$ and m/z 129 $[(CD_3)_3As]^+$ indicates that the cleavage of the H_3C-As bond is insignificant. When both DMA and L-methionine-methyl-d_3 are added to the growing culture of *A. humicola*, the mass spectrum of the produced trimethylarsenic species exhibits the ions at m/z 123 $[(CH_3)_2(CD_3)As]^+$.

The addition of L-methionine-methyl-d_3 does not alter the production of the arsenic metabolites by *A. humicola*.

This is the first time that methionine has been demonstrated to be involved in the production of the non-volatile methylarsenic intermediates which have been proposed in Challenger's pathway. Thus this work strongly reinforces the suggestion that methionine via SAM is the source of the $[CH_3]^+$ shown in Challenger's pathway.

The arsenic accumulation in cells of a unicellular marine alga *P. peniculus* was found to be proportional to the concentration of the arsenic substrates with the exception of arsenate. Arsenate at high concentrations (10 ppm) is more toxic to *P. peniculus*, and affects the bioaccumulating and biomethylating ability of the alga. When arsenate, arsenite and MMA are used as substrates for *P. peniculus* the principal methylation product is DMA. No trimethylarsenic species are found in the cells or in the growth medium. Exposure of *P. peniculus* to arsenate yields arsenite (in cells and in the media) and DMA (in cells and in the medium spiked with 0.9 ppm arsenate). The reduction of arsenate to arsenite by the alga is a rapid process. When the alga is treated with arsenite, MMA and DMA are detected in the cells; the metabolite DMA can also be found in the growth medium spiked with 0.9 ppm arsenite. The substrate MMA is transformed by *P.*

peniculus to produce DMA in the cells. When DMA is used as a substrate, trace amounts of the demethylation product MMA are detected in the cells. When *P. peniculus* is transferred from arsenic enriched media to arsenic free media, the accumulated arsenicals in the algal cells are excreted into the "fresh" media. Biotransformation of arsenic, including methylation and demethylation, also takes place in this media. No complex arsenic compounds, such as arsenosugars, are produced by *P. peniculus*.

The alga transforms arsenate (1 ppm) to a deuterated dimethylarsenic species in the presence of L-methionine-methyl-d_3. The mass spectrum of the dimethylarsenic species obtained by using the HG-GC-MS system shows the presence of ions at m/z 112 $[(CD_3)_2AsH]^+$, and m/z 109 $[(CH_3)(CD_3)AsH]^+$. The CD_3 label is incorporated intact by the cells to form deuterated DMA. This is the first time that methionine, or SAM, has been shown to be the source of the methyl groups in the biological alkylation of arsenic in marine algae.

These results suggest that the alga follows the biomethylation pathway proposed by Challenger for microbial processes involving alternating oxidation and reduction steps and the use of carbonium ions for the alkylation of arsenic. In the case of *P. peniculus*, DMA is the end product of this methylation. A model for the methylation of arsenate by the cells of *P. peniculus* is proposed to explain the results obtained.

The semi-continuous HG-GC-MS system has proved to be very useful for the identification of antimony hydrides produced from the trimethylantimony compounds $Me_3Sb(OH)_2$ and Me_3SbCl_2. The identification of the molecular rearrangement products of trimethylstibine is achieved by using this system. The possible causes of the molecular rearrangement of trimethylstibine were investigated. The appropriate operational procedures have been established to effectively eliminate the causes of the

molecular rearrangement of trimethylstibine during the hydride generation processes. The system has been successfully used to analyze the extracts of plant samples collected from Kam Lake and Keg Lake (Yellowknife). These samples were found to contain methylantimony compounds. This is first time that convincing evidence is provided for the existence of methylantimony compounds in the environment.

The HG-GC-AA and HG-GC-MS developed during the course of this project have proved to be useful in the determination and identification of the arsenic metabolites produced by the microorganisms and a marine alga. The biotransformation processes of antimony in organisms have not been fully investigated. Therefore, it is also possible to use the two systems to study the interactions between antimony and biota including microorganisms, algae, and plants.

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APPENDIX

DETERMINATION OF HYDRIDE-FORMING AND "HIDDEN" ARSENICALS IN THE SEAWATER SURFACE MICROLAYER BY USING MICROWAVE DIGESTION FOLLOWED BY HG-GC-AA

A.1 INTRODUCTION

Arsenobetaine (AB), arsenocholine (AC), tetramethylarsonium salts (Me_4As^+), and arsenosugars, the so called "hidden" arsenicals, are known to be present in marine organisms in significant concentrations.[94-100] The presence of "hidden" arsenicals in seawater has not been well documented, partly because of the low concentration of these compounds in the water column and the limitation of analytical techniques. It is possible that arsenobetaine, arsenosugars, and their breakdown products may in fact be present in the water column.

The seawater surface microlayer (about 50 μm in depth) represents an interface between the atmosphere and ocean systems. Large microlayer phytoplankton enrichments have been found. They frequently occur at densities 10-100 times greater than the underlying phytoplankton, and play an important role in the productivity of many waters. As a result, arsenic speciation in the microlayer may be markedly different from that in the bulk seawater.

A convenient batch-type hydride generation system which was developed to determine trace amounts of hydride-forming arsenic compounds in seawater surface microlayer samples collected from coastal sites of British Columbia (Canada). When used in conjunction with batch-type microwave digestion, this system can detect "hidden" arsenic species in these samples.

A.2 EXPERIMENTAL

A.2.1 Sample Collection and Pretreatment

Seawater surface microlayer samples were collected from coastal sites of British Columbia (Canada) in April of 1989. A glass plate of convenient size (20 cm square by about 4 mm thick) was introduced vertically through the surface. The glass plate was taken out of the water, held by hand and the surface film and water layer adhering to the plate were removed from both sides with a Teflon wiper blade and collected in a Teflon bottle. Samples were preserved in Teflon bottles by rapid freezing on dry ice, and then stored at -20^{o}C prior to analysis.

A.2.2 Analytical Procedures

The apparatus for arsine production, collection, and determination is shown in Figure A.1. The sample (30-50 mL) and the acid (3 mL of 4.0 M hydrochloric acid or 2 mL of 4.0 M acetic acid) were introduced into the reaction vessel (250 mL Erlenmeyer flask) by using a pipette. The $NaBH_4$ solution (4.0% w/v) was then introduced into the reaction vessel by using the peristaltic pump. The volatile arsines were then collected, separated, and quantified as previously described in Chapter 2. The optimum conditions for the determination of hydride forming arsenicals are shown in Table A.1.

A Sharp Model microwave oven operating at maximum power (500 W) was used for sample digestion. The sample solution (30-50 mL) and 2-5 mL of potassium persulphate solution (2.0-6.0%) were introduced into a 250 mL Erlenmeyer flask. The flask was loosely covered with a Teflon cap, placed in the microwave oven, and irradiated for 2-5 min at full power. After digestion, 2-5 mL of ascorbic acid (10% w/v),

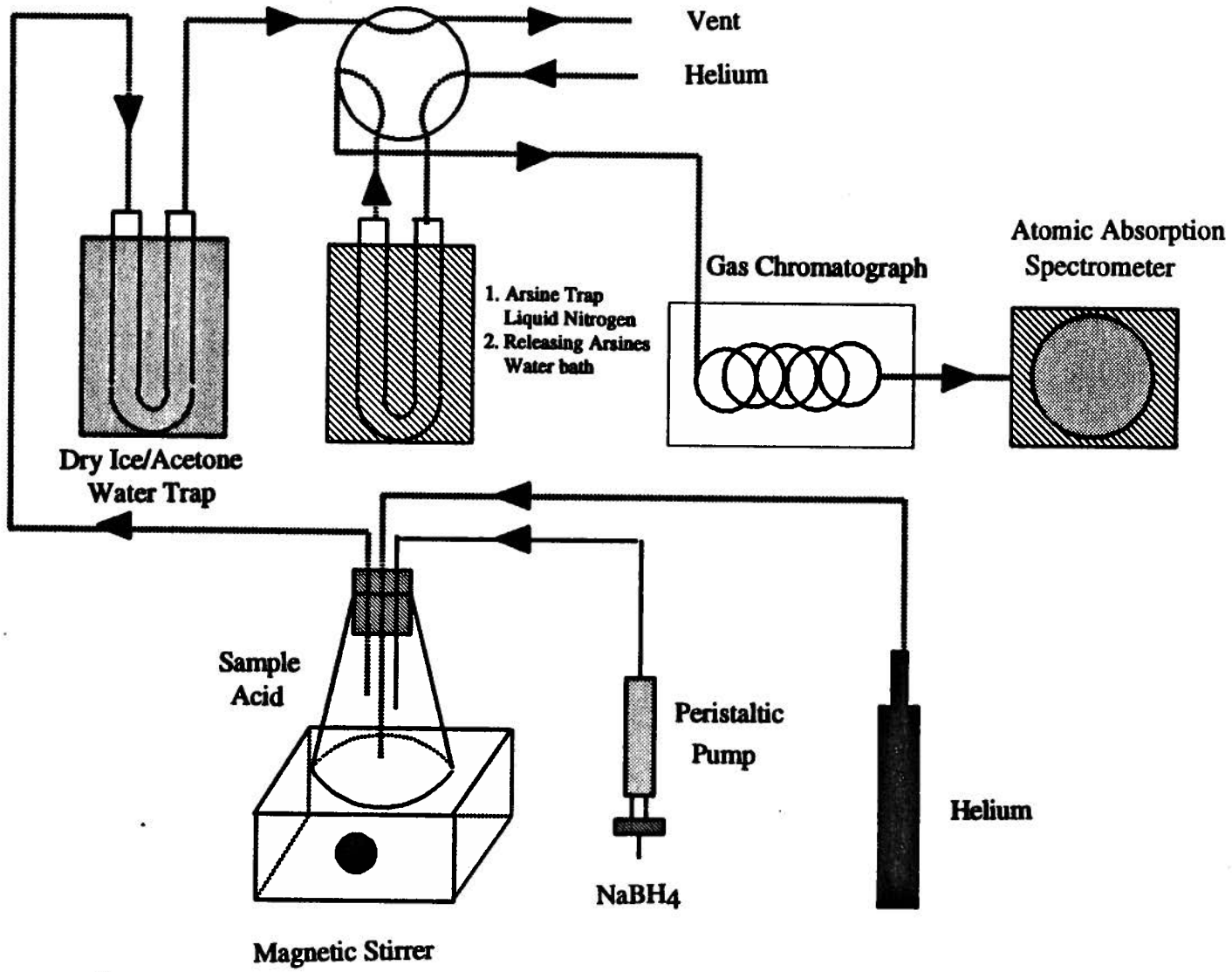

Figure A.1 Schematic diagram of a batch type hydride generation-gas chromatography-atomic absorption spectrometry.

Table A.1 Optimum conditions for the determination of hydride-forming arsenicals in a batch type hydride generator

	In hydrochloric acid	In acetic acid
Sample volume	50 mL	50 mL
Arsenic concentration	1 ppb	1 ppb
Acid concentration	1 M	4 M
Acid quantity	3 mL	2 mL
$NaBH_4$ concentration (w/v)	4%	4%
Peristaltic pump rate	2 mL/min	2 mL/min
Reaction time	2 min	2 min
Arsenicals to be determined	arsenate, arsenite, MMA	arsenite, DMA, TMAO

3 mL of 4.0 M HCl, and an appropriate amount of deionized water were added to the flask to bring the total volume to 50 mL. The samples were then cooled to room temperature prior to HG-GC-AA analysis by using the system described above. The optimum conditions for the decomposition of "hidden" arsenicals are displayed in Table A.2.

A.3 RESULTS AND DISCUSSION

A.3.1 Determination of hydride-forming arsenicals

The batch type hydride generator described here (Figure A.1) uses a peristaltic pump for the introduction of the $NaBH_4$ solution and a magnetic stirring bar for mixing of the reactants, thus improving the analysis efficiency, reducing the potential operational inconsistency, and significantly reducing the analysis time. A complete

analysis takes about 7 min.

Table A.2 Optimum conditions for the decomposition of "hidden" arsenicals

	in a standard solution	In a seawater sample
Sample volume	50 mL	30 mL
K_2S_2O8 concentration (w/v)	2%	6%
K_2S_2O8 volume	2 mL	5 mL
Microwave digestion time	2 min	5 min
Ascorbic acid concentration (w/v)	10%	10%
Ascorbic acid volume	2 mL	2 mL

The quantity of hydrochloric acid, acetic acid, and $NaBH_4$ used for hydride generation was optimized by using the single parameter variation method. The peak area of the signals from arsine, methylarsine, dimethylarsine and trimethylarsine was chosen as the response to be optimized. A 50 mL of 1 ppb each of arsenate (or arsenite), MMA, DMA, and TMAO aqueous solution was used for the optimization.

The effect of hydrochloric acid (1.0 M), acetic acid (4 M), and $NaBH_4$ on the determination of arsenate, arsenite, MMA, DMA and TMAO is shown in Figures A.2, A.3, A.4, A.5. The optimum conditions for the determination of these arsenicals are shown in Table A.1.

The calibration curve for each arsenic compound is linear from 0.1-5 ppb of As in a 50 mL of aqueous solution. In the presence of hydrochloric acid, the detection limits were determined as 0.3 ng (of arsenic) for arsenate, arsenite, and MMA; and 0.4 ng for DMA and TMAO. In the presence of acetic acid, the detection limits were 0.3 ng

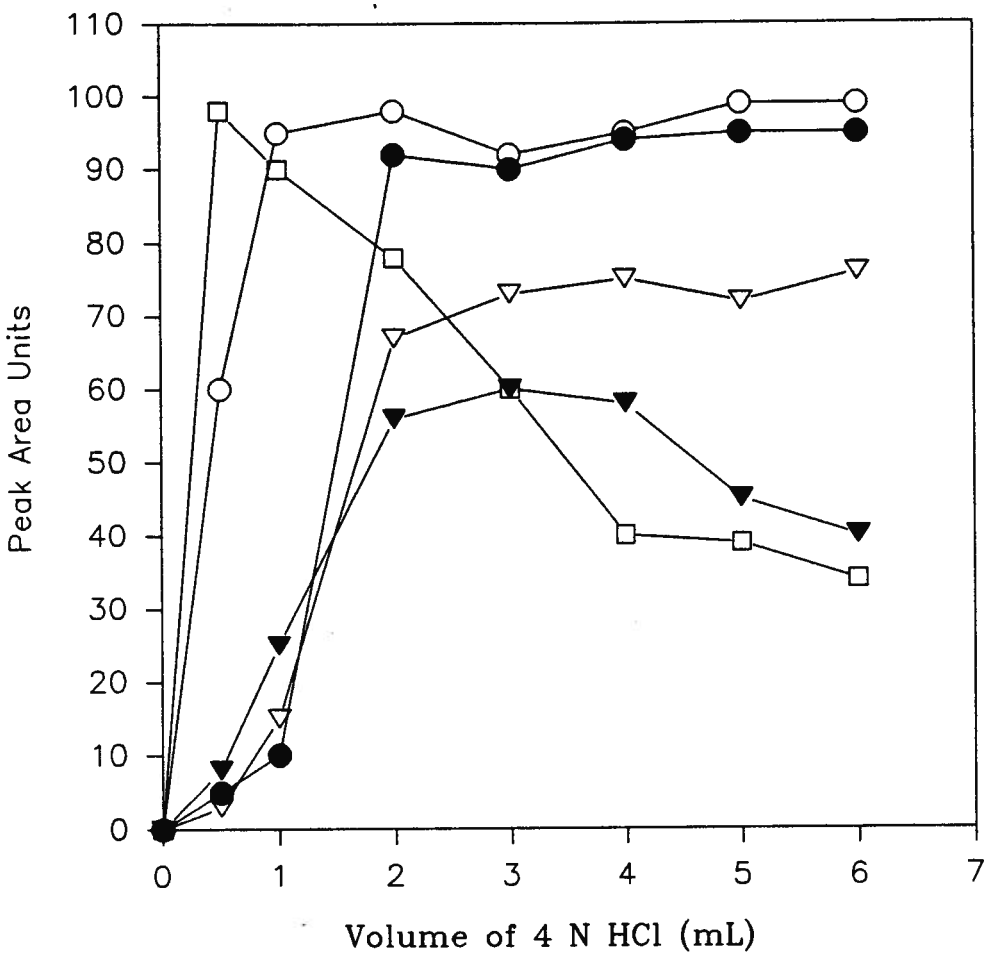

Figure A.2 Effect of the concentration of hydrochloric acid on the determination of hydride-forming arsenicals. The $NaBH_4$ solution (4.0% w/v) was added to the reaction system at a rate of 2 mL/min for 3 minutes. (O) arsenite (●) arsenate (▽) MMA (▼) DMA (□) TMAO

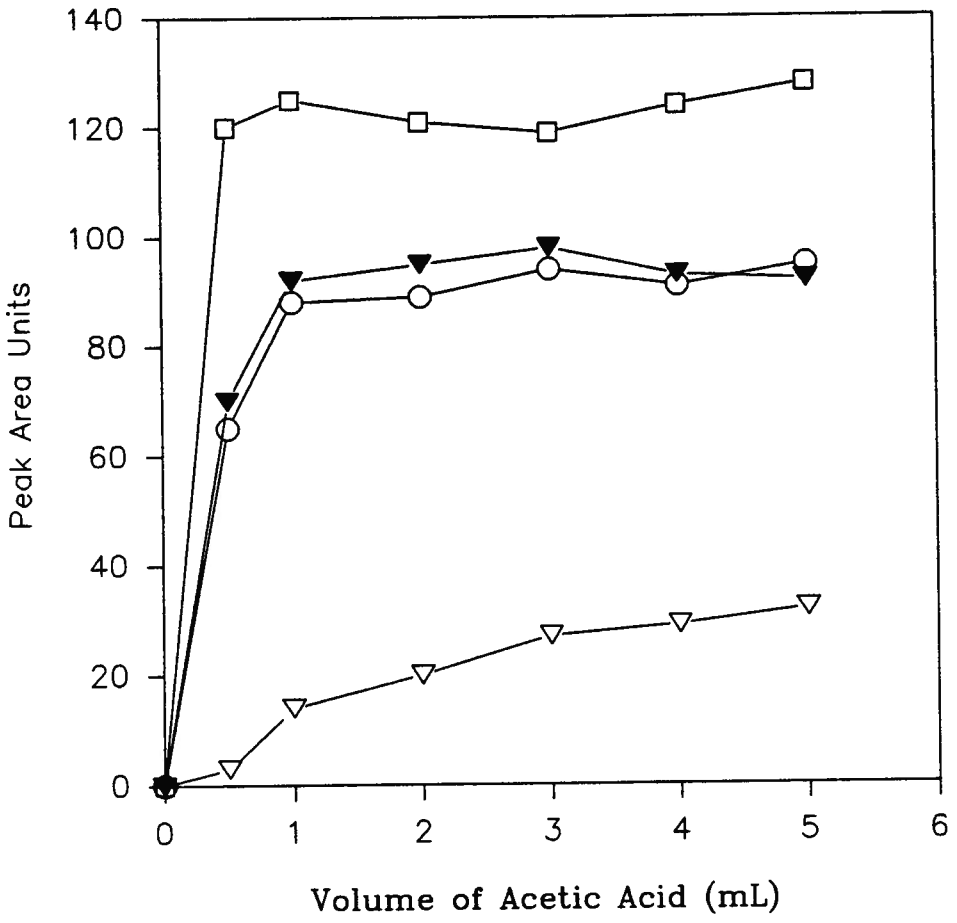

Figure A.3 Effect of the concentration of acetic acid on the determination of hydride-forming arsenicals. The $NaBH_4$ solution (4.0% w/v) was added to the reaction system at a rate of 2 mL/min for 3 minutes. (O) arsenite (●) arsenate (▽) MMA (▼) DMA (□) TMAO

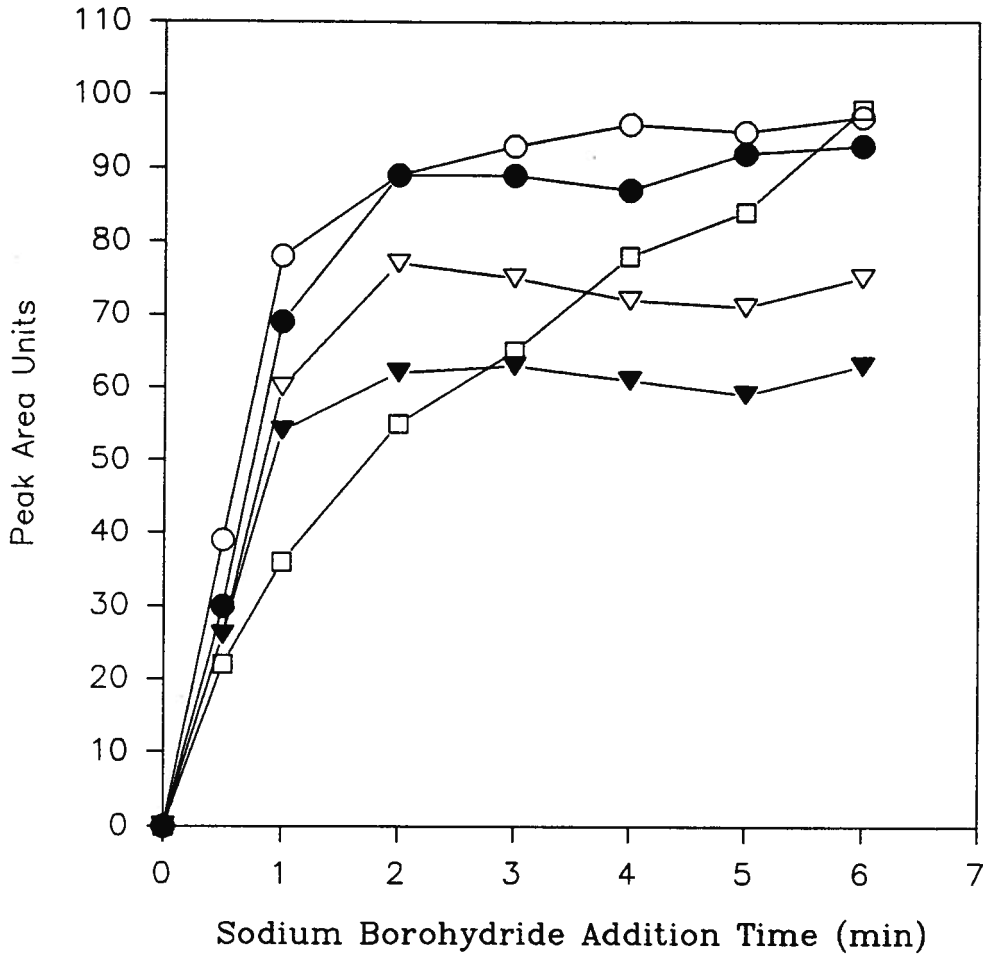

Figure A.4 Effect of $NaBH_4$ concentration on the determination of hydride-forming arsenicals. A 3 mL of 4.0 M HCl was added to the reaction system to acidify the sample. (O) arsenite (●) arsenate (▽) MMA (▼) DMA (□) TMAO

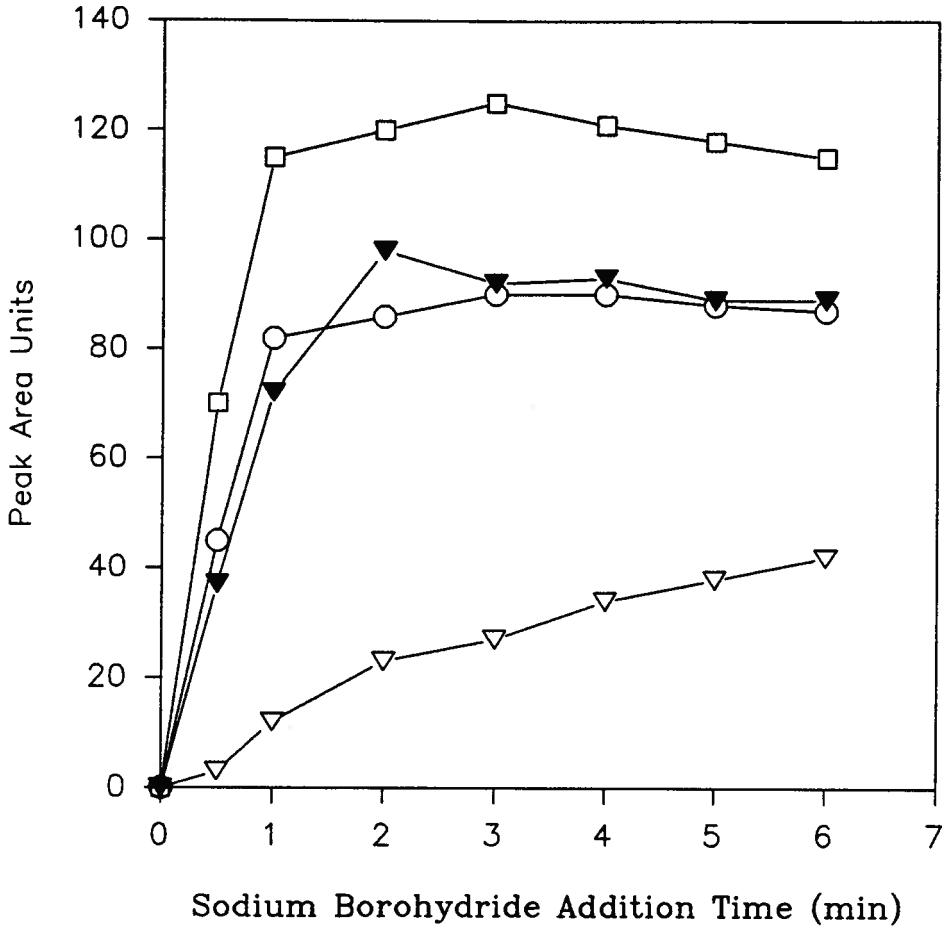

Figure A.5 Effect of $NaBH_4$ concentration on the determination of hydride-forming arsenicals. A 2 mL of 4.0 M acetic acid was added to the reaction system to acidify the sample. (O) arsenite (●) arsenate (▽) MMA (▼) DMA (□) TMAO

for arsenite, DMA, and TMAO; and 0.5 ng for MMA.

The reproducibility was 6% for arsenate and arsenite, 5% for MMA, and 10% for DMA and TMAO in the presence of either hydrochloric acid or acetic acid.

The interference from the seawater matrix in the determination of arsenate, arsenite, MMA, DMA, and TMAO was eliminated by adding 3 mL of 10% (w/v) EDTA. Calibration curves obtained from standard solutions of arsenicals were used for quantitative analysis of seawater samples.

Determination of the total amount of inorganic arsenic (arsenate and arsenite) and the amount of MMA was achieved when the seawater surface microlayer samples were acidified by using hydrochloric acid. The amount of arsenite and DMA in the seawater surface microlayer samples was quantified when acetic acid was used. The results are shown in Table A.3.

Table A.3 The concentrations of hydride-forming arsenicals (ppb) in seawater surface microlayer samples

Stations	Arsenite	Arsenate	MMA	DMA	Total
*RB1	0.30	0.12	< L.O.D.	d	0.42
*R19	0.35	0.35	< L.O.D.	d	0.70
*PR1	0.22	0.39	< L.O.D.	d	0.61
*MSA1	0.26	0.37	< L.O.D.	d	0.63
*MSA2	0.27	0.12	< L.O.D.	d	0.39
*RS1	0.25	0.36	< L.O.D.	d	0.61
*RS3	0.21	0.34	< L.O.D.	d	0.55

d: detectable
L.O.D.: limit of detection

RB1: Desolation Sound; R19: Rupert Inlet; MSA1: Moira Sound, Alaska; MSA2: Moira Sound, Alaska; RS1: Rennell Sound; RS3: Rennell Sound;

A.3.2 Determination of "hidden" arsenic species

Initially the aqueous solutions (1 ppb, 50 mL) of arsenobetaine, arsenocholine, tetramethylarsonium ion were used to establish the optimum digestion conditions. The single parameter variation method was used to study the factors affecting the decomposition efficiency, in particular the digestion time and the amount of $K_2S_2O_8$. The decomposition efficiency is presented as the arsenate peak area of the signals from the "hidden" arsenicals relative to that from the same amount of arsenate, thus the arsenate peak area produced from 50 ng of AB was compared with the peak area from 50 ng of arsenate.

The effect of microwave irradiation time and the amount of $K_2S_2O_8$ on digestion efficiency is shown in Figures A.6 and A.7.

Any excess $K_2S_2O_8$ and NaOH remaining in the flask after the microwave assisted digestion could affect the hydride generation reaction since they can react with the $NaBH_4$ and HCl added to the reaction vessel. The addition of ascorbic acid (2 mL, 10% w/v) which reacts with both $K_2S_2O_8$ and NaOH, could solve this problem. The optimum conditions for the decomposition of "hidden" arsenicals are shown in Table A.2.

The calibration curve of arsenate was used for quantitative analysis of the "hidden" arsenicals.

Most environmental samples contain a complex matrix, which could affect the decomposition efficiency of "hidden" arsenicals in the microwave assisted digestion. Initial attempts to achieve a complete decomposition of organoarsenicals in a seawater sample matrix were made by optimizing quantity of persulfate, and the microwave digestion time. A known amount (50 ng) of AB, AC, or Me_4As^+ were spiked into a seawater matrix (50 mL) and recoveries were evaluated. The effect of the amount of

potassium persulfate and the digestion time on the decomposition of AB, AC, Me_4As^+ is shown in Figures A.8 and A.9. The maximum recoveries of the "hidden" arsenicals in 50 mL of seawater is 65-75%. Complete recovery is achieved when the sample volume is reduced to 30 mL. The optimum conditions for the complete decomposition of AB, AC, and Me_4As^+ are shown in Table A.2.

Arsenate prepared in distilled water was chosen as the standard for quantitative analysis in the work.

By using microwave digestion-HG-GC-AA, it was found that some seawater surface microlayer samples contain "hidden" arsenic as shown in Table A.4. In these studies, the samples which were filtered through a 0.45 μm pore-size glass filter show a similar concentration of arsenic to those without filtration. This indicates that the "hidden" arsenicals are dissolved in seawater, and the amount of arsenicals present in phytoplankton is insignificant.

Table A.4 The concentrations of arsenic (ppb) in seawater surface microlayer samples with and without microwave assisted digestion

Stations	RB1	R19	PR1	MSA1	MSA2	RS1	RS3
Without microwave digestion	0.42	0.70	0.61	0.63	0.39	0.61	0.55
With microwave digestion	0.45	0.66	0.79	0.65	0.59	0.60	0.57

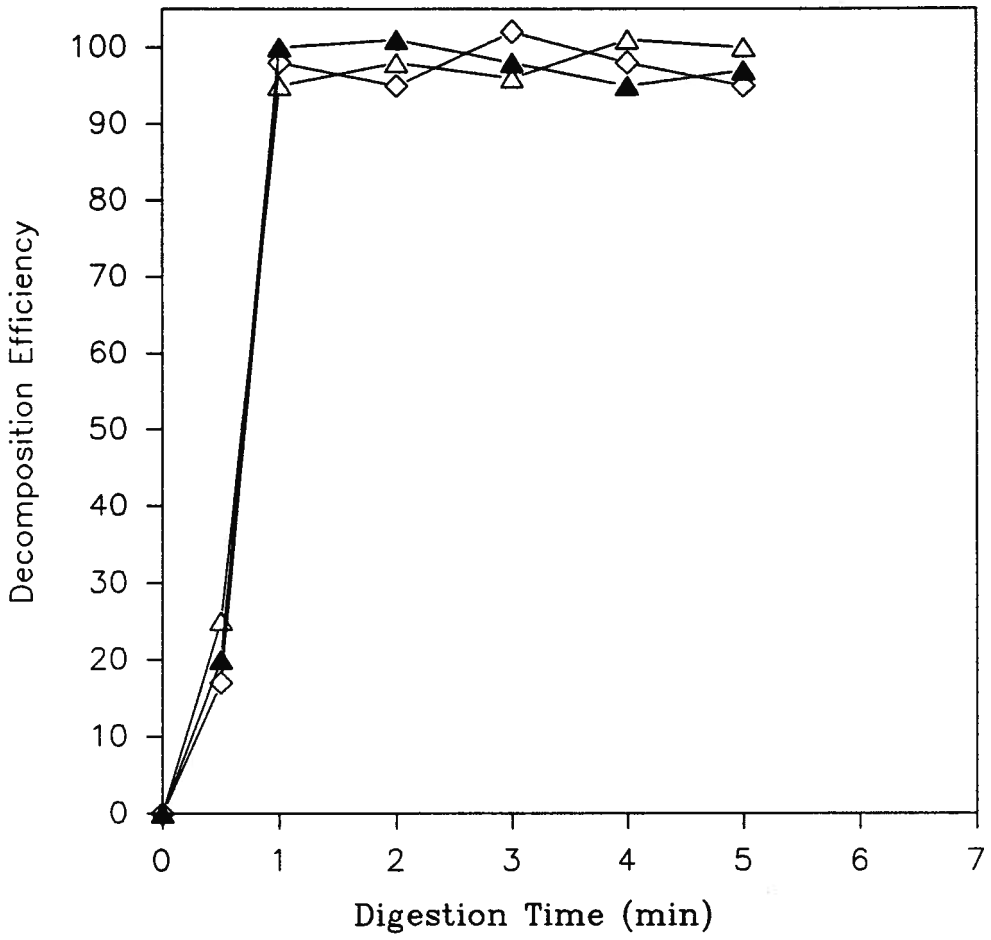

Figure A.6 Effect of microwave digestion time on the decomposition efficiency in the presence of 2 mL of 2.0% (w/v) $K_2S_2O_8$. The sample solution (50 mL) contained 50 ng each of AB (Δ), AC (▲), and Me_4As^+ (◊).

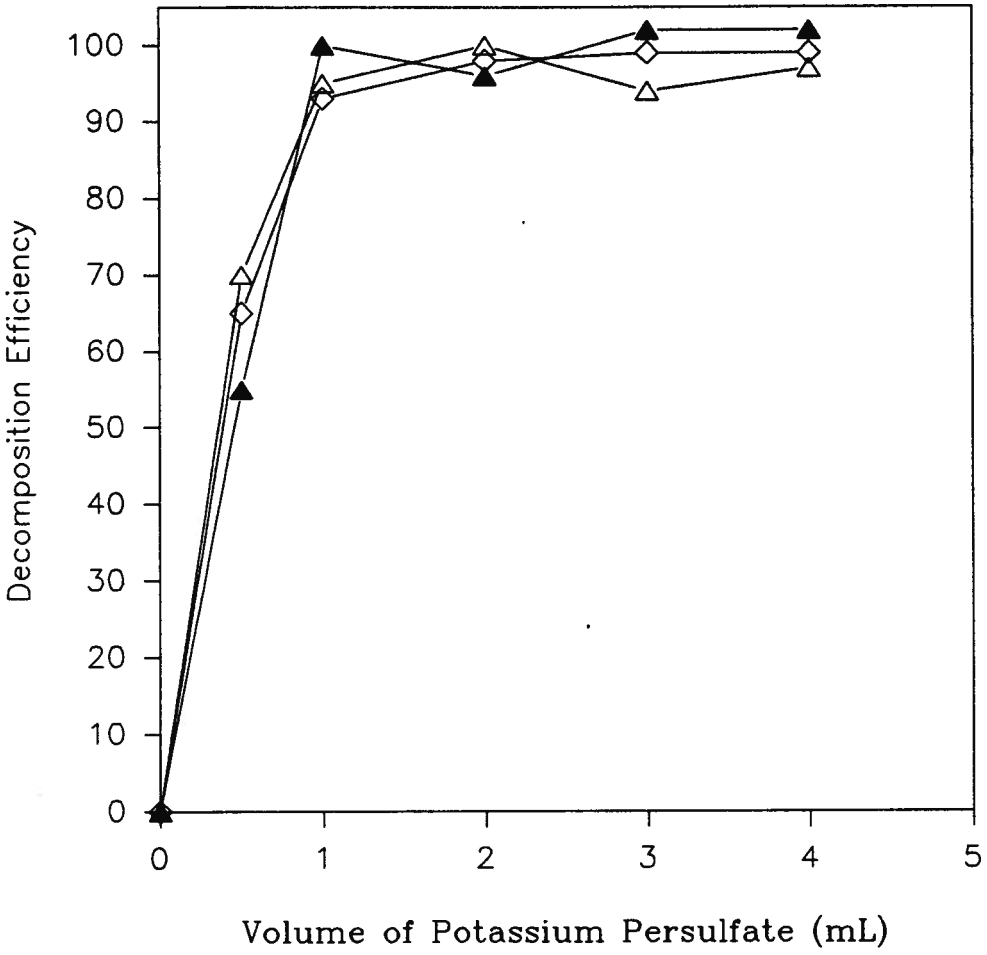

Figure A.7 Effect of the quantity of potassium persulfate (2.0% w/v) on decomposition efficiency. The sample solution (50 mL) contained 50 ng each of AB (Δ), AC (▲), and Me_4As^+ (◊).

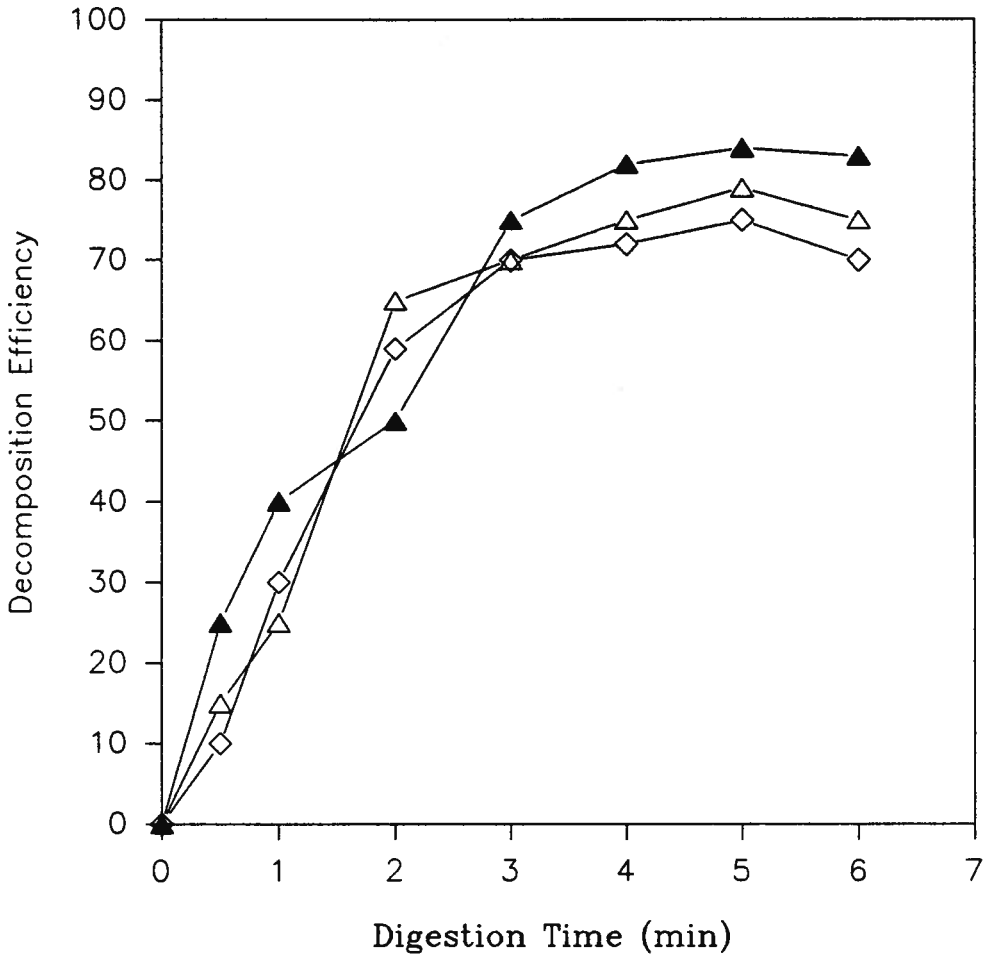

Figure A.8 Effect of microwave digestion time on the decomposition efficiency in the presence of 5 mL of 6.0% (w/v) $K_2S_2O_8$. A 50 mL of seawater sample was spiked with 50 ng each of AB (Δ), AC (▲), and Me_4As^+ (◊).

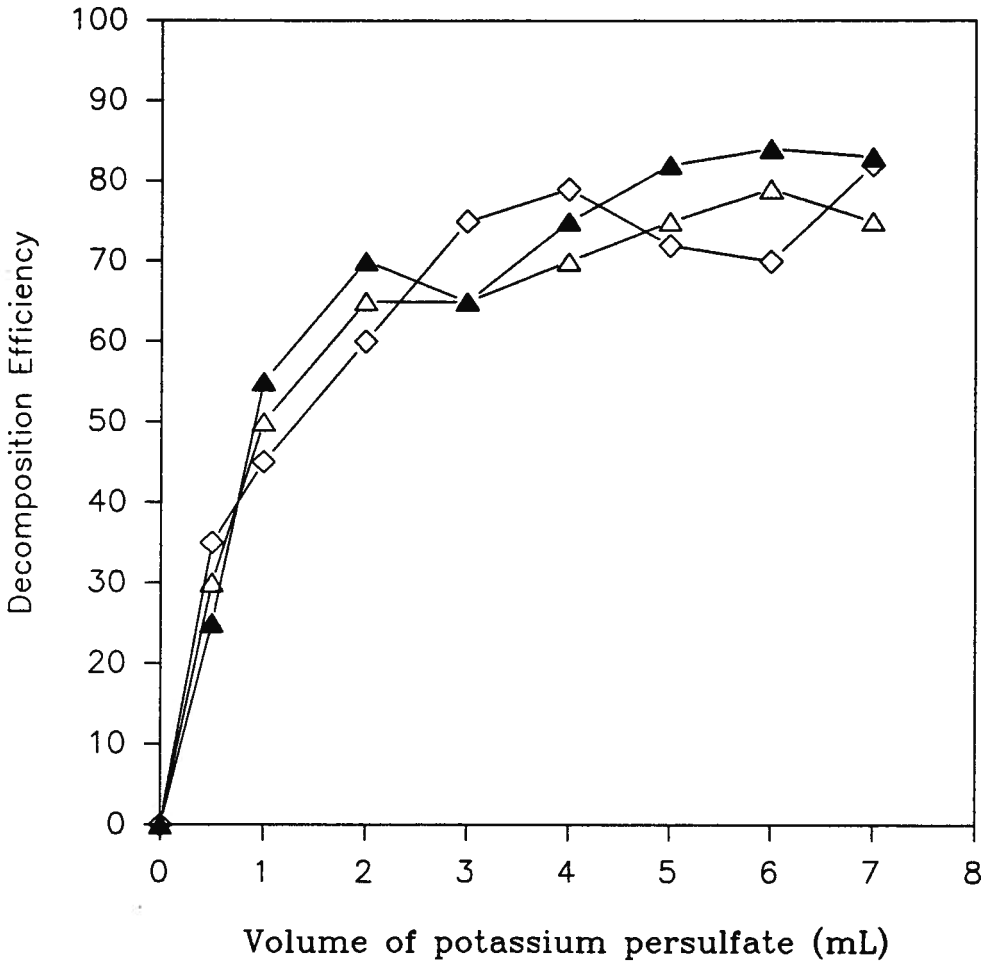

Figure A.9 Effect of the quantity of potassium persulfate (6.0% w/v) on decomposition efficiency. A 50 mL of seawater sample was spiked with 50 ng each of AB (Δ), AC (▲), and Me_4As^+ (◊), and irradiated in the microwave oven for 5 min.