

ARSENIC SPECIATION ANALYSIS IN FOOD-RELATED AND ENVIRONMENTAL SAMPLES

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ABSTRACT

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Metallomics approaches based on the combined use of elemental and molecular mass spectrometry for arsenic speciation analysis in phytoremediating plants, marine algae, cut tobacco and cigarette smoke total particulate matter have been developed. Size-exclusion chromatography (SEC)-ICP-MS is proposed to use as a powerful tool for selecting both the appropriate extractant as well as optimum extraction conditions. Comparative SEC-ICP-MS As profiles and total As concentrations in the extracts were used to identify the optimum condition for As speciation studies as a compromise between extraction efficiency and preservation of compound identity.

Methodologies have been developed to gain a better grasp of the factors involved in the uptake and distribution of As in the hydroponically grown *Arabidopsis thaliana*. The effect of the presence of Se on the As and Hg incorporated into the leaves was investigated here for the first time; Se in the growing media was found not to affect As and Hg concentrations in the leaves. Results also revealed the presence of small amounts of As-PC₃, As-PC₄ and As-PC₅ complexes in the leaves, which were characterized by ESI-Orbitrap MS to minimize ambiguity in species identification. The application of an *in vitro* dialysis method for predicting the As bioaccessibility in selected edible marine algae, was investigated here for the first time. Results showed low As dialyzability (10-20%) and no transformation of As species, primarily arsenosugars, observed following *in vitro* gastrointestinal digestion. Knowledge of the distribution of arsenic species in cut tobacco, obtained by sequential extraction, provides an insight into the transformation of arsenic species during the combustion process when the cigarette is burnt. The combustion of organic compounds present in tobacco resulted in the change of redox state and As-species distribution in tobacco smoke. Both the hyphenated MS and XANES techniques were used to obtain information about arsenic speciation in smoke condensates. The results showed that the tobacco smoke contained a mixture of As(III) and As(V); As(III) being found as arsenite and, possibly, thio-arsenite by HPLC-ICP-MS. The reduction of As(V) to As(III) during dynamic cigarette smoke formation can be explained by the overall smoke redox properties in accordance with the cigarette combustion process.

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GLOSSARY

ASE	Accelerated solvent extraction
MeCN	Acetonitrile
Å	Angstrom
AE	Anion exchange
As(V)	Arsenate
As ^{III} -PCs	Arsenic phytochelatin complexes
As(III)	Arsenite
AsB	Arsenobetaine
AsC	Arsenocholine
atm	Atmosphere
API	Atmospheric pressure ionization
BF	Bioconcentration factor
CRMs	Certified reference materials
cigt	Cigarette
CID MS/MS	Collision induced dissociation tandem mass spectrometry
C/RC	Collision/reaction cell
CPS	Count per second
°C	Degree celsius
DNA	Deoxyribonucleic acid
MA ^{III} -(GS) ₂	Di(glutamylcysteinyl-glycyl)methyl-dithio-arsonite
DMA	Dimethylarsinic acid
DMAA	Dimethylarsenoacetate
EtOH	Ethanol
GSSG	Disulfide glutathione
eV	Electron volt
ESI	Electrospray ionization
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
EDTA	Ethylene diamine tetra acetic acid
EFSA	European Food Safety Authority
EXAFS	Extended X-ray absorption fine structure
Ext Cal	External calibration
GC	Gas chromatography

DMA ^{III} -(GS)	γ -glutamylcysteinyl-glyciny dimethyl thioarsinite
Hz	Hertz
HCD	Higher energy collision induced dissociation
HMW	High molecular weight
HPLC-ICP-MS	High performance liquid chromatography coupled to inductively coupled plasma mass spectrometry
HR-ICP-MS	High resolution inductively coupled plasma mass spectrometry
h	Hour
HGAAS	Hydride generation atomic absorption spectrometry
HG-AFS	Hydride generation atomic fluorescence spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
Int Std	Internal standard
IDMS	Isotope dilution mass spectrometry
K	Kelvin
kDa	Kilo Dalton
LD ₅₀	Lethal dose 50; in toxicology, the dose required to kill half the members of a tested population after a specified test duration
LODs	Limits of detection
LC or HPLC	Liquid chromatography or high performance liquid chromatography
LC-MS	Liquid chromatography coupled to mass spectrometry
LMW	Low molecular weight
m/z	Mass per charge
MeOH	Methanol
MT	Metallothionein I
MAE	Microwave assisted extraction
mEq	Milli equivalent
min	Minute
M	Molarity
MW	Molecular weight
MMA	Monomethylarsenic acid
PAR	Parabolic aluminized reflector

ppb	Part per billion
ppm	Part per million
ppt	Part per trillion
PA	Peak area
PFA	Perfluoroalkoxy polymer resin
PMSF	Phenylmethanesulfonyl fluoride
PCs	Phytochelatin or ([γ -glutamate-cysteine] _n -glycine, n=2-11)
PIPES	Piperazine-N,N-bis(2-ethane-sulfonic acid) disodium salt
PEEK	Polyether ether ketone
PTFE	Polytetrafluoroethylene
Psi	Pounds per square inch
Q-TOF	Quadrupole time-of-flight
QC	Quality control
RF	Radio frequency
GSH	Reduced glutathione
RSD	Relative standard deviation
t _r	Retention time
RP	Reversed phase
rpm	Revolutions per minute
sec	Second
SEC	Size exclusion chromatography
SC	Smoke condensate
SON	Sonication
SDS	Sodium dodecyl sulphate
SD	Standard deviation
SOD	Superoxide dimutase
MS/MS	Tandem mass spectrometry
TMAH	Tetramethylammonium hydroxide
TeMA	Tetramethylarsonium ion
TDS	Total dissolved solid
TPM	Total particulate matter
TMAO	Trimethylarsine oxide
As ^{III} -(GS) ₃	Tris(glutamylcysteinylglycyl)trithioarsenite
UV-Vis	Ultraviolet visible

W	Watt
WHO	World Health Organization
XANES	X-ray absorption near edge spectroscopy
XAS	X-ray absorption spectroscopy

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AIMS & OBJECTIVES

Aim- To develop improved extraction techniques for arsenic species quantification in selected environmental samples and food related products including *Arabidopsis thaliana* leaves, edible marine algae, cut tobacco and tobacco smoke using ICP-MS hyphenated methods.

Objectives

- Optimize extractants, sample size to extractant volume ratio and extraction times.
- Explore extraction approaches such as ultrasonication and microwave assisted extraction (MAE) and compare them in terms of extraction efficiency and species preservation.
- Evaluate the distribution of As species according to their polarity.
- Develop a sequential extraction to permit characterization of As from different origins.

Aim - To develop improved methodologies for species identification.

Objectives

- Evaluate sample pretreatment procedures for arsenic speciation to enable better sensitivity and improve detection capability specifically for ESI-tandem MS.
- Develop chromatographic separations to enable the on-line detection of organoarsenicals and/or arsenic containing biomolecules by ICP-MS and ESI-tandem MS.

Aim - To apply the developed speciation methodologies to :

- Study factors involved in metal uptake and translocation by plants and to investigate the inter-elemental effect of selenium on arsenic and mercury incorporation into the leaves of *Arabidopsis thaliana*.
- Investigate the potential transformation of arsenosugars in edible marine algae following *in vitro* gastrointestinal digestion and to evaluate the bioaccessibility of arsenic by means *in vitro* dialysis method.
- Investigate the transformation of water-soluble arsenic species from cut tobacco to cigarette smoke total particulate (TPM) samples with the combined use of available data obtained by XANES and the Eh-pH diagram for the prediction of arsenic forms.

CHAPTER 1

INTRODUCTION

Elemental speciation analysis has received an increasing interest over the last decade since the determination of total concentration is not sufficient for gaining insights into its actual impact on the surroundings and human health. Toxicokinetic information on the absorption, distribution, biotransformation and elimination of metal(loid) species in environmental and biological samples is needed to assess the risks associated with human consumption. A number of issues, such as the potential toxicity or benefit of elemental-containing compounds and factors affecting mineral bioavailability, can be addressed by speciation analysis, i.e. the identification and quantification of individual chemical species in a sample. To obtain such information, knowledge of the diverse chemical forms existing in a particular media is necessary since the behaviour of chemicals is largely dependent on the physical properties of the media in which they are contained. Initially, the development of the elemental speciation methodologies was dedicated to the determination of low molecular-weight metal(loid) species (e.g. organometallic compounds). Current trends in speciation analysis are now focused on bioinorganic applications, particularly the investigation of high molecular-weight metal species in relation to biological processes.

The term metallome was defined as the entirety of metal and metalloid species present in a cell or tissue type [1]. The research field coping with the study of metallomes and their connections with genomes and proteomes was referred to as metallomics [2]. Given by Mounicou *et al* (2009), the definition of metallomics is the study of a metallome, interactions and functional connections of metal ions and their species with genes, proteins, metabolites and other biomolecules within organisms and ecosystems [3]. The emergence of metallomics makes it possible to elucidate the interaction of metal(loid)s and endogeneous or bio-induced biomolecules such as organic acids, proteins, sugars or DNA fragments among different cell compartments.

Metallomics has a significant impact on different areas of life science such as geochemistry, clinical biology, nutrition and plant and animal physiology. Since metals are considered to be a significant link in the chemistry of the atmosphere and oceans from which life has evolved, understanding how organisms respond, adapt and utilize these metals in their metabolisms is required. In plant physiology, studies of plant mechanisms for (i) metal uptake from soil, (ii) metal translocation in plants and

(iii) sequestration of metals in cellular compartments, are of particular interest. Such knowledge acquired is essential to gain a better grasp of the factors involved in metal uptake and translocation by plants, which may be useful to produce better phytoremediating plant. In clinical chemistry, although metals play a significant role as regulatory cofactors, they could be detrimental to humans and cause several diseases. The detection of biomarkers to identify a disease and track its progression is also an interesting and important topic of research. In nutrition, the prevention of certain diseases associated with low levels of mineral consumption could be effective by supplementing foods or mineral elements. Knowledge of the characterization of chemical forms, their bioavailability in food products, as well as transport and fate in the target organs are all important to determine the dietary requirement and related legislation. The development of a metallomics approach for identification and quantification to elucidate biological pathways and to contribute to better understanding the species specific biological effect of elements is still a challenge.

Arsenic has generated a great deal of interest for a century because of its recognized toxicity. An obvious example of this is that many countries, in particular some regions of Bangladesh, West Bengal and Nepal have suffered from arsenic-contaminated soils and ground waters. It is believed to be caused by the dissolution of geological strata, in which high levels of As-containing mineral complexes such as arsenopyrite (FeAsS) and orpiment (As_2S_3) are contained. The degradation and dissociation of the minerals in the strata by ground water result in the release of inorganic arsenic and its contamination of domestic ground water. This has affected people who ingest polluted food and water unavoidably, thereby leading to serious diseases such as diabetes, anemia, cardiovascular disease, neurological and immunological effects, including cancers (bladder, skin, lung and prostate).

Studies indicate that the degree of arsenic toxicity depends on its oxidation state and chemical form. For example, inorganic arsenic is the most toxic form and classified as a human carcinogen; arsenite is more harmful than arsenate; while organic forms have varying toxicity levels. Since prolonged low level exposure to arsenic has resulted in chronic effects, it is necessary to regularly perform surveys of As species and total arsenic in its main source to consumers (e.g. foodstuffs). Most surveys have been done for total As since it is still difficult to reliably measure As species, especially inorganic As. The implication of this is that, if a decision were made for European-wide legislation to focus specifically on the toxic species (rather

that “total” As), there may not be sufficient analytical methods capable of providing reliable data to support the new law enforcement.

The provisional tolerable weekly intake value established by the World Health Organization (WHO) at $15 \mu\text{g kg}^{-1}$ body weight per week to inorganic As for human intake has not been succeeded by any international implementation of regulatory limits in foodstuffs [4]. Asked by the European Commission, European Food Safety Authority, (EFSA, 2009) has published a scientific opinion on possible health risks associated with As contaminants in food products [5]. It emphasized the need for more information on levels of inorganic and organic arsenic in a wide diversity of food stuffs and the relationship between levels of As intake and possible health effect. Since food is the major source of As exposure for Europeans, EFSA suggested that exposure to inorganic As should be reduced. The main contributors to inorganic As exposure in diet were listed as following: cereal grains, cereal-based products, rice, rice-based products, fish, vegetables, food for special dietary uses (such as algae), bottled water, coffee and beer. However, there are presently no harmonized maximum levels for As in food products in Europe. This would increase the demand for control analysis, which would in turn emphasize the need for standardized methods. Such methods for the measurement of inorganic As in food are not available and equally important, no food CRMs with certified values for inorganic As has become commercially available. The U.S. Environmental Protection Agency (USEPA) Office of Pesticide Programs (OPP) has designed DMA as “not carcinogenic up to doses resulting in regenerative proliferation” (USEPA 2006, p.17) [6]. The presence of arsenosugars found in seaweed and some bivalves has received a considerable concern due to studies of the human metabolism indicating its metabolism to DMA. The estimation of human health risk from seafood consumption by arsenic speciation studies is therefore required in risk assessment for As-contaminated sites.

There are three important steps to study chemical speciation using the metallomics approach: sample preparation, separation and detection. Sample preparation is the most important of these since errors can be caused by non-homogeneous sampling, contamination, volatilization, species decomposition and transformation. As a result, analytical procedures must be chosen with a view to species preservation and homogeneity. Speciation analysis generally uses hyphenated techniques which couple together separation and detection. HPLC coupled with ICP-MS (HPLC-ICP-MS) is becoming the most powerful means of speciation analysis due

to its simplicity, reliability and reproducibility. Since HPLC gives excellent reproducibility and ICP-MS offers not only high sensitivity and selectivity but also a wide linear range of detection, HPLC-ICP-MS is regarded as an effective coupling technique for arsenic speciation analysis. Although arsenic is monoisotopic, ^{75}As , it has possible spectral interference from $^{40}\text{Ar}^{35}\text{Cl}$. This technique allows us to obtain accurate results by using a mathematical correction, a collision cell to eliminate the interference prior to mass spectrometric detection, and buffer systems to prevent arsenic species from co-eluting with chloride. In addition, this technique makes it possible to characterize arsenic species in diverse samples and to gain structural information using electrospray MS combined with ICP-MS.

In order to meet the requirement for risk assessment, speciation analysis should emphasize toxic or metabolically important species and provide adequate information about the element species in the sample (water-soluble, lipid-soluble, and insoluble fractions). Due to the inherent capability of As(III) and As(V) to interchange, it is advisable that any future certification of inorganic arsenic should be based on the sum of the two species rather than trying to aim individual values for each of the species. Advances in the speciation analysis of arsenic would serve as a basis for future work. Efforts should be put in the development of sample clean-up procedures and analyte preconcentration in order to enable the detection of low levels species using molecular MS techniques.

In this thesis, the development of As speciation methodologies for individual samples to enable the reliable identification and quantification of As-species is discussed. This involves the use of optimized methods to apply in several fields of metallomics research. Phytoremediation has gained an increasing interest for coping with the hazardous consequences from high levels of toxicants, especially heavy metals in contaminated soil. Arsenic speciation in a non-hyperaccumulating plant, *Arabidopsis thaliana*, hydroponically grown in a controlled condition, is investigated to gain a better grasp of factors affecting the mechanism controlling the uptake of species, metabolism and detoxification processes. The effect of the presence of Se on the As and Hg uptake by the plant leaves is also discussed. The consumption of edible marine algae raises a significant concern because of the presence of large amounts arsenosugars, whose metabolism pathways are still not established. Questions associated with possible toxic aspects of the algae consumption arise. The investigation of the potential transformation of arsenic species in the selected marine

algae occurring in gastrointestinal digestion is performed by the *in vitro* dialysis method. By this means, the bioaccessibility of arsenic in the marine algae is also evaluated. Furthermore, knowledge on the distribution of arsenic species in cut tobacco is fundamental to understanding the transformation of arsenic species during the combustion process in a burning cigarette. The combustion of the abundance of organic compounds present in tobacco can lead to the change of redox states and therefore influence the distribution of arsenic species in tobacco smoke. The complementary use of the hyphenated MS and XANES techniques to obtain essential information on the speciation of As in cut tobacco and the mainstream smoke is discussed in this thesis.

CHAPTER 2

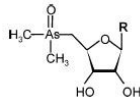
LITERATURE REVIEW

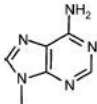
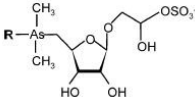
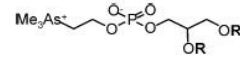
2.1 ARSENIC

2.1.1 Physical and chemical properties

Being a mono-isotopic element and metalloid, arsenic (^{75}As) is naturally present in the environment occasionally as a component of inorganic compounds. Depending on pH and the ionic environment, arsenic salts can be dissolved in HCl, HNO_3 , H_3PO_4 , H_2SO_4 , HF aqueous matrix, water, and NH_4OH . Arsenic is known to have several oxidation states (+3, +5, 0, and -3), and be able to exist in a variety of both inorganic and organic forms [7]. Arsenic species characterized in environmental and biological samples are listed in Table 2.1.

Table 2.1 List of arsenic species characterized from biological and environmental samples [8].

No.	Chemical name	Formula	MW
1	Arsenous acid (arsenite, As^{III})	$\text{OH-As}(\text{OH})_2$	127
2	Arsenic acid (arsenate, As^{V})	$\text{O=As}(\text{OH})_3$	141
3	Monomethylarsenic acid (MMA)	$\text{CH}_3\text{AsO}(\text{OH})_2$	140
4	Dimethylarsenic acid (DMA)	$(\text{CH}_3)_2\text{AsO}(\text{OH})$	138
5	Trimethylarsenic oxide (TMAO)	$(\text{CH}_3)_3\text{AsO}$	136
6	Tetramethylarsonium ion (TeMA)	$(\text{CH}_3)_4\text{As}^+$	135
7	Arsenobetaine (AsB)	$(\text{CH}_3)_3\text{As-CH}_2\text{-COOH}$	179
8	Trimethyl (2-carboxyethyl) arsonium inner salt	$(\text{CH}_3)_3\text{As-CH}_2\text{-CH}_2\text{-COOH}$	193
9	Arsenocholine (AsC)	$(\text{CH}_3)_3\text{As-CH}_2\text{-CH}_2\text{-OH}$	165
10	Dimethyloxarsylethanol	$(\text{CH}_3)_2\text{AsO-CH}_2\text{-CH}_2\text{-OH}$	166
11	Dimethylarsinylacetic acid	$(\text{CH}_3)_2\text{AsO-CH}_2\text{-CH}_2\text{-COOH}$	180
12	N-[4-(dimethyl-arsinoyl) butanoyl] taurine	$(\text{CH}_3)_2\text{AsO}(\text{CH}_2)_3\text{CONH}(\text{CH}_2)_2\text{SO}_3\text{H}$	315
13	5-dimethylarsinoyl 2,3,4-trihydroxy pentanoic acid	$(\text{CH}_3)_2\text{AsOCH}_2(\text{CHOH})_3\text{COOH}$	270
14	5-dimethyl arsinoyl 2,3-dihydroxy pentanoic acid	$(\text{CH}_3)_2\text{AsO}(\text{CH}_2)_2(\text{CHOH})_2\text{COOH}$	254
15	4-dimethylarsinoyl-2,3-dihydroxy butanoic acid	$(\text{CH}_3)_2\text{AsOCH}_2(\text{CHOH})_2\text{COOH}$	240
Dimethyl arsenoyl ribosides (Arsenosugars):			
			
16	5-dimethylarsinoyl-β-ribofuranose	R = H	238
17	5-dimethylarsinoyl-β-ribofuranosol	R = OH	254
18	A: 3-[5'-deoxy-5'-(dimethylarsinoyl)-β-ribofuranosyloxyl-2-hydroxypropane sulphonic acid (Arsenosugar 3)]	$\text{OCH}_2\text{CHOHCH}_2\text{SO}_3\text{H}$	392
19	B: 3-[5'-deoxy-5'-(dimethylarsinoyl)-β-ribofuranosyloxyl-2-hydroxypropylene glycol (Arsenosugar 1)]	$\text{OCH}_2\text{CHOHCH}_2\text{OH}$	328
20	C: 3-[5'-deoxy-5'-(dimethylarsinoyl)-β-ribofuranosyloxyl-2-hydroxypropyl hydrogen sulfate (Arsenosugar 4)]	$\text{OCH}_2\text{CHOHCH}_2\text{OSO}_3\text{H}$	408
21	D: 3-[5'-deoxy-5'-(dimethylarsinoyl)-β-ribofuranosyloxyl-2-hydroxypropyl 2,3	$\text{OCH}_2\text{CHOHCH}_2\text{OPO}_4\text{CH}_2\text{CHOHCH}_2\text{OH}$	482

	hydroxypropyl phosphate (Arsenosugar 2)		
22	E: 2-amino-3-[5'-deoxy-5'-dimethylarsinoyl]-ribose	$\text{OCH}_2\text{CHNH}_2\text{CH}_2\text{SO}_3\text{H}$	391
23	ribose-1-sulphonic acid		
24	Methyl 5-deoxy-5-(dimethylarsinoyl)- β -D-ribose	OCH_3	268
25	3-[5'-deoxy-5'-(dimethylarsinoyl)- β -ribofuranosyloxy]-2-acetic acid	OCH_2COOH	312
26	1-O-[5'-deoxy-5'-(dimethylarsinoyl)- β -D-ribosyl]mannitol		418
27	N-[5'-deoxy-5'-dimethylarsinoyl]- β -ribose-2-carboxylic acid	$\text{OCONHCH}_2\text{COOH}$	355
28	3-[5'-deoxy-5'-(dimethylarsinoyl)- β -ribofuranosyloxy]-2-hydroxypropanoic acid	$\text{OCH}_2\text{CHOHCOOH}$	342
	Dimethylarsinyladenosine		374
Trialkyl arsenio ribosides:			
			
29	R=		
30	CH_3		391
	$\text{CH}_2\text{CHCOOHCH}_2\text{OCH}_2\text{CHOHCH}_2\text{OH}$		554
Trimethyl arsenio phosphate containing chains:			
			
31	Glyceryl phosphorylarsenocholine	R=	318
32	Phosphatidylarsenocholine	H	
		$\text{CO}(\text{CH}_2)_n\text{Me}$	

2.1.2 Sources of arsenic

Being the 20th most abundant element in our environment, arsenic is ubiquitous in water, soil, atmosphere and living organisms. Having more than 245 mineral ores, arsenic is found in the ratio: 60% arsenates, 20% sulfides, and sulfosalts, 20% arsenides, arsenites and elemental arsenic [9]. The most common arsenic minerals found in the terrestrial crust are in the forms of arsenopyrite (FeAsS). Arsenic minerals are mobilized *via* leaching with water, which is reliant on several factors such as pH and redox potential. The contribution of arsenic for natural and anthropogenic sources to the atmospheric deposition was approximately reported to be 60:40 [9]. It is suggested that two dominant sources of natural release are volcanic activity and erosion process of soils [10]. These processes give rise to the natural contamination of groundwater. The main anthropogenic sources of arsenic releases to the environment are various industrial processes such as semiconductor and wood preservative industries, non-ferrous smelters, coal combustion, and agricultural activities [9]. The metal processing industries are considered to be responsible for the main amount of As release from anthropogenic sources. Agricultural industries have

extensively used arsenic compounds for insecticides or herbicides, as growth promoter. In addition, the possible source of arsenic is the use of phosphate fertilizers, depending on the phosphate rock used (approximately $8 \mu\text{g As g}^{-1}$ rock). Chromated copper arsenate (CCA) is widely used in wood preservative industries against biological decay [9].

2.1.3 Toxicity

It is generally accepted that arsenic toxicity is dependent on its chemical form as well as oxidation states. Studies of arsenic toxicity have indicated that inorganic forms are more poisonous than organic forms and trivalent forms are more detrimental to human body than pentavalent forms. It is interesting to note that acute toxicity decreases with increasing degree of methylation (Table 2.2). Acute toxicity of arsenic species decreases in the order $\text{AsH}_3 > \text{As(III)} > \text{As(V)} > \text{MMA} > \text{DMA} > \text{AsB, AsC, TMAO}$. The toxicity of As(III) can be explained by strong affinity for sulfhydryl groups of biologically active protein, which may result in inhibition of metabolic enzymes [11]. Human are subjected to inorganic arsenic through air, water, and food. A number of studies indicated that both ingestion and inhalation of arsenic can increase the risk of human cancers in lung, skin, bladder, kidney, liver, and stomach, which are potentially caused by lipid peroxidation, protein and enzyme oxidation, or glutathione depletion [12-14]. The ingestion of As contaminated water and food by humans can cause diabetes, cardiovascular disease, anemia, neurological and immunological effects. Long term cigarette consumption causes the lack of glutathione, contributing to the oxidative stress generated in lungs, thereby resulting in tissue-damaging effect [13].

Table 2.2 Experimental LD₅₀ values of arsenic species [7].

Arsenic species	LD ₅₀ (g.kg ⁻¹)
As(III)	0.0345
MMA	1.8
DMA	1.2
TeMA	0.89
TMAO	10.6
AsC	>6.5
AsB	>10.0

2.1.4 Arsenic route to human exposure

Exposure of arsenic by contaminated drinking water is considered to be an ecological and a health problem, particularly in Bangladesh and West Bengal [15], due to geologically contaminated groundwater. In the contaminated area, inorganic arsenic levels in well water or in mine drainage areas were reported in the range from 10 to 5,000 $\mu\text{g L}^{-1}$, while the current WHO provisional tolerable weekly intake (PTWI) level for inorganic arsenic from food and water is at 15 $\mu\text{g kg}^{-1}$ body weight [4]. Many methods such as coagulation, filtration, lime softening, activated alumina, ion-exchange, reverse osmosis, nanofiltration and electrodialysis are proposed to remove inorganic arsenic from drinking water. The levels of total arsenic in foods greatly vary from country to country, depending on type of soil, water, geochemical activity, use of arsenical pesticides. Several arsenicals such as arsanilic acid and roxarsone are allowed to be used for growth promoters in animal feeds in the USA [16]. The important sources of arsenic exposure are considered to be daily products, meat, fish and poultry. Although seafood typically contain the highest concentrations of total arsenic due to biological magnification, these levels are not considered to be hazardous, because the majority of arsenic species belong to non-toxic organoarsenicals like arsenobetaine. However, the consumption of Hijiki or *Hizikia fusiforme*, a species of seaweed, should be avoided, due to the presence of high percentage of inorganic arsenic (80% on a dry weight basis) [17, 18]. Arsenic exposure, through the contamination of crops, fruits and vegetables, by humans is thought to occur through spraying or root uptake. Nevertheless, the ordinary levels of arsenic in the edible parts are found not to be toxic and low, as plants accumulate most of arsenic in their roots. Inhalation of arsenic from air is often taken for granted due to a minor route of arsenic exposure. In mainstream cigarette smoke, the total arsenic contents were approximately estimated to be 40-120 ng As cigt^{-1} [19]. However, provided that 20 cigarettes are daily consumed, the intake of arsenic is expected to be 0.8-2.4 μg . Furthermore, a significant occupational exposure to arsenic occurs primarily among industrial workers in the areas associated with melting copper, burning arsenic rich coal and producing arsenical pesticides.

2.1.5 Redox states for prediction of arsenic forms

The pH and redox potential (Eh) play an important role in determining arsenic speciation. Arsenic species can undergo transformation in a variety of redox states, as a result of gaining or losing electrons in a redox reaction together with biotic processes. The diagram shown in Fig 2.1, indicates that arsenate appears to be the predominant form under aerated and oxidizing condition, while arsenite becomes dominant under reducing environments (Eh < 200 mV). The presence of sulfide in reducing environments results in the precipitation of As_2S_3 in river, and marine sediments. The factors affecting the conversion rates involves not only the Eh and pH of matrix, but also other physical, chemical, and biological factors. The diagram can be used for predicting arsenic species present in a sample. For example, the most stable species in normal soil at moderate or high Eh within pH 4-7 are H_2AsO_4^- , HAsO_4^{2-} (pH > 7), and H_3AsO_3 (pH < 9), while the most thermodynamically stable arsenic species in a plummet of the redox potential below +0.3 V at pH 4 or below -0.1 V at pH 8 appear to be as H_3AsO_3 species in the absence of complexing ligands and methylating organisms [20, 21].

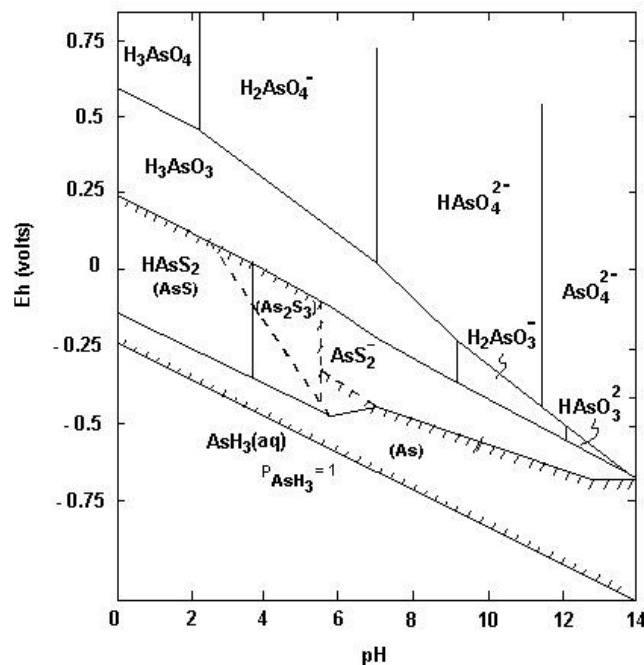
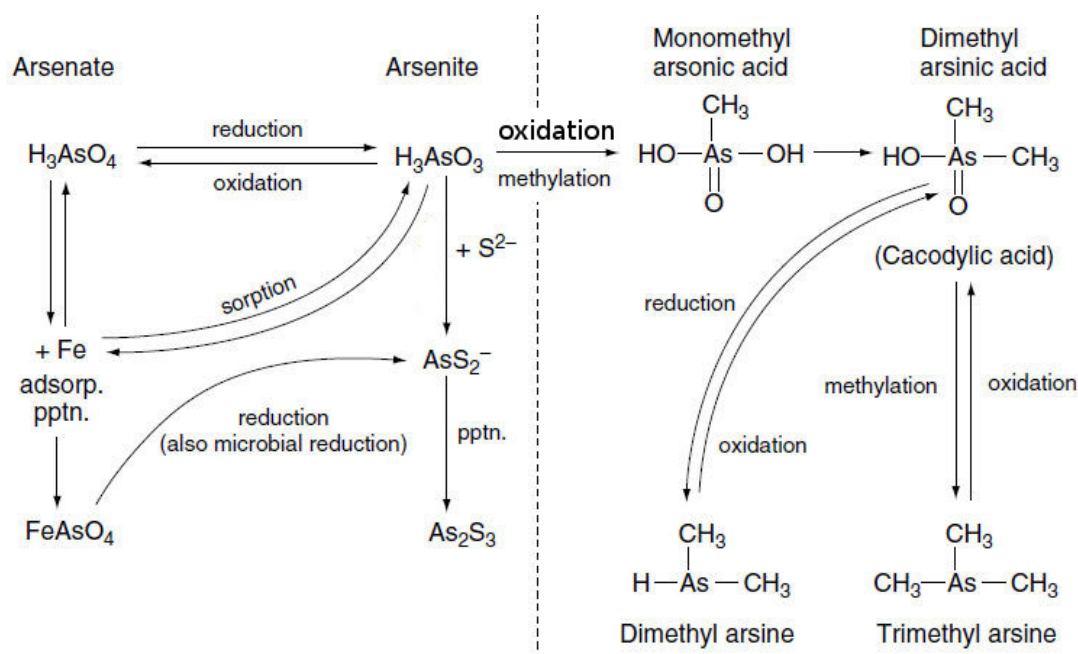


Figure 2.1 The Eh-pH diagram for arsenic at 25°C and 1 atm [21].



2.2 TRANSFORMATION OF ARSENIC SPECIES IN BIOLOGICAL SYSTEM

The well-known plants considered to be capable of a high degree of tolerance and accumulation can be listed as following: *Pteris vittata* (Brake fern) and *Pteris cretica* (Cretan brake) for As, *Brassica juncea* (Indian Mustard) for As and Se, *Allium sativum* (garlic), *Arabidopsis thaliana* and *Astragalus* plants for Se, *Thlaspi caerulescens*, *Alyssum lesbiacum* and *Sebertia acuminata* for Ni. Plants able to

tolerate and accumulate a considerable concentration (>1000 mg/kg) of metals are referred to as hyperaccumulating plants. The degree of accumulation efficiency can be evaluated using the bioconcentration factor (BF), which is the ratio of metal concentration in the plant biomass to that in the soil. Hyperaccumulators are those that possess a $BF > 1$. Insights into metal accumulation pathways are critical to improve the ability of accumulators to be effective. Proposed by Pickering *et al* (2000) [23], the mechanism of arsenic accumulation in *Brassica juncea* is illustrated in Fig 2.3. The most common valence state of arsenic in aerobic waters and soils, arsenate, is taken up from the soil by the root and a small fraction is exported to the shoot *via* the xylem as the oxyanions arsenate and arsenite [24]. Once in the shoots, the arsenic might be stored as As^{III} -tris thiolate. Indeed, the majority of arsenic remains in the roots, also as As^{III} -tris thiolate and during this process, the reduction of arsenate to arsenite, which is the most toxic but less bioavailable, occur. The promotion for more efficient arsenic transportation to the shoots and leaves can be performed by adding a dithiol arsenic chelator (Dimercaptosuccinate).

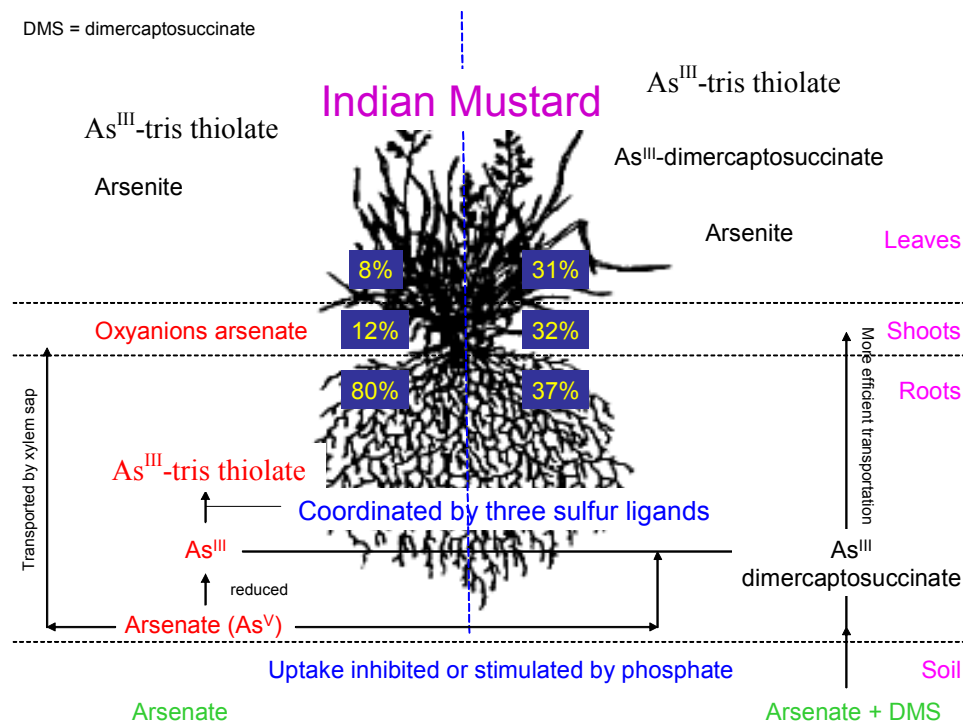


Figure 2.3 Proposed arsenic accumulation in the hyperaccumulator, *Brassica juncea* (diagram based on *As* accumulation pathways as proposed by Pickering *et al*, *Plant Physiology*, 2000, **122**, 1171-1177).

In *Pteris vittata*, Zhang *et al* (2002) demonstrated its capability of rapidly and efficiently accumulating large concentrations of As from a moderately contaminated soil (97 ppm As) [25]. The distribution of As in the plants was varied, showing that total As concentration were 3894, 2610, 2336, 728 and 168 $\mu\text{g/g}$ for old fronds, young fronds, fiddle heads, rhizomes and roots respectively. In the speciation studies of *Pteris vittata* grown in an arsenic-enriched soil, the presence of negligible levels of stable organoarsenic compounds were found [25]. However it is possible that intermediate arsenic-biomolecule complexes may have decomposed on extraction and/or separation. Although these As species were not detected, such complex formations may be accountable for accumulating such high arsenic concentrations. The strong evidence showed the conversion of As^{V} to As^{III} in the course of arsenic translocation. In the fronds, 60-74% of arsenic was found as As^{III} , whereas merely 8.3% was found in the roots. It was hypothesized that arsenic is taken up as arsenate and then converted to arsenite. The study demonstrated the ability of *Pteris vittata* as an efficient accumulator with rapid transfer of As from the soil to above-ground biomass with only minimal arsenic concentration in the root. The conversion of MMA to DMA in roots of xylem has been reported in other studies on *Pteris vittata*. It has been held that microbes involving arsenic oxidation and reduction were not attributed to this process [26].

Nevertheless, the differences in the capability of accumulating arsenic phytochelatin complexes between *Pteris vittata* and other hyperaccumulating plants such as *Helianthus annuus* (sunflower) and *Thunbergia alata*, later reported to contain a variety of the complexes [27-29], have received a significant attention since it might lead to the key factor of the translocation mechanism of arsenic from roots to fronds. In order to test this hypothesis, Liu *et al* (2010) studied the effect of the PC-deficient (*cad1-3*) and the GSH-deficient (*cad2-1*) mutants of *A. thaliana*, isolated by their phenotype of Cd sensitivity, in comparison with the wild type plants [30]. The study in transgenic plants showed the reduction in As^{III} -PC complexation in the plant roots, but the enhancement in arsenic mobility. It was noticeably found that both the $\text{As}(\text{III})$ translocation from roots to shoots and the $\text{As}(\text{III})$ efflux to the external medium were significantly enhanced.

The wide variation of arsenic accumulation in different plants is commonly known [31]. The distinct differences in translocation of arsenic from roots to shoot between hyperaccumulating and non-hyperaccumulating plants were reviewed [32].

The root-to-shoot translocation of arsenic is occasionally limited in most of plant species, except for hyperaccumulators. Zhao *et al* (2009) proposed the use of schematic diagram of arsenic uptake in roots to explain the translocation differences in both types of plants [32], as illustrated in Fig 2.4.

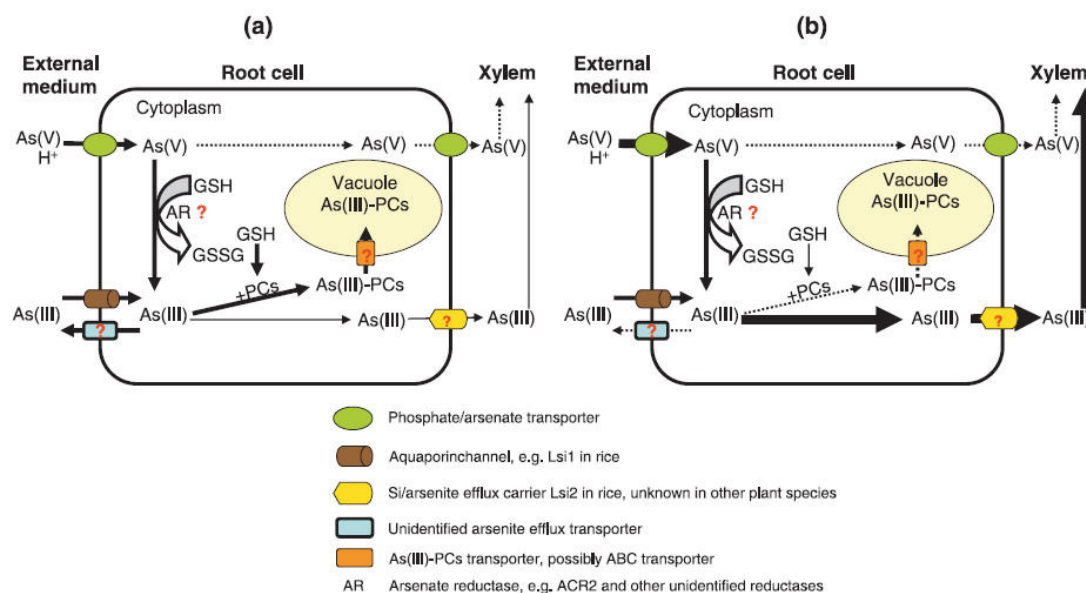


Figure 2.4 Schematic diagram of arsenic (As) uptake and metabolism in roots of (a) non-hyperaccumulators and (b) hyperaccumulators. Line thickness relates to flux rate, with the dotted line indicating the slowest rate [32].

2.2.2 Marine organisms

In the aquatic environment, the majority of arsenic speciation studies in both flora and fauna have been reported, as they are known to accumulate high levels of inorganic arsenic from their surrounding water, sediments and food sources. Although inorganic arsenic is found to be dominant in seawater and sediments in the marine ecosystem, it is occasionally transformed in living marine organisms with a variety of organoarsenic species being predominantly found. It is believed that the transformation of organoarsenical species in living organisms involves complex metabolism, typically *via* biomethylation to give MMA, DMA, TeMA, and TMAO [20]. The proposed pathways for the transformation of organoarsenical compounds are illustrated in Fig 2.5. Marine algae can convert inorganic arsenic to arsenosugars, followed by further metabolic biotransformation to form AsB [33], while the presence of complex organic species such as AsB, AsC, TMAO, TeMA, and arsenosugars

through the marine food chain have been identified in marine animals [34-36]. Several key factors such as redox reaction, methylation and biosynthesis by the organism are accountable for the biotransformation of arsenic species in living organisms. The biotransformation of inorganic arsenic is generally considered to be the detoxification mechanism, as the end products methylated arsenicals are less harmful with tissue constituents.

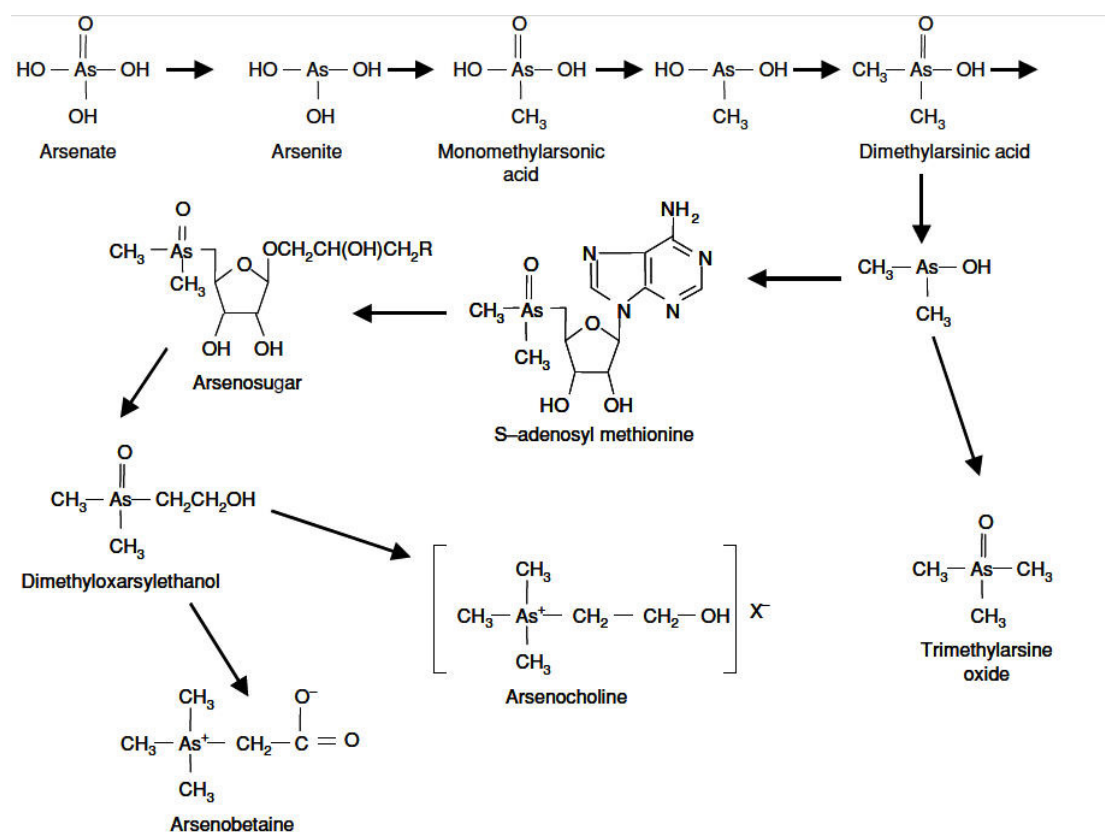


Figure 2.5 Potential pathways for the reduction and methylation of arsenic in living organisms [37].

2.2.3 Human

When ingested, arsenic is absorbed from the gastrointestinal tract to bloodstream, and distributed to body tissues, following the first pass through the liver. Most of arsenic is excreted after the metabolism *via* urine within 3-4 days [38]. A certain portion of arsenic is distributed in different parts of body and then excreted *via* hair, skin scale, nails, feces and sweat. More than 90% of inorganic arsenic can be absorbed when the ingested arsenic is soluble [39]. The studies revealed that 60-75% of the inorganic As

intake was excreted with the following percentage of urinary metabolites: 10-15% inorganic As, 10-15% monomethylated arsenical, and 60-80% DMA dimethylated arsenical [40]. Inorganic arsenic can be metabolized through consecutive reduction and oxidative methylation reactions, as schematically illustrated in Fig 2.6. It is methylated to MMA and DMA by alternating reduction of pentavalent As to trivalent form, and addition of methyl groups, which is believed to be derived from S-adenosyl methionine [11]. The conversion of inorganic arsenic to methylated forms such as MMA and DMA can occur in the liver [41]. The presence of two trivalent arsenicals, MMA^{III} and DMA^{III} were reported in the urine of people who were drinking As contaminated water [42-44] and tissues [45, 46]. These methylated trivalent species may play a significant role in the toxic effects from chronic arsenic exposure. Arsenic metabolism studies revealed that MMA^{III} and DMA^{III} were more toxic than the precursor in different human cell types [22, 47-49].

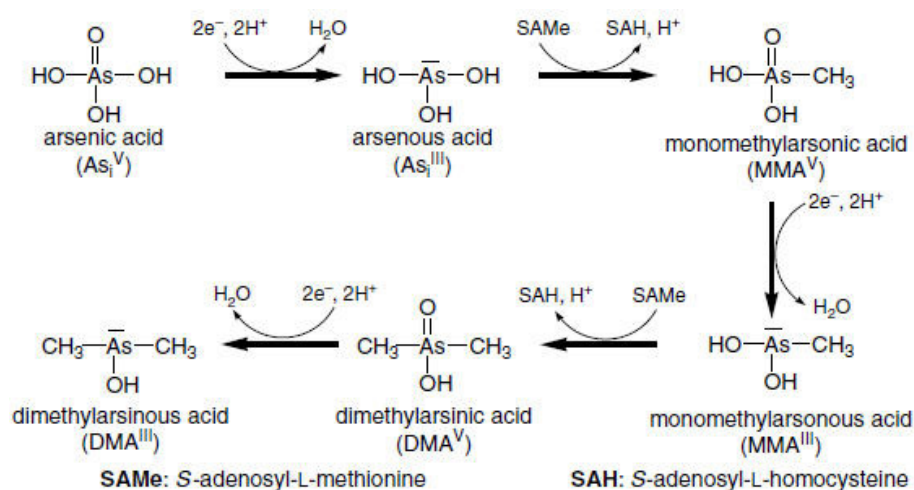


Figure 2.6 Proposed metabolic pathway for inorganic arsenic [50].

The absorption of organoarsenicals is also high. Some organoarsenicals such as AsB and AsC are rapidly excreted intact in human after the ingestion of seafood [40]. Unlike AsB and AsC, the ingestion of arsenosugars leads to the increasing concentration of various arsenic metabolites, mainly DMA^{V} in urine [51-53]. The studies of the transformation of arsenosugars have gained interest for elucidation of the metabolic pathways, since they might promote carcinogenic activity of DMA^{V} , which could be mediated by its reduced form DMA^{III} . It is known that the

consumption of edible algae is the important route of the arsenosugar exposure to human. The main route of the excretion of inorganic and organic arsenic is urine, which is generally considered to be used as a biomarker for arsenic exposure since the arsenic concentration in urine represents recent exposure [51].

The study of human metabolism *via* human urine following arsenic ingestion of an oxo-arsenosugar was investigated by Raml *et al* (2005) [52]. Seven arsenic metabolites, accounted for 88% of total As in urine collected within 61 h, were DMA, oxo-dimethyl arsenoethanol (oxo-DMAE), trimethylarsine oxide (TMAO), oxo-dimethyl-arsenoacetate (oxo-DMAA), thio-dimethylarsenoacetate (thio-DMAA), thio-dimethylarsenoethanol (thio-DMAE) and the thio-arsenosugar, as illustrated in Fig 2.7, were identified.

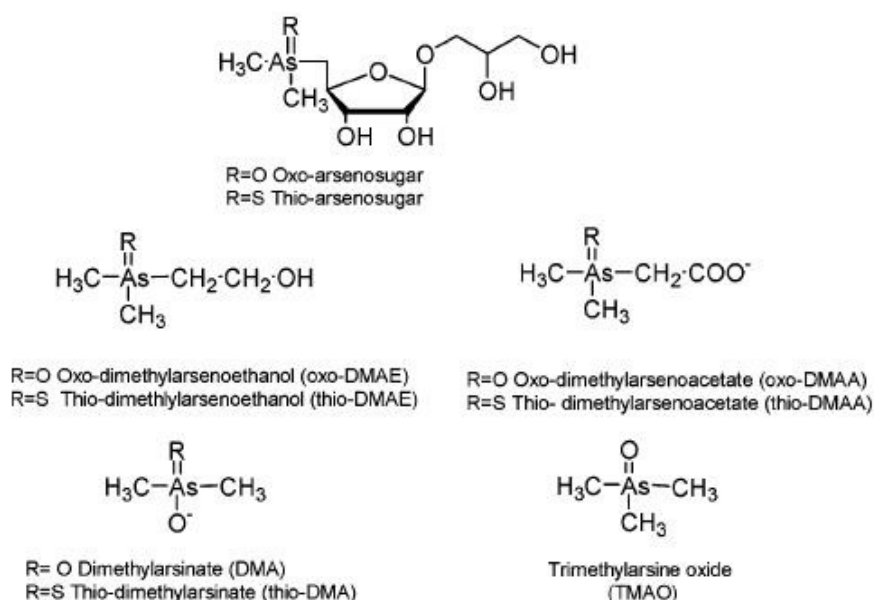


Figure 2.7 Structures and abbreviations of arsenic compounds [52].

As shown in Fig 2.8, possible transformation of the oxo-arsenosugar was given in this study through time profile of the excretion; arsenosugar was first metabolized to oxo-DMA, oxo-DMAA, and their thio-analogues, which were further degraded to DMA and thio-DMA. Furthermore, cytotoxicity was also studied in all metabolites by incubation with HepG2 cells and the results revealed no toxicity even at 10 mM, except for DMA.

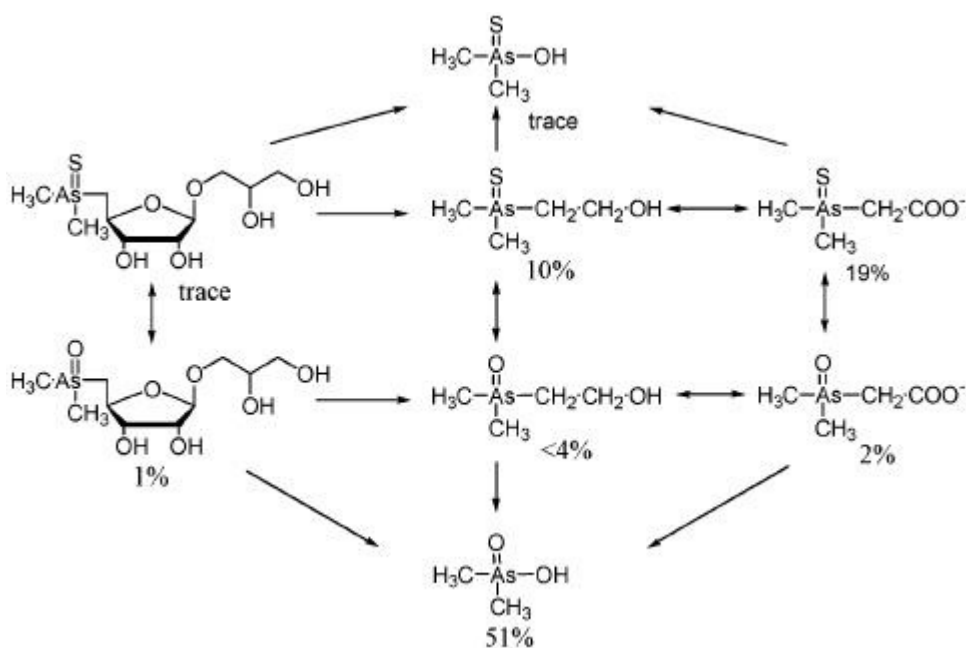


Figure 2.8 Possible interconversions of identified metabolites in urine after ingestion of the oxo-arsenosugar [52].

2.2.4 Sheep

In common with the urinary metabolites in human, the presence of DMA [53], oxo DMAE [53], oxo-DMAA [53], thio-DMAA [54] and thio-DMA [55] was also found in the related studies with sheep following feeding with algae.

Feldmann *et al* (2000) found large amounts (>86%) of arsenic were retained and bioavailable and only 13% of the total ingested was excreted by North Ronaldsay (NR) sheep under a controlled feeding trial [56]. They showed that significant amounts of arsenic ingested were preferably accumulated in the lipid tissues such as muscle fat, liver fat and kidney fat. This study led to fractionation studies and an enzymatic hydrolysis method in the different tissues and faeces following the feed with *Laminaria digitata* [57]. Although the major arsenic species bound to the lipids of the seaweed were arsenosugar 1, the result revealed the presence of DMA as the predominant species bound to lipid tissues and in their faeces. With the use of phospholipase D, a specific enzyme, arsenosugar 1 and DMA, found as the major hydrolyzed components in *L. digitata* and muscle, kidney lipids, could be a good indication of the presence of phospholipid as their structure of the corresponding lipid

part. Two arsenolipid structures believed to be present in the sheep lipid tissues and its faeces are presented in Fig 2.9.

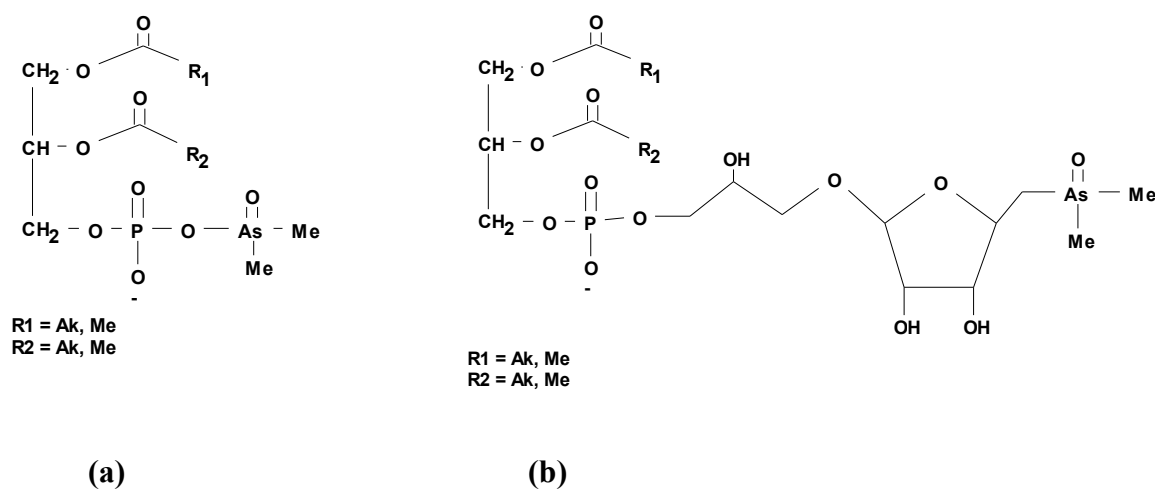


Figure 2.9 Structures of (a) phosphatidyl dimethylarsenic acid; (b) phosphatidyl arsenosugar.

2.3 ARSENIC BIOACCESSIBILITY BY *IN VITRO* METHODS

Bioavailability studies should be determined by measurements *in vivo*. Human *in vivo* methods are the best way to study the bioavailability of minerals and trace element, however, are time-consuming and expensive, rather complicated to perform and sometimes yield quite variable results. In laboratory animal *in vivo* studies, rat is often used as a model for man. These experiments are less expensive but are limited by uncertainties with regard to differences in metabolism between animals and man. As an alternative to human and animal *in vivo* studies, bioavailability of minerals and trace elements has also been estimated through simple, rapid and inexpensive *in vitro* methods.

2.3.1 Solubility method

Most of solubility methods involve determining the solubility of the trace element in various solutions including dilute acids. The solubility of minerals and trace elements under simulated conditions of the stomach (pH 1-2, 37°C) is used as an index of bioavailability, while those under the simulation of the conditions in the stomach (pH 1-2, 37°C) and small intestine (pH 6.5-8, 37°C) is used as a measure of *in vitro* bioavailability.

The occurrence of DMA found in the urinary metabolites following the intake of arsenosugars might be accountable for gastrointestinal digestion. Gamble *et al* (2002) applied simulated gastric juice with HCl (pH 1.1) in four purified arsenosugars 1-4 to investigate the possible degradation of four arsenosugars (1-4) [58]. They reported the slow degradation rate of all major arsenosugars ($1.4\% \text{ h}^{-1}$, at 38°C and $12\% \text{ h}^{-1}$ at 60°C), giving the same product, the free dimethylarsinoylribose. In boiled *Laminaria*, after 4 hours of incubation, the relatively higher rate of arsenosugars degradation to the similar product was found in the range from 32 to 86% [59]. These studies showed that gastric enzyme pepsin does not play a role in the degradation of arsenosugars to DMA found in the *in vivo* studies [52, 53, 60].

The study of arsenic speciation in raw and cooked edible algae following an *in vitro* digestion (pepsin, pH 2 and pancreatin-bile extract, pH 7) was performed by Almela *et al* (2005) [61]. It was found that various treatments (soaking, baking, boiling, or toasting) did not alter the arsenic forms exist in the algae extracts. There was no evidence of arsenosugar degradation, as a consequence of the *in vitro* digestion. Irrespective of cooking methods, the high bioavailability of arsenosugars (>80%) in all of algae investigated was reported.

2.3.2 Dialyzability method

The dialyzability method is based on the simulation of a gastrointestinal digestion through membrane-based separation. The proportion of an element diffusing across a semi-permeable membrane during the intestinal stage is used as a prediction of the availability of minerals. Semi-permeable membranes that hold back large molecules and colloids but pass small molecules and ions through are called dialysis membranes. For this purpose, the dialysis membrane may be cellophane, collodian, or/and animal bladder. Dialysis reliant on simple diffusion is this selective passage of small molecules and ions through such membranes, and it obviously resembles osmosis.

The *in vitro* dialysis method developed by Miller and Schriker (1981) is one of the most extensively used for bioavailability studies; however, more precise standardization was required [62]. Several modifications to this method have been proposed for the availability of intrinsic or supplemented mineral from food [63-66]. These methods employ NaHCO_3 as a dialyzing solution, whose concentration is determined through the estimation of titratable acidity with KOH to obtain pH 7.5, for pH adjustment during pancreatic digestion. However, Wolfgor *et al* (2002) indicated

that the use of NaHCO_3 might affect the uncertainty of final dialyzate pH, as there is a likelihood of CO_2 loss occurring in the dialysis due to agitation and general manipulation [67]. The differences in the final dialyzate pH values can lead to an error in the bioavailability measurement. The pH adjustment during pancreatic digestion is largely dependent on the food matrix and assay conditions. For example, when performing in food with high protein contents, the occurrence of acid or base further generated during hydrolysis leads to the difficulty for determining base concentration. Wolfgor *et al* (2002) proposed the use of PIPES buffer [piperazine- $\text{N,N}'$ -bis(2-ethane-sulfonic acid), $\text{pK}_a=6.80$] to achieve a uniform final pH of 6.5 in digest/dialyzate system [67]; the authors believed that a pH range from 6.3-6.9 gives results, which are in good agreement with *in vivo* experiments. The calculation of buffer concentration can be achieved by measuring HCl mEq required to obtain pH 2 and HCl mEq included in the aliquot of pepsin suspension, which avoids the step of measuring titratable acidity. This method can compensate for the effect of acid or base generated by hydrolysis due to the PIPES buffer capacity. However, the low buffer capacity (<0.1 M) may not adequately tolerate the pH change due to the hydrolysis. In order to prevent the capacity falling to 0.1 M, the use of 0.15 M PIPES buffer with pH adjustment to obtain the optimum pH is used [68]. The pH regulation procedures were differently reported. Kapsokefalou and Miller (1991) proposed the use of 0.15 N PIPES buffer, pH 6.3 for the study of dialyzability in certain iron compounds [69]. Haro-Vicente *et al* (2006) proposed to select the optimal pH of PIPES buffer, where the final pH values of dialyzate and residual fractions provide the values close to a physiologically relevant pH (pH 6.5-7.5). They employed 0.15 N PIPES buffer, pH 8.5 for the dialyzability study in citric fruit juices fortified with different iron compounds. Using the same approach, Dominguez-Gonzalez *et al* (2010) employed a 0.15 N PIPES solution, pH 7.5 for estimating the bioavailability of Co, Cr, Mn and V in edible seaweeds [70].

The *in vitro* dialysis method is performed by the batch system. In principle, aliquot of peptic digest is added to a flask then the dialysis bag, filled with PIPES buffer, is placed in the flask and incubated for 30 min in a shaking water bath at 37°C . After 30 min, the pancreatin-bile mixture is added. The incubation last for further 2 h. Following the dialysis process, the dialysis bag is removed and rinsed with water. The pH of both the peptic digest and dialyzate inside the membrane are checked to ensure

they are in the range between 6.5 and 7.5. The percentage of dialyzed minerals (dialyzability) is used to express the bioaccessibility.

2.4 DIRECT SPECIATION ANALYSIS WITH X-RAY SPECTROSCOPIC TECHNIQUES

2.4.1 X-ray absorption spectroscopy (XAS)

X-ray spectroscopic methods such as X-ray absorption near edge spectroscopy (XANES) and extended X-ray absorption fine structure region (EXAFS) have gained increasing popularity for elemental speciation analysis. Although these methods have occasionally been used for geological samples [71-73], they are also applicable for the analysis of biological samples with high levels of analyte of interest [28, 75, 76]. X-ray spectroscopy is based on the absorption of X-ray photons by an atom usually produced using a synchrotron source and the measurement of the emitted fluorescence intensity. Briefly, X-ray absorption spectra can be obtained using intense X-ray beams by tuning the photon energy around the absorption edge of a specific element. When the energy of the X-ray beams are scanned across the precise binding energy of individual core electron in an atom, the absorption is abruptly increased. The analysis of oscillations in X-ray absorption versus photon energy that are led to the scattering of the X-ray excited photoelectrons reveals local structural information on the absorbent element. According to the X-ray energy, the X-ray absorption spectrum can be divided into two regions: the edge region and the higher edge region. The former of the spectrum referred to as XANES provides information primarily about oxidation number, and geometry, whereas the latter of the spectrum referred to as EXAFS provides the structural information regarding coordination number, identity of ligand atoms, and bond distance. The measurement of the transmitted photons through the sample is often conducted in XAS, but the fluorescence mode is selected when measurement is performed in diluted analytes due to its higher sensitivity. Owing to the poor selectivity, this technique is appropriate for the identification of a limited number of dominant species present by comparing with the standard of the oxidation state or coordination center. It is generally used for the confirmation of the presence of predicted species in a sample, but it is proved to be difficult to identify new species.

The recent development of hard XAS optics with more powerful focused beams has successfully applied to the single cell, particularly by μ -XANES [74]. This allows the direct speciation analysis of elements in subcellular compartments without cell fractionations which might lead to the alteration of chemical species. Detection limits of chemical species using synchrotron XAS microprobe are typically 100 ppm [75].

2.4.2 Examples of application for arsenic speciation

Suess *et al* (2009) demonstrated the use of XANES to discriminate thioarsenites, and thioarsenates from arsenite and arsenate in sulfidic water [76]. The absorption near edge energies measured for the synthesized thioarsenate standards were found to decrease in the order: arsenate (11872.3 eV) > thioarsenates > arsenite (11868.2 eV) > thioarsenites, as illustrated in Fig 2.10. Interestingly, the As K-edge energies from mono- (11871.3 eV) to di-(11870.3 eV) to tetra-thioarsenate (11869.3-11869.8 eV) decreased in a row. The accuracy of the determination of the As-K edge energies was approximately 0.3 eV, which preclude the misidentification of thioarsenate species from arsenite and arsenate. They indicated that the shift of the K-edge energies reflects the more covalent character of As-S bond compared to the As-O bond. The EXAFS spectra showed bond distances of As^V-S and As^V-O in thioarsenates (2.13-2.18 Å and 1.70 Å, respectively) obviously shorter than As^{III}-S and As^{III}-O in thioarsenites (2.24-2.34 Å and 1.78 Å), with error ± 0.02 Å. This study also showed the sensitivity of EXAFS inferior to XANES; the limits of quantification for XANES and EXAFS were estimated to be 0.5 mmol As L⁻¹ and 5 mmol As L⁻¹, respectively.

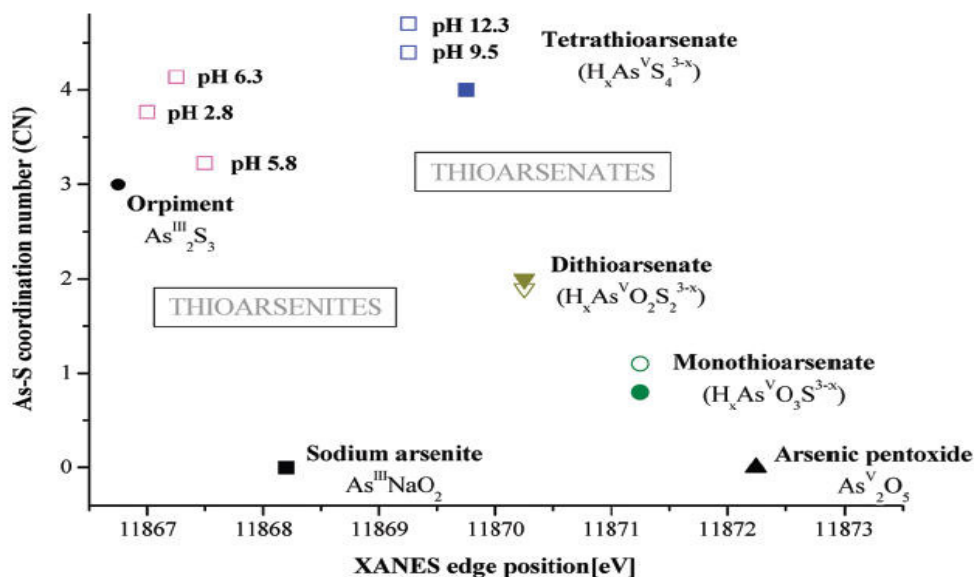


Figure 2.10 Arsenic K-edge XANES edge position versus EXAFS-derived As-S coordination number [76].

In metallomics approach, XAS is useful to ensure there is no degradation of the original species occurring following sample handling and to investigate the oxidation state such as As(III) and As(V), which are readily converted during any extraction steps. Bluemlein *et al* (2008) used XANES and EXAFS to confirm that formic acid can be used for quantitative extraction of arsenic peptides in plants without significant decomposition or *de-novo* formation using HPLC-ICP-MS [28]. The XAS data showed that 53% of total arsenic in the root of *T. alata*, freshly exposed to 1 mg arsenic L^{-1} as arsenate, were As(III) bound to sulfide of proteins, whereas 38% and 9% were arsenite and unchanged arsenate, respectively. Due to the low levels of arsenic in the sample ($<10 \text{ mg kg}^{-1}$), signal accumulation with the signal to noise ratio good enough for EXAFS analysis was achieved by increasing the number of scans (8 scans of 45 min each). The EXAFS data enabled the identification of the occurrence of As-S (at $2.27 \text{ \AA}^0$) and As-O bonds (at $1.75 \text{ \AA}^0$) in the plant roots. These XAS results were found to be in agreement with those obtained by LC-ICP-MS/ESI-MS.

2.5 METALLOMICS APPROACH FOR ARSENIC SPECIATION STUDIES

2.5.1 Sample collection/ storage

As arsenic can exist in various forms (+5, +3, 0 and -3) on an oxidation-state basis, it is easily prone to transforming into another one. In order to obtain reliable results, well-organized procedures for sampling, storage and sample pretreatment are of paramount importance. Sampling is the first step in the analytical process and involves a number of factors such as geological location, ecosystem and season. It is necessary to plan and design specific protocols, which will avoid changes in oxidation state, changes to induced microbial activity and losses by volatilization or adsorption [7]. The bacteria naturally existing in samples play a significant role in altering species forms in a biological sample, since they can transform inorganic arsenic to methylated species. Storing samples at low temperature is required to minimize any bacterial and enzyme degradation. Methods for water removal by freeze drying prior to being homogenized have been used occasionally to facilitate sample handling and to preconcentrate the sample. The selection of storage materials is important to prevent the possible wall effects, which can cause adsorption or losses of analyte. Sample containers such as acid-washed glass, PTFE or polyethylene containers have been observed not to be subject to losses. However, the residual nitric acid used for rinsing many types of containers can lead to oxidation of arsenic species. The pretreatment of samples with nitric acid needs to be carefully considered because it can cause a change in the species present [77].

One of the most important aspects in analytical procedure is to remove contaminants attached to samples prior to the analysis. Following harvesting and separating plants into different parts (e.g. root, shoot and leaves), many studies followed Meharg and MacNair (1992) [78] by washing roots with 10 mM potassium hydrogen phosphate and distilled water, grinding under liquid nitrogen and sub-sampling before extraction [27-29, 82]. In algae work, the detection of low signals of some arsenic species such as AsB may derive from epiphytes or bacteria [79, 80]. It is therefore important to ensure all possible artifacts removed in order to minimize the risks of erroneous results or misinterpretation. Epiphytes can be removed by knife milling and sieving [80], or by scraping with razor blades [81, 82] or tweezers [83]. Algae samples are subsequently rinsed with deionized water or ethanol to remove epiphyte and checked by optical microscopy [80]. The study on thio-arsenosugars in marine algae pronounced the importance of cleaning that higher levels of thio-

arsenosugars were detected in the unbrushed samples [84]. Before homogenization, thermal treatments (room temperature to 60°C, 18-48 h) and freeze-drying are commonly used for eliminating water in algae samples.

Limited information on appropriate storage conditions for arsenic speciation is available. The interconversion of As(III) and As(V) in water samples was commonly known to be matrix dependent, rapid and randomly interconvert [85, 86]. Without additives, Bednar *et al* (2002) reported As(V) and As(III) in pure water, ground water and mine drainage samples remained stable under light and dark condition for at least 24 h; however, after 4 days, As(V) were entirely converted to As(III), which was believed to be ascribed to microbial activity [87]. They reported the addition of EDTA and H₂SO₄ can effectively preserve the identity of As(III) and As(V) for up to 5 days. However, using the Eh-pH diagram (Fig 2.1), it should be aware of the possibility of acid reduction from As(V) to As(III). Hall *et al* (1999) suggested that natural water samples, which kept at 5°C can preserve 20 µg L⁻¹ of As(III) and As(V) concentrations for 1 month [88]; at lower levels, it was recommended to keep aqueous solutions in the dark at 4°C [89]. The transformation of AsB in marine sample matrix to TMAO and two other species following 4°C storage for 9 months was also reported [90].

2.5.2 Sample preparation

Due to the complexity of real-world samples, low levels of analytes and often the presence of chemically labile species, appropriate sample preparation is regarded as a critical step in any speciation procedure. In order to preserve the natural distribution of a multitude of metallospecies, the sample preparation step should be therefore performed under “mild” conditions. It is clear that no single extraction method is appropriate for all matrices and all arsenic species; the extraction of species of interest to obtain the highest recovery still remains challenging.

2.5.2.1 Terrestrial plants

Water extraction using sonication and heating, enzymatic hydrolysis, and microwave digestion have been occasionally reported for the arsenic extraction from terrestrial plants. The extraction efficiency is dependent on plant structures, the degree to which they are macerated, the species under investigation and extraction methods. Bohari *et al* (2002) investigated the use of varying concentrations of water-methanol mixtures

(1:9, 1:1, 9:1 (v/v)) in terrestrial plants and found the capability of extracting arsenic decreased with increasing methanol contents [91]. It was thought that the majority of arsenic in terrestrial plants mainly occurs as inorganic forms. Yuan *et al* (2005) revealed that water-MeCN with shaking, offered satisfactorily extraction efficiencies for As^{III} and As^{V} from *Oryza sativa L*, while the extraction of MMA^{V} and DMA^{V} required water-EtOH to achieve similar extraction efficiencies [24]. Water-MeCN was also considered to give acceptable extraction using ultrasonic extraction. Using microwave assisted extraction, the most desirable extraction medium tested was water-EtOH, although higher efficiencies were found for As^{V} using water-MeCN. However, the use of microwave extraction and sonication for the determination of total arsenic with water-EtOH provided efficiencies of 85.4 and 49.2%, respectively. Mattusch *et al* (2006) compared efficiencies from enzymatic extraction to water extraction to determine As species from *Tropaeolum majus* [92]. It was found that the use of enzymes makes it possible to digest cellulose and other constituents of cell walls, leading to a higher concentration of As species. However, the amount of extracted arsenic was much lower than the total. Further development of the enzymatic extraction needs to be carried out.

The method and solvent utilized for the extraction of arsenic species is of paramount importance for maintaining species integrity. The stability of trivalent arsenic-glutathione species ($\text{As}^{\text{III}}(\text{GS})_3$, $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{DMA}^{\text{III}}(\text{GS})$) extracted from *Holcus lanatus* by sonicating and leaching for 24 hr at 4°C was investigated [93]. Sonication at ambient temperature of the previously cooled sample with water-MeOH (1:1), water-MeCN (1:1) or 1% formic acid caused disintegration of $\text{DMA}^{\text{III}}(\text{GS})$, suggesting its instability in this complex. $\text{As}^{\text{III}}(\text{GS})_3$, $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{DMA}^{\text{III}}(\text{GS})$ were all present in the extract when leaching method was applied. The highest recoveries obtained using water-methanol and water-acetonitrile were in the range of 75-85%. Moreover, results also suggested the storage at 4°C after extraction and early measurement is required for successful detection. Difference in As extraction yields when applied to different plant areas have also been reported [25]. Arsenic extraction from *Pteris vittata* revealed recoveries of 85-100% for most parts of the plants by water-methanol, except for the roots where a recovery of 60% was found [25]. Sample preparation involving freezing under liquid N_2 (to break cell walls) with immediate homogenization has also been reported to increase extraction efficiency. Extraction with tetramethylammonium hydroxide (TMAH) revealed recoveries of

~90% total As. However, a milder extraction medium is required for speciation analysis as the ability of TMAH to reduce or destroy the integrity of the species has been reported, especially As-S species. Montes-Bayon *et al* (2004) found that the use of 25 mM ammonium acetate is suitable for As extraction from *Brassica juncea*, and no significant differences in total extracted arsenic at different pH values, providing recoveries ranged from 47-60% [94]. The extraction techniques employed for arsenic speciation analysis in terrestrial plants are illustrated in Table 2.3.

The storage of the fresh plant extracts was tested. It was found that arsenic peptide complexes were stable in 1% formic acid at 1°C for 3 days; however, they were degraded even at -20°C [95]. Furthermore, kinetic disintegration studies showed that As-PC complexes are more stable than arsenic glutathione complexes [95]. It was indicated that the arsenic glutathione complex has short half life being only a few hours, while As^{III}-PC₃ is considerably more stable with 23 h half-life [96]. The stability of the trivalent arsenic glutathione species was reported to decrease with the decreasing number of thiol groups in the order As^{III}-(GS)₃ > MA^{III}-(GS)₂ > DMA^{III}-(GS) [93, 97]. Nevertheless, the partially purified As^{III}-(GS)₃ standard following the storage at 1°C for 2 months remained stable [95].

Table 2.3 Techniques employed for the extraction of arsenic species from terrestrial plants.

Plant Species	Extraction Technique	Extractant	As Species Extracted	Extraction Procedure
<i>Brassica juncea</i> [94]	Leaching	25 mM ammonium acetate buffer (pH 4.4, 5.6 or 7.8)	As-PC species	0.15 g ground material dissolved in 2 mL extractant, ultra-centrifuged for 30 min at 4°C and filtered ^a
	N/A	N/A	As-PC species	Sap collected for 1 h by decapitating plant just above the root. Sap, roots and leaves were frozen in liquid nitrogen immediately after collection ^b
<i>Arabidopsis thaliana</i> [30]	Leaching	1% formic acid	As ^{III} -thiol complexes	1 g plant material to 2 g extractant, extracted for 1 h at 4°C
<i>Oryza sativa L</i> [24]	Sonication	H ₂ O, MeCN 1:1 H ₂ O:EtOH 1:1	As ^{III} , As ^V , MMA ^V , DMA ^V	1 g sample and 10 mL extractant were vortex mixed, sonicated for 30 min and centrifuged for 15 min. This was repeated twice more with 5 mL extractant

	Shaking		As ^{III} , As ^V , MMA ^V , DMA ^V	1.0 g sample and 20 mL extractant were shaken by horizontal rotator shaker for 6 h, centrifuged and the supernatant removed. 5 mL extractant was added to the residue and shaken for further 2 h.
	Microwave assisted extraction		As ^{III} , As ^V , MMA ^V , DMA ^V	1 g sample and 10 mL extractant were microwave digested. Programme: increase from room temp – 80°C over 15 min, remain at temp for 5 min and then cool down room temp over 10 min. Samples centrifuged, the supernatant removed and 5 mL water added and extraction process repeated twice more. All extractants were combined prior to analysis
<i>Tropaeolum majus</i> [92]	Enzyme assisted	Viscozyme 0.1% (v/v)	As ^{III} , As ^V	Fresh plant treated with 10 mL acetate buffer (pH 5.4) and 10 µL of extractant and horizontally shaken for 24 h
	Water extraction	Distilled Water	As ^{III} , As ^V	2 g fresh plant hacked and suspended in 10 mL extractant and horizontally shaken for 24 h
<i>Pteris vittata</i> [25]	Sonication	MeOH:H ₂ O (1:1)	As ^{III} , As ^V	10 mg sample, 5 mL extractant sonicated for 2 h. 2 extraction cycles were conducted. Residue was rinsed with H ₂ O and added to extractant ^c
<i>Cucumis sativus</i> L [98]	N/A	N/A	DMA, As ^{III} , As ^V	Sap collected with micropipettes for 1.5 h into PE vessels immersed in an ice bath and diluted with H ₂ O prior to analysis
<i>Holcus lanatus</i> [93]	Leaching	MeOH:H ₂ O (1:1) MeCN: H ₂ O (1:1)	As ^{III} (GS) ₃ , MA ^{III} (GS) ₂ , DMA ^{III} (GS)	Leaching for 24 h at 4°C

^a extraction efficiencies ranged from 47-60% compared to microwave digestion.

^b detection by x-ray spectroscopy.

^c extraction efficiencies of 85-100% compared to microwave digestion.

The use of trifluoroacetic acid for the extraction of arsenic species from plant (rice [99]) samples should be applied with caution as reduction of As^V to As^{III} can occur [100]. Oxidation of As^{III} to As^V was also found by ASE methods [24]. ASE is reported to result in low extraction efficiencies when samples mainly contained inorganic arsenic and extraction by these methods have been found not appropriate for arsenic speciation studies [24]. Koch *et al* (2000) suggested that following 1:1 MeOH/water extraction, non-extracted arsenic, which was greater than 50% of the total, was possibly strongly bound to lipids, cytosolic proteins and/or to cell walls

components such as lignin, pectin and cellulose [101]. In order to improve extraction efficiencies, enzymatic extraction with the use of α -amylase for apples [102], Viscoenzyme[®], used for digestion of cellulose and other cell wall constituents, for algae and terrestrial plants [92], and trypsin and pancreatine for baby food [103] were employed. The application of a microwave assisted treatment with modified protein extraction at 90°C for 20 min was reported to provide complete extraction efficiency for a variety of freeze-dried plant materials [104]. However, in shoot and root materials of *Holcus lanatus* and *Arabidopsis thaliana*, which were grown in arsenic contaminated medium, the results showed the presence of most of arsenic existed as inorganic arsenic with a negligible amount of MMA and DMA. This might be seen as resulting in a loss of speciation information. The use of sequential extraction has been proposed to apply in yeast [105, 106] and garlic [107] for characterizing elemental bindings among different origins (e.g. water-soluble, cell wall bound, and protein bound).

2.5.2.2 Marine algae

The vast majority of arsenic speciation studies in seaweed have emphasized a water-soluble fraction. Water and varying concentrations of methanol mixtures have been occasionally used for extracting water-soluble arsenic species. Despite the fact that the use of water can extract arsenic species in fish tissues almost completely because most of arsenic species are arsenobetaine, it does not provide the effective recoveries in marine algae; occasionally lower than 80% of the total extracted arsenic is achieved [8]. One of the most widely used extracting agents in marine algae, mixtures of methanol/water have been proposed to use with varying extracting conditions such as sample size and extracting volume ratios, extraction methods (shaking, sonication, MAE and ASE), extraction time and temperature to achieve good extraction efficiency [108]. Using a chemometric approach, the studies on factors affecting the arsenic extraction from macroalgae were reported that there were two critical factors such as solvent composition and sample mass, while extraction temperature and time did not significantly influence [109]. Shibata *et al* (1992) reported that 85-100% of arsenic can be successfully extracted in samples of oyster and red and brown green using a methanol/water extraction, but not for blue-green algae (only 34% arsenic could be extracted) [110]. It is important to point out that most studies have used extraction in MeOH:H₂O based on the misconception that all organoarsenicals will

prefer methanol to water as an extraction solvent because they are organic. However, most arsenic species identified so far are polar and very water-soluble (some are hygroscopic) and they would mostly favour water over methanol as an extraction solvent [86]. Rubio *et al* (2010) suggested that the difficulties of designing extraction procedures are accountable for the differences in morphology, structural complexity, and chemistry among a variety of algae (green, brown, or red) [108]. On this basis, studies for gaining insights into these parameters are required to develop appropriate well-defined protocols for the specific species extraction of algae. Furthermore, Francesconi *et al* (2003) commented that the selection of extraction conditions should be considered accordingly to the polarity of the target species, since the use of single extraction procedure to achieve complete extraction efficiency from biological samples seems to be unrealistic [111]. The incomplete arsenic extraction obtained from methanol or methanol/water might suggest a possibility of the presence of arsenolipids in algae, which requires less polar solvents such as chloroform and acetone. The presence of arsenosugar 1 as a major hydrolyzed arsenic species bound to algae lipids were reported in chloroform extracts of *Undaria pinnatifida* [112] and *Laminaria digitata* [57], suggesting the structure of phosphatidylarsenosugars. Despite the fact that hydrolysis of lipid-bound or protein-bound fractions may provide information associated with speciation, direct analysis of arsenolipids is thought to be more feasible to prove their identities [113]. An example of the use of a sequential extraction scheme consisting of three main fractions: non-polar, polar and inorganic arsenic species to obtain high As recoveries was proposed as in dry seafood products [114].

The stability of four arsenosugars investigated under the treatment with TMAH in different conditions indicated that arsenosugar 4 was substantially more labile than the other three arsenosugars; it was found to be almost entirely degraded to DMA [115]. A slight degradation of arsenosugars isolated from algae sources over time were found [116]. Madsen *et al* (2000) found arsenosugars 2 and 4 can hydrolyze to arsenosugar 1 in solution [117]. It is thought that microbial activity in the extracts may be accountable for the degradation. They recommended the frozen storage of dry extracts, although storage as solution at 20°C for 2 days did not alter arsenic speciation results. They reported that freeze-dried extracts stored at 60°C for 10 days can maintain the stability of arsenosugars. The brief descriptions of extraction techniques for arsenic speciation analysis in algae are summarized in Table 2.4.

Table 2.4 Techniques employed for the extraction of arsenic species from marine algae.

Algae species	Extracting solutions	Extraction methods	Species extracted
<i>Undaria pinnatifida</i> <i>Sargassum Fulvellum</i> <i>Sargassum piluliferum</i> <i>Hizikia fusiformis</i> <i>Pelvetia wrightii</i> <i>Myelophycus simplex</i> [118]	H ₂ O	Sonication for 15 min (extraction efficiency: 9.3-91.2%)	As(III), As(V), AsC, AsB, MMA, DMA, TMAO, TeMA, Arsenosugars2-4
<i>Ceramium sp.</i> <i>Gelidium sp.</i> <i>Cystoseira barbata</i> <i>Enteromorpha sp.</i> <i>Fucus virsoides</i> <i>Padina pavonica</i> <i>Polysiphonia sp.</i> <i>Ulva rigida</i> [119]	H ₂ O	Shaking overnight (extraction efficiency: 20.7-97%)	As(III), As(V), AsB, DMA, Arsenosugars1-4
<i>Fucus vesiculosus</i> [84]	H ₂ O	Sonication for 1 min	Thio-arsenosugars1-4
Commercially available marine algae “kelp”, Kelp, <i>Laminaria digitata</i> [120]	H ₂ O	Shaking for 10 min (extraction efficiency: 86-92%)	Thio-arsenosugars1-4
<i>Chlorella vulgaris</i> , <i>Hizikia fusiformis</i> <i>Laminaria digitata</i> [121]	H ₂ O	Microwave assisted extraction at 90°C for 5 min (extraction efficiency: 78-88%)	As(III), As(V), MMA, DMA
<i>Phyllophora Antarctica</i> <i>Iridaea cordata</i> [80]	1:4 (v/v) MeOH/H ₂ O	Shaking top over bottom for 40 h (extraction efficiency: 68-115%)	As(V), AsB, DMA, Arsenosugars1-2
<i>Alaria marginata</i> <i>Sargassum muticum</i> [122]	30:70 MeOH/H ₂ O	ASE, 500 psi, ambient temperature, 1 min heat step, 1 min static step, 90% vol. flush, 120 s purge (extraction efficiency 25.6-72.6%)	As(III), As(V), DMA, Arsenosugars1-4
<i>Porphyra</i> [123]	1:1 (v/v) MeOH/H ₂ O	Sonication for 15 min (repeat twice; extraction efficiency: 90-96%)	Arsenosugars1-2
<i>Laminaria</i> [124]	1:1 (v/v) MeOH/H ₂ O and 9:1 (v/v) MeOH/H ₂ O	Sonication for 3 h	Arsenosugars1-4
<i>Zostera sp.</i> [125]	1:1 (v/v) MeOH/H ₂ O, followed by 2% (v/v) HNO ₃	Microwave assisted extraction at 70°C for 15 min (repeat 3 times), and at 95°C for 6 min	As(V), Arsenosugar 1

Brown algae Kelp powder [126]	1:1 (v/v) MeOH/H ₂ O	Shaking for 12 h at room temperature	Arsenosugars
<i>Porphyra</i> <i>Hizikia fusiforme</i> [127]	3:1 (v/v) MeOH/H ₂ O	Homogenization for 2 min with a dispersion unit	As(V), DMA, Arsenosugars1-4
<i>Undaria pinnatifida</i> <i>Laminaria sp.</i> <i>Hizikia fusiformis</i> [128]	0.25 M H ₃ PO ₄	Shaking for 12 h	As(III), As(V), MMA, DMA, Arsenosugar1-3
<i>Hijiki fuziforme</i> [129]	Three extracting solutions: 9:1 (v/v) MeOH/H ₂ O, 1.5 M H ₃ PO ₄ and H ₂ O	All extractions performed under a cross-shaped rotor at 45 rpm at 25°C for 14 h (extraction efficiencies for H ₃ PO ₄ , MeOH/H ₂ O, and water were 76%, 33%, and 65%, respectively)	As(III), As(V), DMA, Arsenosugars1-4
<i>Undaria pinnatifida</i> [112]	Lipid extraction: 1:1 (v/v) CHCl ₃ /MeOH	Immersion for 2 days, Hexane/acetonitrile separation for chloroform fraction, and alkaline digestion	Arsenosugar2
<i>Laminaria digitata</i> [57]	Lipid extraction: 2:1 (v/v) CHCl ₃ /MeOH	Sonication, separation and enzymatic hydrolysis of lipid fraction with phospholipase D	As(III), As(V), DMA, Arsenosugar1
<i>Dunaliella tertiolecta</i> , <i>Phaeodactylum</i> [130]	Lipid extraction: 2:1 (v/v) CHCl ₃ /MeOH Water extraction: hot water	Lipid soluble fraction: vortex and rotation for 4 h Water-soluble fraction: extraction for 1 h	Lipid-soluble fraction: As(V), DMA, arsenosugar1 Water-soluble fraction: As(V), MMA, DMA, arsenosugars1-2
<i>Posidonia australis</i> [82]	Lipid extraction: 2:1 (v/v) CHCl ₃ /MeOH Water extraction: hot water (100°C)	Lipid extraction: as described in [130] Water extraction: as described in [130] (extraction efficiencies were 20-31% and 13-20% for lipid soluble and water soluble fractions)	Inorganic As, AsC, AsB, DMA, Trimethylated glycerol arsonioribose Arsenosugars1-2
Brown algae (from the Yellow sea) [114]	Step1 (non-polar As): acetone Step2 (polar As): 1:1 (v/v) MeOH/H ₂ O Step3 (Inorganic As): HCl	Sequential extraction by shaking for 5 min, sonication for 20 min, centrifugation (repeat 3 times, extraction efficiency 92%)	As(III), As(V), AsB, DMA

2.5.3 Hyphenated techniques for arsenic speciation analysis

In metallomics research, high performance liquid chromatography (HPLC) has been proved to be a powerful separation technique since it offers high resolution and suitably for low-volatile species in ultra-trace levels, whereas gas chromatography has a limited use in some cases, e.g. determination of sulfur- and selenium-containing amino acids (after derivatization), or of volatile sulfur and selenium species. Due to the high complexity of biological samples containing a large number of metallospecies, multi-dimensional chromatography, i.e. at least two (liquid-based) chromatographic approaches, is often employed to achieve the selectivity needed for a sufficient signal to noise ratio for species identification by ESI-MS/MS. Size-exclusion chromatography (SEC) is often first used prior to a finer separation with anion exchange (AE) or reversed phase (RP).

2.5.3.1 Terrestrial plants

The reported work on As speciation analysis of terrestrial plants has employed chromatography (size exclusion, anion/cation exchange and reversed phase) in combination with either ICP-MS or HG-AFS for element-specific detection. The chromatographic methodology employed for such analysis is dependent upon the plant type and species under investigation. The selection of chromatographic conditions is of paramount importance to maintain integrity of arsenic glutathione species. Work conducted by Raab *et al* (2004) reports disintegration of the $\text{As}^{\text{III}}(\text{GS})_3$, $\text{MMA}^{\text{III}}(\text{GS})_2$, $\text{DMA}^{\text{III}}(\text{GS})$ using ion chromatography with carbonate or formate buffers [93]. However, all species found were stable and well separated when utilizing reversed phase chromatography with formic/acetonitrile gradient. Moreover, $\text{As}^{\text{III}}(\text{GS})_3$ and $\text{MMA}^{\text{III}}(\text{GS})_2$ were found to be more stable than $\text{DMA}^{\text{III}}(\text{GS})$, showing a slight degradation with a column temperature of 25°C and much more stable when the temperature reduces to 6°C. Using a normal mode ICP-MS, the analysis of As^{III} -PCs or free PCs *via* their sulfur is troublesome due to the high detection limits of sulfur caused by its low ionization efficiency and the considerable background arising from O_2 (m/z 32 and 34). Several strategies to overcome these problems and to allow sulfur detection such as the use of Xe (a heavy collision gas), the use of HR-ICP-MS, and the use of a desolvation system were discussed [28, 131]. The individual methodologies which have been employed for these analyses are illustrated in Table 2.5.

Table 2.5 Separation and detection techniques employed for arsenic speciation analysis in terrestrial plants.

Plant Species	Separation	Detection	As Species	Column Type	Mobile Phase
<i>Brassica juncea</i> [94]	Size-Exclusion	ICP-MS	As-PC species	TSK Gel-2000 GSXW 300 × 7.8 mm	25 mM ammonium acetate at pH 4,4 ^a , 5.6 and 7.8.
<i>Arabidopsis thaliana</i> [30]	Reversed phase	ICP-MS ESI-MS (Orbitrap)	As ^{III} -thiol complexes	Ace 5 C18 4.6 x 250 mm x 5 µm	Gradient of 0.5% formic acid and 0.5% formic acid in methanol; up to 20% MeOH in 20 min
<i>Thunbergia alata</i> [28, 96]	Size-Exclusion	ICP-MS	As-PC species (GS)As ^{III} PC ₂	Superdex 75	25 mM ammonium acetate (pH 5.6)
	Reversed phase	ICP-MS ESI-MS	As ^{III} (PC ₂) ₂ As ^{III} PC ₃ As ^{III} (PC ₄) (GS)As ^{III} PC ₂	Ace 5 C18, 250 x 4.6 mm x 5 µm	Gradient of 0.5% formic and 0.5% formic in methanol up to 20% methanol within 20 min
<i>Holcus lanatus</i> [93]	Reversed phase	ICP-MS ^b ESI-MS ^c	As ^{III} (GS) ₃ , MMA ^{III} (GS) ₂ , DMA ^{III} (GS)	Spherisorb S5 ODS2, 250 × 4.6 mm	A = 0.1% formic in H ₂ O, B = MeCN Gradient: 0-20 min up to 5-30% B, then 10 min 5 % B 1 mL min ⁻¹
<i>Oryza sativa L</i> [24]	Anion Exchange	HG-AFS	MMA ^V , DMA ^V , As ^{III} , As ^V	Dionex IonPak AS11 250 × 4.0 mm	A = H ₂ O, B = 100 mM NaOH. 0-5min, 90% A, 10%B. 5-6min from 90% to 0% A, from 10% to 100% B. 6-10 min, 100% B. 10-11 min from 0% to 90% A, from 100% to 10% B. 1 mL min ⁻¹ .
<i>Tropaeolum majus</i> [92]	Anion Exchange	ICP-MS	As ^{III} , As ^V	IonPak AG7/As7 250 × 4 mm	Gradient elution of 0.4 mM HNO ₃ , and 50 mM HNO ₃
<i>Pteris vittata</i> [25]	Anion Exchange	ICP-MS HG-AFS	As ^{III} , As ^V	PRP X-100, 250 × 4.6 mm x 10 µm	Potassium phosphate (0.015M for KH ₂ PO ₄ and KH ₂ PO ₄), pH 5.9 1 mL min ⁻¹
<i>Cucumis sativus L</i> [98]	Anion Exchange	HR-ICP-MS	As ^{III} , DMA ^V , MMA ^V , As ^V	PRP X-100, 250 × 4.6 mm x 10 µm	20 mM NH ₄ H ₂ PO ₄ pH 5.6 1.5 mL min ⁻¹
	Cation Exchange	HR-ICP-MS	As ^V , As ^{III}	LC SCX-100, 250 × 4.1 mm x 10 µm	25 mM pyridine pH 2.7 1.5 mL min ⁻¹

^a 101% As recovery (calculated by mass balance).

^b addition of 3% oxygen to plasma gas.

^c post column detection by their molecular peaks (M+H)⁺ or (M+2H)⁺.

2.5.3.2 Marine algae

A fast separation of arsenicals such as As(III), As(V), MMA and DMA was demonstrated by Wangkarn and Pergantis using of a narrow bore reverse-phase HPLC column with ion pairing to reduce sample size and solvent consumption [132]. McSheehy and Szupunar (2000) proposed the use of two-dimensional chromatography for fractionation of organoarsenic species and matrix removal by first separation with size-exclusion column, followed by AE-HPLC prior to detection of arsenosugars with ICP-MS and ESI-MS/MS [133]. Anion-exchange mechanisms using Hamilton PRP-X100 with carbonate or phosphate buffers as mobile phase have occasionally been used for the separation of As(III), As(V), MMA, DMA and oxo-arsenosugars [118, 134, 135], while cation exchange can be used to separate the cationic arsenic species such as AsB, AsC, TeMA and TMAO [136, 137]. However, sodium or potassium phosphate buffers are generally not desirable due to the residue left upon the sampler cone of the ICP-MS, while ammonium carbonate has become more popular owing to little residue left with low detection limits (17-29 pg As g⁻¹) [138]. Little signal drift following the prolonged use of ammonium carbonate buffered anion-exchange systems was observed [139]. Kohlmeyer *et al* (2002) [140] developed method based on Londesborough *et al* (1999) [103] using anion-exchange column (Ionpak AS7) with nitric acid eluents and benzene 1,2-disulfonate as ion-pairing agent. This method allows cationic arsenic to form negatively-charged ion pairs with benzene 1,2-disulfonate. As a result, the separation of cation species could be retarded by the column, allowing the determination of anions and cations in a single chromatographic run. However, Francesconi and Kuehnelt commented that there were several suspects of the results from this method, which remains to be justified such as reported matrix effects and peak assignments [86]. In order to achieve the separation of a series of thio-arsenosugars, which require up to 40% MeOH in the mobile phase for conventional AE chromatographic conditions, Nischwitz *et al* (2006) proposed to use reversed phase anion pairing HPLC with a low methanol content (5%) in 5 mM tetrabutylammonium hydroxide (TBAH) for ICP-MS detection [120]. Gradient elution using the conventional AE column with 20 mM NH₄HCO₃ by increasing the concentration of methanol up to 40% was used for the detection of 50 organoarsenic species including thio-arsenosugars by ESI-MS/MS [141]. Chromatographic conditions for separating a range of arsenic species present in marine algae are summarized in Table 2.6.

Table 2.6 Separation and detection techniques employed for arsenic speciation analysis in marine algae.

Authors	Separation	Detection	As Species	Column Type	Mobile Phase
Wangkarn and Pergantis (2000) [132]	Reversed phase narrow bore	ICP-MS	As(III), As(V), DMA, MMA, p-arsanilic acid and 4-hydroxyphenyl arsenic acid	Discovery C18, 2.1 x 150 mm	5 mM tetrabutylammonium hydroxide (pH6), 0.7 mL min ⁻¹
McSheehy and Szpunar (2000) [133]	Size-Exclusion	ICP-MS	Organoarsenicals	Superdex Peptide HR 10/30, 300 x 10 mm x 13 µm	1% acetic acid (pH 3), 0.6 mL min ⁻¹
	Anion Exchange	ICP-MS ESI-MS	Four oxo-arsenosugars, As(III), As(V), AsB, DMA, MMA	Supelcosil SAX1, 250 x 4.6 mm x 5 µm	5 to 30 mM phosphate buffer (pH 6) within 22 min, 1 mL min ⁻¹
Van Hulle <i>et al</i> (2002) [134]	Anion Exchange	ICP-MS ESI-MS	Four oxo-arsenosugars, As(III), As(V), DMA	Hamilton PRP-X100, 250 x 4.1 mm x 10 µm	(1) 20 mM (NH ₄) ₂ HPO ₄ (pH7) (2) 20 mM NH ₄ HCO ₃ (pH7.5) (3) 20 mM NH ₄ HCO ₃ (pH7.7) in 20% MeOH 1 mL min ⁻¹
	Cation Exchange	ICP-MS	Oxo-arsenosugar I, 3 and DMA	Dionex Ionpak CS-10 4 x 50 mm x 10 µm	20 mM pyridine (pH2.4) 1 mL min ⁻¹
Kohlmeyer <i>et al</i> (2002) [140]	Anion Exchange	ICP-MS	17 different As species such as As(III), MMA, DMA, As(V), AsB, TMAO, AsC, TeMA and oxo-arsenosugars	Dionex IonPak AS7, 250 x 4.0 mm with IonPak AG7, 50 x 4.0 mm guard column	Mop A: 0.5 mM nitric acid and Mop B: 50 mM nitric acid, Both are in 0.05 mM benzene-1,2-disulfonic acid dipotassium salt, 0.5% MeOH, gradient elution, 1 mL min ⁻¹
Wahlen (2004) [135]	Anion Exchange	ICP-MS	As(III), As(V), AsB, MMA, DMA	Hamilton PRP-X100, 250 x 4.1 mm x 10 µm	2.2 mM (NH ₄) ₂ CO ₃ and 2.5 mM tartaric acid with 1%MeOH (pH8.4), 1 mL min ⁻¹
Nischwitz <i>et al</i> (2006) [120]	Reversed phase	ICP-MS	Oxo-arsenosugars and thio-arsenosugars	Discovery C18, 4.6 x 150 mm x 5 µm	5 mM tetrabutylammonium hydroxide (TBAH) in 5% MeOH (pH7.5), 1 mL min ⁻¹
Nischwitz and Pergantis (2006) [141]	Anion Exchange	ESI-MS	50 organoarsenic species including 4 thio-arsenosugars	Hamilton PRP-X100, 250 x 4.1 mm x 10 µm with two cation exchange pre-columns (PRP-X800)	Mop A: 20 mM NH ₄ HCO ₃ (pH10) Mop B: 20 mM NH ₄ HCO ₃ with 40% MeOH (pH10), gradient elution, 1 mL min ⁻¹

Wuilloud <i>et al</i> (2006) [142]	Anion Exchange	ICP-MS ESI-MS	Oxo-arsenosugars, As(III), As(V), DMA, MMA	Dionex Ionpak AS7, 250 x 4.0 mm with IonPak AG7, 50 x 4.0 mm guard column	Mop A: 25 mM NH ₄ HCO ₃ (pH10), Mop B: Deionized water, gradient elution, 0.8 mL min ⁻¹
	Cation Exchange	ICP-MS	Oxo-arsenosugars	Hamilton PRP- X200, 150 x 4.1 mm x 10 µm	4 mM pyridine (pH2.4) with formic acid, gradient elution, 0.8 mL min ⁻¹
Hirata and Toshimitsu (2007) [118]	Anion Exchange	ICP-MS	Four oxo- arsenosugars	Hamilton PRP- X100, 250 x 4.1 mm x 10 µm	20 mM NH ₄ HCO ₃ (pH 8.4), 1 mL min ⁻¹
	Reversed phase	ICP-MS	As(V), MMA+As(III), DMA, AsB, TMAO, AsC, TeMA	Capcell Pak C18 ODS, 250 x 4.6 mm x 5 µm	2 mM malonic acid, 0.3 mM 1-butanedisulfonate sodium and 5 mM 1-hexanesulfonic sodium in 0.5% methanol (pH 3.0), 1 mL min ⁻¹

For speciation studies, excellent sensitivity of element-specific detection is regarded as one of the most desirable factors. There are presently more than a few spectroscopic techniques capable of coupling to chromatography such as Flame atomic absorption spectrometry (FAAS), Graphite furnace atomic absorption spectrometry (GFAAS), Atomic fluorescence spectrometry (AFS) and Inductively coupled plasma mass spectrometry (ICP-MS). FAAS gives the highest LODs in the level of ppm, which are not sufficient to exploit in speciation studies, which require at least ppb levels. Even though GFAAS provides relatively higher sensitivity, it is found that a long analytical cycle, which consists of drying, ashing and atomizing steps, is required. It is therefore difficult for continuously monitoring detection. Similarly, although the sensitivity obtained from AFS technique is considered to be excellent, it needs a high intensity excitation source, which is currently not commercially available [143]. In addition, special treatment of samples is required to generate volatile hydrides for the purpose of elimination of the matrix and improvement of sensitivity, but with the limitation of hydride generation in the case of arsenic speciation, some organoarsenicals such as AsB, AsC, TeMA and arsenosugars do not form AsH₃. Consequently, samples sometimes need to be irradiated by UV rays [144-146] or microwave digested [147] to degrade unachievable forms before hydride generation. Also, the efficiency of hydride generation can be influenced by the matrix. Therefore, it is recommended to perform determination using standard additions in comparison with external calibration to check for possible matrix-induced

interferences. Amongst element-specific detectors, ICP-MS has received the most considerable attention for speciation studies because it offers not only detection limits at the part-per billion and part-per-trillion levels, but also multi-elemental and multi-isotopic capability. Linearity of the calibration obtained from ICP-MS technique is usually 4-11 orders of magnitude, while merely 1-2 orders of dynamic range are normally observed in other traditional spectrometric techniques. Furthermore, the coupling of chromatography to ICP-MS is straightforward.

In bioinorganic speciation analysis, however, not only the identification of the inorganic moiety, but also the characterization of the organic composition is required. Since ICP-MS is a destructive method, the elucidation of the structural information is therefore not possible. ESI-MS is normally used for structural elucidation of metal complexes, characterization and quantification of small organometallic species after multi-dimensional separation. This technique is ideal to detect metal(loid) containing biomolecules and able to study metal(loid) accumulation in plant or animal tissues, due to the softness of the ionization process that allows labile complexes to be transferred intact into the gas-phase [148]. Identification of arsenic species has been conducted using either retention time matching with standards by HPLC-ICP-MS or organic mass spectrometry such as ESI-Q-TOF-MS/MS following fraction collection and preconcentration. Since retention time matching only is not sufficient for full identification, the use of ESI-MS/MS is required. Prior to the use of ESI-MS/MS for identifying such species, the prerequisite is to clean up sample, which is regarded as time consuming and challenging. The development of more promising methods for better pretreatment procedures and improvement of the sensitivity of ESI-MS/MS including the selectivity of chromatographic separations for ICP-MS detection is necessary. Arsenic species in *Brassica juncea* which have been identified are illustrated in Table 2.7.

Table 2.7 Identification of species detected in *Brassica juncea* by ESI-Q-TOF-MS after As accumulation [94].

Sample Introduction	Species identified	Structure/Molecular formulae	Molecular Ion and Fragmentation upon Collision Induced Dissociation
Infusion of pre-concentrated fractions collected <i>via</i> HPLC-ICP-MS	PC ₂	C ₁₈ H ₂₇ N ₅ O ₁₀ S ₂	m/z 538.14 (m/z 540) ^a = [M+H] ⁺ m/z 409.10 (loss of Glu) m/z 334.06 (loss of Glu-Gly) m/z 306.06 (loss of Glu-Cys) m/z 212.86 (loss of Glu-Cys-Gly)
	PC ₃	C ₂₆ H ₃₉ N ₇ O ₁₃ S ₃	m/z 770 (m/z 772) ^a
	PC ₄	C ₃₄ H ₄₈ N ₉ O ₁₈ S ₄	m/z 1000.26 (m/z 1004) ^a = [M+H] ⁺ m/z 925.22 (loss of Gly) m/z 871.23 (loss of Glu)
	As-PC ₄	As bound to three of the SH groups of PC ₄ and one free SH	m/z 1076.173 (m/z 1076.145)

^a protonated molecular ions were expected at m/z 540, 772 and 1004 for PC₂, PC₃ and PC₄, respectively. However, due to an internal disulfide bridge formation (internal oxidation), different molecular ion masses were found.

Although the coupling of LC to ICP-MS (or ESI-MS) allows us to operate in a continuous-mode for on-line detection, the presence of low concentrations of analytes in high levels of matrix requires preconcentration/matrix-removal step for the successful detection. Using size-exclusion, followed by fraction collection and freeze-drying, off-line approach by two-dimensional separation has frequently been applied to overcome these problems. However, species degradation has occasionally been found in certain labile metal(loid) complexes. For example, Bluemlein *et al* (2009) reported that the use of freeze-drying process caused the degradation of most As-PC₃ complexes (90%) in plant extracts [96]. Therefore, other sample-handling methods need to be considered. For such unstable metal(loid) species, the on-line hyphenated technique is preferred.

2.6 ANALYTICAL QUALITY CONTROL IN SPECIATION ANALYSIS

2.6.1 Method validation

Studies of speciation analysis by HPLC-mass spectrometry deals with three steps: quantitative extraction, effective separation and selective detection of species. The information on the mass balance and preservation of species identity is of paramount importance. Extraction efficiency is estimated by establishing a mass balance of analyte of interest. This involves the quantification of amount of extracted analyte plus that found in the extracted sample residue against total amount of analyte in the

intact sample. Extraction efficiency can inform what extents all extracted metal species from the solid sample (biota) transfer into a liquid solution, while chromatographic recovery can imply what extents the non-eluting element species remain in the column. Chromatographic recovery can be expressed as a percentage of the total quantity of arsenic eluting from the column and the quantity of arsenic actually injected onto the column.

Although the question of species preservation during sample preparation and analysis is not easily approached, spiking the species of interest in the extraction solution with and without sample are required to verify that chemical species are not altered during all the steps of speciation analysis. Information on identification and quantification of the species by HPLC-ICP-MS can lead us to calculate possible species transformation in the course of extraction. The use of isotopically labeled species is considered to be beneficial for species integrity testing.

The comparison of retention times of species standards with that of analyte peaks may result in the wrong assignment, because the sample matrix often affects the retention time slightly. Spiking of sample with standards and the use of at least two different chromatographic methods are therefore necessary for preliminary identification.

2.6.2 Inter-laboratory comparisons

Inter-comparison studies cope with the collaboration of different laboratories to determine one or more chemical species in a common sample to compare the results from a range of methods used in the different laboratories [8]. It is accepted that acquiring a good statistical agreement from inter-comparison studies can suggest the best approximation of true values. These studies are proved to be useful for determining systematic errors, particularly contributed by analytical procedure. However, there have been scarce reports on the inter-laboratory comparisons, with the exception of several on certification campaigns for producing CRM marine animals. One report on the inter-laboratory trial on determination of water soluble arsenic species in five algae samples using BCR 279 Sea lettuce as a QC sample was found [149]. For this trial, all participants used the same extraction protocol and followed the guidelines for the storage condition of extracts. The arsenic speciation analysis was performed by HPLC-ICP-MS following the internal method of the individual

laboratory. In this sense, variability of extraction efficiency, separation techniques, including species identified and quantified were discussed.

2.6.3 Certified reference materials

Certified reference materials (CRMs) are required for validating a method and demonstrating the accuracy of measurement. One or more property values, expressed on a certificate with an uncertainty at a stated level of confidence, are certified by procedures, which establish traceability to an accurate realization of the unit [8]. Although a significant number of CRMs with arsenic values for the total measurement are commercially available, the number of existing speciated CRMs is limited. Moreover, the current number of arsenic-species standards is in fact relatively much lower than that of arsenic species found in real samples. Such standards, once properly characterized, are needed for species quantification and for confirmation of species identity. For validation of speciation methods in real samples, it is essential to perform the analysis of matrix reference materials certified for arsenic species. Examples of CRMs commercially available for total arsenic and arsenic speciation are listed in Table 2.8. A table listing all the existing CRMs per matrix per element for speciation analysis is available on the EVISA website, www.speciation.net. There is an urgent need to develop more speciated CRMs in a range of matrix particularly for marine algae and terrestrial plants.

One of the greatest challenges for producing CRMs for arsenic speciation in a biological matrix is that results obtained largely rely on the analytical method used. Due to the lack of speciated algae CRMs, a homogeneous extract of *Fucus serratus* algae was prepared for quality assessment to facilitate analysts to gain benefits from assessing the quality of extraction and quantification of four oxo-arsenosugars in algae extracts [117]. The comparative results of arsenic speciation analysis with independent different techniques in the homogeneous extract from *F. serratus* are presented in Table 2.9.

Table 2.8 List of selected certified reference materials used to check the accuracy of total arsenic and arsenic speciation analysis [7, 108].

Matrix	BCR626	BCR627	BCR710	DORM-2	BCR279	IAEA-140/TM
	Solution	Tuna fish	Oyster tissue	Dogfish muscle	<i>Ulva lactuca</i>	<i>Fucus sp.</i> Plant homogenate
Total As (mg kg ⁻¹)	-	4.8 ± 0.3	25.7 ± 2.7	18 ± 1.1	3.09 ± 0.20	44.3 ± 2.2
DMA (mg kg ⁻¹)	-	2 ± 0.3 μmol kg ⁻¹	0.82 ± 0.18	-	-	-
AsB (mg kg ⁻¹)	1031 ± 6	52 ± 3 μmol kg ⁻¹	33 ± 7	16.4 ± 1.1	-	-
TeMA (mg kg ⁻¹)	-	-	-	0.248 ± 0.054	-	-

Table 2.9 Reported results on arsenic speciation in the homogeneous extract from *Fucus serratus* [117] in comparison with those obtained by independent different techniques.

Arsenosugar	Means arsenic contents in μg g ⁻¹ (% RSD)			
	HPLC-ICP-MS [117]	HPLC-ESI-MS [117]	HPLC-HG- ICP-MS [150]	HPLC- thermooxidation- HG-AFS [61]
Arsenosugar 1	0.10 (4.8%)	0.088 (21%)	0.098 (4.1%)	0.098 (1.6%)
Arsenosugar 2	0.086 (2.9%)	0.075 (2.7%)	0.082 (6.5%)	0.084 (0.4%)
Arsenosugar 3	0.62 (3.8%)	0.57 (2.2%)	0.58 (5.3%)	0.65 (3.4%)
Arsenosugar 4	0.40 (3.1%)	0.41 (2.6%)	0.39 (5.9%)	0.39 (2.5%)
DMA	0.005 (20%)	-	-	-
Arsenate	~ 0.001	-	-	-
unknown	~ 0.01	-	-	-

Mean values (RSD%) on the extract.

Total As determined by ICP-MS: 1.22 μg g⁻¹ (3.2%), and HGAAS: 1.27 μg g⁻¹ (2%).

2.7 ANALYTICAL CHALLENGES

Despite the fact that the complementary use of HPLC-ICP-MS and ESI-MS/MS is considered to be the most promising means to obtain full information of elemental speciation, the majority of studies have always dictated by “analytical convenience”, which most of species of interest in samples should have to be extracted into an aqueous phase to be amenable to chromatographic separation and ICP-MS detection. The differences of physicochemical properties of individual species such as solubility, polarity and electrical charge including their chemical labile make it difficult to obtain high recovery in a single step. In order to extend a range of metal species, at least two extracting solutions (polar, and non-polar) are needed to extract water-soluble and lipid-soluble species. The main challenges of current speciation methods include the extraction of compounds from the complex matrix, for which there is a tradeoff between extraction efficiency and preservation of compound entity. Pretreatment procedure offering selective and effective extraction of the elemental species without causing instabilities of species is required for the quantification and characterization for elucidation of metabolic route of the elements in biological system. In addition, special care for the selection of the separation conditions is required to avoid species transformation and to ensure the compatibility of the column effluents with ICP-MS operation. Appropriately multi-dimensional chromatographic systems are needed for complicated samples. Furthermore, the low concentration of target compounds demands for enhancement of the detection capability (sensitivity and selectivity) of mass spectrometric detection, which are the most desirable for ultra-trace elemental speciation analysis. The lack of standards and reference materials required to valid the quantitative speciation method is also a remaining challenging. Since arsenic is a mono-isotopic element, its accurate quantification by primary methods (e.g. IDMS) is not possible. It is important to ensure whether standard addition with available standards is the most adequate calibration technique or external calibration would be fit for purpose. Further efforts should be pursued to understand the impact of toxic elements, which is of special concern to human health.

CHAPTER 3

INSTRUMENTATION

The different analytical instruments that were employed in the experimental work are briefly described in this chapter. Most of instrumentation was based in LGC Science & Technology Division (Teddington, UK). However, sample preparation for the *in vitro* dialysis and collecting mainstream smoke total particulate matter (TPM) were carried out in other laboratories. In order to estimate the bioaccessibility of arsenic, the simulated *in vitro* gastrointestinal procedure was carried out in the Department of Analytical Chemistry, University of Santiago de Compostela, Spain. For smoke TPM collection using a smoking machine and an impaction trapping device, the procedure was conducted by the British American Tobacco company (BAT).

3.1 EXTRACTION TECHNIQUES

In order to extract arsenic species from a sample matrix, a variety of organic solvents of different polarity and extraction systems have been performed. The ultimate goal of the extraction method must extract arsenic species from the matrix in a quantitative manner without altering their chemical and physical forms. Sample extraction procedures should be tailored to the specific application and goals of the study. Extraction methods vary with individual study depending on a range of factors such as whether the target analyte is incorporated into the matrix and stored in the biological tissues, or loosely bound to the matrix by adsorption to particulate matter, and how stable the analyte of interest in the matrix is. Owing to the complex chemical matrix of real-world samples, low concentration of the target, and often the presence of chemically labile species, suitable sample preparation is regarded as a critical step in any speciation procedure. To preserve the natural distribution of a multitude of arsenic species in samples, sample extraction/ pretreatment should be therefore carried out under relatively “mild” conditions. The extraction of species of interest with the highest recovery therefore remains challenging. Specific instrumentation employed for the various applications in this work is described below.

3.1.1 Sonication-Assisted Extractions

Emerging as an alternative to rapid extraction methods, sonication-assisted extraction has occasionally been preferred over the simple extraction, which requires a long mechanical shaking time. In principle, the ultrasonic electric generator inside the sonication device produces intense, high frequency signal (usually around 20 kHz) that powers a transducer. This transducer converts the electric signal into a mechanical vibration *via* piezoelectric crystals. The vibration can be directed to liquids resulting in intimate mixing and strong chemical and physical reactions. This causes chemical effects through the phenomenon of cavitation, creating microscopic bubbles into the solution and producing shock waves, when a large negative pressure is applied to it. Sonicators are commonly used in laboratories for a range of purposes. They are usually employed to disintegrate compounds or cells for further examination. When sonication is applied to a suspension of solid particles, particle disruption can occur, which leads to an increased surface area for reaction and accelerating the ability of the extractant to leach metals.

3.1.2 Microwave-Assisted Extractions

Unlike conventional heating techniques where heat is transmitted by conduction (e.g. heating blocks and hot plates), in theory, the use of microwave-assisted techniques, which heat and extract a sample in a microwave field, offer uniform and directed heating of sample solution. However, the microwave field is occasionally non-uniform and localized superheating occurs, owing to uneven absorption by the object being heated. Microwave-assisted digestion/extraction utilizes non-ionizing radiation which causes the migration of ions and rotation of dipoles. Different compounds can convert microwave radiation to heat by different amounts.

Microwaves are electromagnetic waves with frequency from 300 MHz to 300 GHz, but 2.45 GHz is the most commonly used for microwave-assisted sample preparation to avoid the interference with typical telecommunication wavelengths [151]. The behavior of microwaves is different when they pass through a substance. Microwaves can be reflected from solid metals, pass through certain plastics and glass, or be absorbed in sample solutions such as water and acid. Teflon PFA vessels are generally favoured for microwave digestion systems, because they are transparent to microwave radiation and inert to mineral acids at high temperatures, allowing very rapid heating and cooling times compared with conventional oven steel-jacketed

digestion bomb [152]. Liquids heat rapidly when exposed to microwave energy, since polar molecules and ions are energized through two mechanisms: dipole rotation and ionic conduction [152]. Absorptive substances are excited and act by molecular motion, whereas free ions present in the solution are moved through the electromagnetic field.

Principally, a microwave vessel containing a known amount of sample and an extracting solution is heated under microwave conditions. The behavior of both sample and extracting solution in the electromagnetic field play an important role in determining the extraction efficiency achievable. However, this approach is not appropriate for compounds which are easily transformed under microwave conditions.

There are two types of microwave extraction systems: open- and closed-vessel systems. Both systems allow users to choose the maximum power supplied, duration of extraction, and maximum temperature used. In open-vessel systems, the digestion pressure cannot be controlled, and therefore the principle process for the extraction is to supply heat to a sample solution in the electromagnetic field, whereas closed-vessel systems are occasionally used for accelerating the extraction or decomposition owing to the accumulating pressure in a sealed microwave vessel. Therefore, the latter provides a relatively higher extraction temperature than the former. Following the extraction, the mixture of sample and extracting solutions are centrifuged and filtered in order to separate the supernatants from the solid matter prior to the analysis.



Figure 3.1 CEM Focused MicrowaveTM Synthesis System, Model Discover.

Microwave-assisted extraction/ digestion of samples were carried out in a compact closed-vessel microwave system (CEM Focused MicrowaveTM Synthesis System, Model Discover) as shown in Fig 3.1. Generally, this system allows a sample

to be digested/ extracted within 10 minutes. A known amount of sample together with an acid/ extracting solution are added to a 10-mL pressurized Pyrex[®] vessel (max 300 psi) with a snap-on cap. Only a single sample can be digested/ extracted at any one time, thereby allowing full pressure and temperature control (ensure reproducible reaction conditions). In this system, automated power control is based on temperature feedback. The microwave condition used for both digestion and extraction can be defined according to maximum temperature and pressure, power supply as well as extraction duration. Actual microwave conditions used are described in more details in the relevant sections (4.2.1 and 6.4.2).

A Paar Physica Multiwave (Anton Paar, Perkin Elmer, Beaconsfield, UK) closed vessel microwave system was used for the digestion of tobacco leaves (Section 6.2.1). This system can accommodate six high-pressure quartz or teflon vessels.

3.2 CHROMATOGRAPHIC SEPARATION TECHNIQUES

Following extraction, a variety of arsenic species are separated from the sample matrix in order to enable quantification of the individual species. In elemental speciation analysis, the sample pretreatment has occasionally been avoided since any clean-up before the actual measurement may lead to selective loss or the change of concentration of some arsenic species and therefore affect the original pattern of speciation. However, clean-up techniques such as filtration, centrifugation, liquid-liquid extraction [153], solid-phase extraction [154-157], and chromatography [133, 158-161] have been employed.

3.2.1 Liquid Chromatography

Liquid chromatography is based on a column packed with a stationary phase and a mobile phase used as an eluent, which is continuously passed through the stationary phase by a pump. When the sample solution is introduced at the beginning of the column, the sample containing the different species is eluted along the stationary phase by the mobile phase used. The interaction between the sample components and stationary phase depend on their chemical and/or physical properties, which vary from polarity to size. Two phases are chosen so that the sample components distribute themselves between the mobile and stationary phases to varying degrees to achieve efficient separation for a large range of analytes.

Liquid chromatography is the most popular technique for the separation of arsenic species, as they are generally readily soluble and non volatile in aqueous solution. Although the use of gas chromatography of derivatized arsenic species is possible, extra steps in the analytical procedure can lead to erroneous results. Due to the high possibility of species misidentification in real-world samples, bi-, tri-, or multidimensional chromatography depending on the number of individual steps is often employed [133, 158, 160]. Such clean-up steps are necessary for most sample matrices, particularly when organic mass spectrometry is used as the detection method.

3.2.2 LC instrumentation

A modern LC apparatus is equipped with degassers which may consist of a vacuum pumping system, a distillation system, a device for heating and stirring. Prior to the HPLC separation, removal of dissolved gases and dust from the mobile phase is required, because they can lead to irreproducible flow rates and band spreading. The mobile phases, generally organic solvents or aqueous solutions, are used to deliver to the analytical column by means of single, dual or quaternary pumps, which are capable of achieving pumping pressures of several hundred atmospheres to achieve reasonable flow rates. The elution of sample components can be accomplished by either isocratic elution (with a single solvent or solvent mixture of constant composition), or gradient elution (at least two solvent systems that differ significantly in polarity used and vary in composition during the separation).

Column dimensions can vary from 5 to 25 cm long. However, sometimes length can be extended by coupling two or more columns. Columns are however typically 10-15 cm long, 4.6 mm id, and packed with 5 μm particles, generating 40,000 to 70,000 plates.metre⁻¹. In order to reduce the volume of solvent used, microcolumns have become available which are 3 cm to 7.5 cm long, 1 mm to 4.6 mm in internal diameters, 3- to 5- μm particles, which achieve as many as 100,000 plates.metre⁻¹. A short guard column is sometimes coupled before the analytical column in order to prevent not only particulate matter and contaminants from reaching the analytical column, but also sample components that bind irreversibly to the stationary phase. Although the most widely used detectors for HPLC are based on absorption of UV-Vis radiation, mass spectrometry detectors such as ICP-MS and MS, MS/MS have

gained popularity especially for trace analysis, where high sensitivity is required or to gain molecular structure information for characterization purposes.

Various chromatographic mechanisms may be used together in some applications. In elemental speciation analysis, the choice of column selection is of paramount importance. The metal-ligand bond in elemental species has to be much stronger than its interaction with the column stationary phase, in order to prevent the alteration of the original species or the undesirable formation of artifact species on the column. In order to separate HMW compounds (greater than 10 kDa), size exclusion chromatography is most frequently used owing to its high tolerance to the sample matrix; however, it is possible to cope with such compounds by reversed-phase chromatography. An additional benefit is that it is primarily used for fast species screening of biological extracts for the presence of stable metal(loid) complexes. For LMW ionic species, ion-exchange chromatography is extensively used due to the presence of ionic functional groups on stationary phases, while reversed-phase chromatography employing packing materials (usually containing a covalently bound C₈ or C₁₈ linear hydrocarbon) principally free of ligands for metal(loid)s is appropriately applied for the separation of metal(loid) containing biomolecules. Owing to problems with retention reproducibility and irreversible adsorption, adsorption chromatography with solid stationary phases has been replaced by normal-phase (bonded-phase) chromatography for separating non-polar species and structural isomers.

An Agilent Technologies 1200 HPLC systems (Palo Alto, CA USA) was used for this research. The HPLC is equipped with a dual pump, a vacuum de-gasser, a temperature-controlled autosampler with positions for up to 100 vials, and a heated column compartment. Chapters 4.4.1, 4.4.3, and 5.4.2 contain more details on the actual liquid chromatographic separations used including mobile phase compositions and analytical columns.

3.3 ELEMENTAL DETECTION : INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS)

The ICP-MS is regarded as one of the most important techniques for elemental (speciation) analysis, because it offers several advantages over other traditional detectors such as multi-element and multi-isotopic detection and more importantly high degree of selectivity with reasonably good precision and accuracy. The majority

of ICP-MS applications involve the analysis of liquid samples, where ICP torch serves as an atomizer and ionizer. In ICP-MS, the sample matrix could seriously affect the elemental determination when sample solutions are analyzed at the high concomitant concentrations about 500 to 1,000 mg mL⁻¹. These effects often lead to a reduction in analyte signal, despite the fact that signal enhancement is observed under certain circumstances. In order to minimize these effects, the sample solution should be diluted or offending species separated from the matrix. Dissolved solid levels have to be carefully controlled for the analysis, typically no greater than 0.2% to avoid matrix deposition on the spectrometer interface. In addition, these effects can be compensated for by the use of appropriate internal standards, which has the mass and ionization potential similar to those of the analyte.

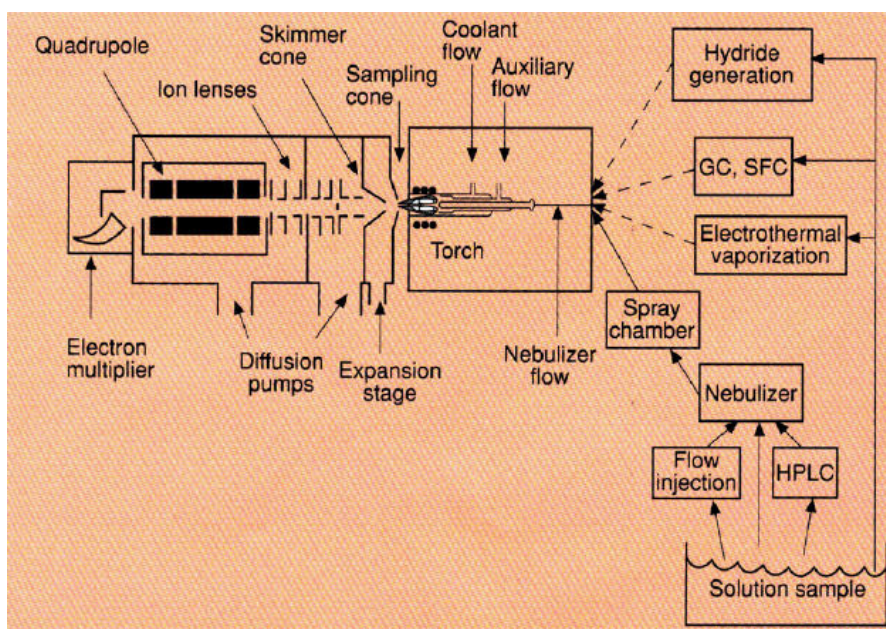


Figure 3.2 Schematic diagram of an ICP-MS system. Dotted lines show introduction of gaseous samples; solid lines show introduction of liquid samples [162].

3.3.1 Sample Introduction System

The components of a commercial ICP-MS system with a selection of sample introduction procedure for the ICP-MS analysis are schematically shown in Fig 3.2. For aqueous samples, the solution is normally pumped at $\sim 1 \text{ mL min}^{-1}$ via a peristaltic pump and introduced into the central channel gas flow, as an aerosol of fine droplets using a nebulizer, to be efficiently ionized in the plasma. Using mechanical forces of gas flow (normally argon at a pressure of 20-30 psi), the pneumatic nebulizer, by far the most common design for ICP-MS, is often chosen. Pneumatic nebulizers include concentric, microconcentric, microflow and crossflow are extensively used, depending on specialized applications. Due to the limited capability of plasma to dissociate large droplets, a spray chamber is used not only to remove droplets produced by the nebulizer that are larger than $8 \text{ }\mu\text{m}$ in diameter, but also to smooth out pulses that occur during the drainage process. Two configurations of spray chambers are used in ICP-MS instrumentation: Scott double pass and cyclonic spray chambers. The former, based on the selection of the small droplets by directing the aerosol into a central tube, is by far the most common, because it is generally considered as the most rugged design for the routine use. Some ICP-MS spray chambers are maintained at low temperature (-5°C to 5°C) depending on the application in order to minimize the solvent load on the plasma, especially volatile organic solvents and to allow more energy to decompose matrix and to facilitate the ionization of analyte (to reduce the formation of oxide species). The sample-introduction part is considered to be the weakest component of the instrument because only 1-2% of the sample is able to pass into the plasma.

3.3.2 Inductively Coupled Plasma (ICP)

The ICP, generated by coupling energy from a radio frequency generator to a suitable gas (usually Ar) by means of a magnetic field, is a high energy and effective ionization source. Depending on the region in the plasma, the plasma typically operates at temperatures of 5,000 to 10,000 K at the atmospheric pressure. A flow of argon carries very fine droplets of sample into an ICP torch, where vaporization, atomization and ionization of the analyte take place nearly simultaneously. The extent of the ionization is a function of the first ionization potential of the element relative to that of argon (15.76 eV). This has a great influence in a number of factors such as sensitivity and susceptibility to certain sample matrix effects. Under hot argon plasma

conditions, more than 90% of the elements in the periodic table can be determined by ICP-MS and singly charged ions are primarily produced at yields ranging from 5% to 100%.

The most important part of the instrument is the interface that couples the ICP to the mass spectrometer. In order to extract analyte ions from the ICP-MS as efficiently as possible to the mass spectrometer, a sampling interface is used to allow the quantitative transfer of ions from atmospheric pressure ($\sim 1,000$ mbar) environment of the ICP into the spectrometer at greatly reduced pressure (10^{-5} - 10^{-9} mbar) under which conditions mass analysis and detection of the analyte ions take place. This coupling is achieved by the interface comprising two cones: sampler and skimmer cones, made out of nickel or platinum. The sampler cone is in direct contact with the hot plasma gas, where ions are sampled through the cone orifice (< 1.0 mm) by the vacuum generated by a rotary pump, which is connected to the interface. In this region, rapid expansion of the gas occurs, leading to being cooled. The analyte ions are subsequently transmitted into the analyzer chamber, maintained at the pressure of the mass spectrometer *via* a small hole of the skimmer cone. In this stage, the positive ions, isolated from electrons and molecular species by a negative voltage, are accelerated, and then focused by magnetic ion lens prior to the entrance orifice of a quadrupole mass analyzer.

3.3.3 Quadrupole Mass Analyzers

Based on a mass to charge (m/z) ratio, quadrupole mass analyzer in the ICP-MS provides high scan rates so that an entire mass spectrum can be obtained in less than 100 msec [163]. The ions entering the mass analyzer are almost exclusively singly charged and equal to the selected m/z ion. As shown in Fig 3.3, quadrupole mass analyzer consists of four cylindrical rods serving as electrodes, which are arranged in two pairs [164]. An electric field is generated in-between the rods by the combination of variable radio-frequency alternating current (AC) and direct current (DC) voltages applied to each pair. In order to obtain a mass spectrum, ions are accelerated into the space between the rods by a potential difference of 5 to 10 V and the AC and DC voltages on the rods are increased simultaneously while maintaining a constant ratio.

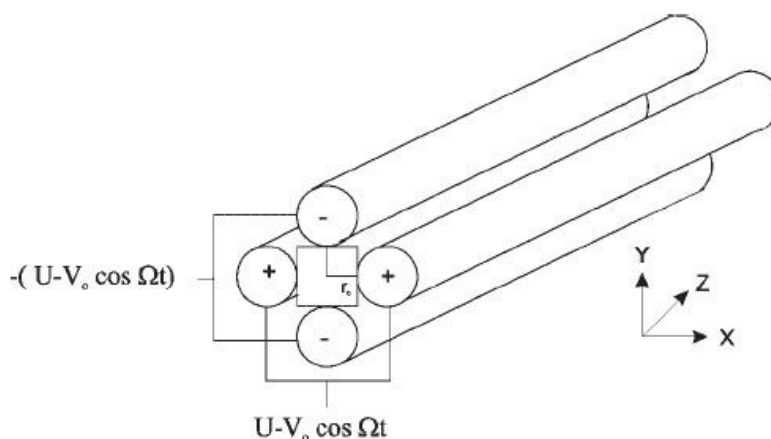


Figure 3.3 Quadrupole mass filter. The ions enter and travel in the z-direction, while oscillating in the x-y plane. The oscillation is controlled by the DC (U) and RF (V) potentials applied to each pair of rods [164].

At this stage, all of the ions with the exception of those having a certain m/z value strike the rods and are transformed into neutral molecules, whilst only ions of certain m/z reach the detector. Typically, quadrupole mass analyzers are capable of easily resolving ions that differ in mass by one unit. The advantages of the quadrupole mass analyzers are that they are compact, less expensive, and more rugged than most other types of mass spectrometers.

3.3.4 Collision and Reaction Cells (C/RC)

In spite of its high sensitivity, ICP-MS suffers from chloride interference from the sample matrix for the determination of arsenic. The chlorine combines with argon to form polyatomic $^{40}\text{Ar}^{35}\text{Cl}$ and $^{38}\text{Ar}^{37}\text{Cl}$ ions having the same nominal mass-to-charge ratio as ^{75}As . Several strategies are used to avoid spectral interferences, although the use of alternative isotope for As determination is hampered by being a mono-isotopic element. In order to overcome these interferences, a mathematical correction approach can be used by monitoring the signal at m/z 75, where the signal is derived from two sources, the arsenic and the argon chloride ions. In addition, argon-based interferences can be reduced by the use of a high resolution ICP-MS or cold plasma conditions for a quadrupole instrument. However, cold plasma conditions are not appropriate to employ with elements having high ionization potentials such as arsenic and selenium. Although offering sufficient resolution to overcome many polyatomic ions, the cost of high resolution ICP-MS is prohibitive. Moreover, any increase in mass resolution has

to be paid for by a decrease in ion transmission. The introduction of ICP-MS equipped with a collision/reaction cell (C/RC) is another approach to minimize interfering ions *via* interactions or chemical reactions using gases such as He and H₂ or their combination.

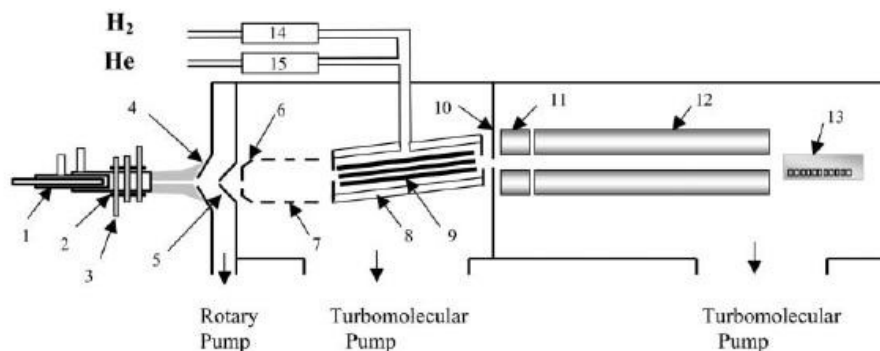


Figure 3.4 Schematic diagram of the ICP-MS with octopole reaction cell. 1, Plasma torch; 2, grounded thin metal; 3, induction coil; 4, sampler cone; 5, skimmer cone; 6, ion lens; 7, triple cylinder Einzel lens; 8, reaction cell chamber; 9, octopole; 10, ion lens; 11, pre-filter; 12, analyzing quadrupole; 13, detector; 14 and 15, mass flow controllers [165].

The collision/reaction cell (quadrupole, hexapole, or octopole) is generally based on the pressurization of a multipole inserted between the ion lens and quadrupole filter, with a gas or mixture of gases to minimize interfering polyatomic species by collisional dissociation or more commonly by ion-molecule reactions. Furthermore, the collision/reaction cell can increase the efficiency of the ion transmission by collisional focusing. The cut-away schematic diagram of the ICP-MS with octopole reaction cell is illustrated in Fig 3.4. The He-mode ICP-MS employs an inert gas (He) to minimize all polyatomic species based on their size. Due to their larger cross section (size) than analyte ions, all polyatomics suffer more collisions with inert gases such as He or Xe. This leads to the reduction in mean kinetic energy of the ions, as they pass through the pressurized region. Therefore the polyatomic species, having distinctly lower kinetic energy than the analyte ions, are prevented from leaving the cell exit by the selection of suitable energy discrimination (ED) voltage at the cell exit, allowing only analyte ions to enter the analyzer. This separation mechanism is known as kinetic energy discrimination (KED).

The C/RC ICP-MS used in his study was an Agilent Model 7500ce (Agilent Technologies, UK) equipped with an octopole reaction cell. The octopoles have a small internal diameter; therefore the cell entrance and exit apertures are narrow. This allows the cell to operate at relatively higher pressure compared to quadrupole or hexapole cells. Octopoles facilitate increased collisions of polyatomic ions with the inert gas, thus reducing the number of polyatomic ions, which exit the cell. In addition, octopoles also provide better focusing efficiency than hexapoles or quadrupoles, since the ion beam is tightly focused, resulting in greater ion transmission.

Another advantage of the use of inert gases is the absence of newly formed interfering species and freedom from analyte signal reduction through loss by reaction. Therefore, the use of an inert-collision gas may be appropriate for variable and complicated sample matrices, where the identity of any potential interfering species is not known. The use of He as the inert collisional gas in an octopole-based C/RC ICP-MS has been applied to the removal of unidentified polyatomic species arising from chloride, sulfide and carbon-based matrices [166].

In order to remove polyatomic ions especially argon-based polyatomic species, the use of hydrogen as a reactive cell gas in combination with cell multipole voltage settings is recommended. Reaction gases such as hydrogen, methane and ammonia are utilized to react with argon ions and neutralize them *via* charge, proton, electron or hydrogen atom transfer. These gases preferably react more rapidly with argon ions rather than with the analyte ions. Therefore, the separation mechanism for chemically removing interferences from the analyte mass is known as ion molecule reaction. Since the use of reactive gas may affect the analysis in each individual sample, it requires a high degree of awareness of the generation of newly formed polyatomic species in the cell gas. The cell conditions need to be set up specifically for each analyte in each sample so as to exclude the new interferences from the mass spectrum.

The optimization of C/RC ICP-MS to minimize polyatomic interferences is performed using a mixture of 50 ng g⁻¹ of As and Se in 500 µg g⁻¹ of chloride for the simultaneous determination of As and Se. A 500 µg g⁻¹ of chloride solution is assigned to a blank solution for optimizing to keep minimal effects of ArCl⁺ and ArAr⁺.

3.4 MOLECULAR DETECTION (1): ELECTROSPRAY IONIZATION – ION TRAP – MS/MS

3.4.1 Electrospray Ionization

Unlike the ICP, electrospray ionization (ESI) is a considerably softer ionization source capable of producing gas-phase ions of thermally stable and non-volatile compounds with little fragmentation. Performed under atmospheric pressures, the electrospray ionization is based on the electro-nebulization of a sample solution, which is introduced to the ion source at a rate of a few microliters per minute *via* a stainless steel capillary needle operating at a high electrical potential. The liquid is ejected from the needle where evaporation of the solvent and attachment of charge to the analyte molecules occur. The droplets become smaller as a result of the evaporation of solvent by a gas stream (e.g. nitrogen), while their charge density becomes greater until leaving ionized droplets. Little fragmentation of large and thermally degradable biomolecules occurs due to a little retained energy by the analyte upon ionization.

The ionized droplets can be detected as either positive or negative ions, depending on the chemical properties of the solvents and modifiers. Volatile substances of a mobile phase, a solvent, and a modifier need to be used in order to avoid in-source blockages, which lead to a serious reduction in signal intensities and signal to noise ratios and then affect the accuracy of results.

3.4.2 Ion Trap Mass Spectrometry

The quadrupole ion trap is a three dimensional analogue of the linear quadrupole mass analyzer, in which gaseous anions or cations can be formed and confined by electric and magnetic fields. As illustrated in Fig 3.5, the quadrupole ion trap comprises a central ring electrode, and a pair of end-cap electrodes [164]. The two end-cap electrodes are grounded, whereas the ring electrode is variably applied with radio-frequency voltage. In the linear quadrupole, stable motion of the appropriate m/z ions are allowed freedom in one dimension, but in the ion trap, the ions are confined and consistently circulated within the system of the three electrodes. When a radio-frequency voltage is supplied, the orbits of heavier ions become stabilized, but those of lighter ions become destabilized, thereby colliding with the wall of the ring electrode.

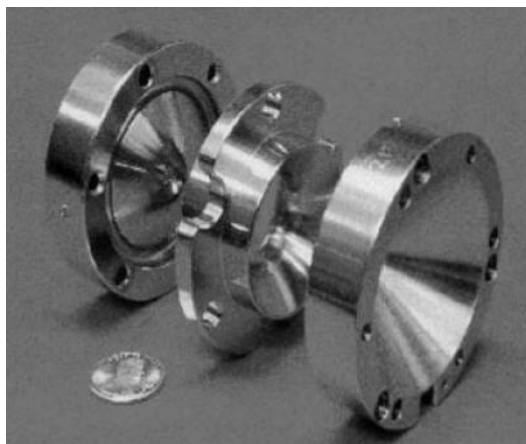


Figure 3.5 The three electrodes of the quadrupole ion trap shown in open array [164].

In chemical analysis, the advantages of the quadrupole ion traps are that (1) they provide high sensitivity, (2) ions of high mass to charge are accessible using resonance experiments and (3) tandem mass spectrometry can be performed using sequential analysis measurement.

With the advent of the quadrupole ion trap, it is possible to perform CID MS/MS experiments directly in the trap through resonant excitation, followed by collision with helium buffer gas atoms, in order to gain information about the observed CID fragments. This can be achieved by the use of a sequence of operations in the scan function. The sequence starts with ionization and the selection of precursor (parent) ions. All other ions are ejected from the trap. By applying an additional rf voltage to the end cap, the precursor ion is excited and then fragmented with the help of helium buffer gas. Practically, the energy required in the fragmentation varies from 10 to 90% of the total available collision energy and can be optimized to preserve the signal of precursor ions in the order of 5 to 10%. Following the CID of these excited ions, the product ions are recorded by scanning the rf voltage to perform the next mass-analysis scan. Using a quadrupole ion trap, the specificity is improved, because a significant reduction in signals arising from other matrix components is eliminated.



Figure 3.6 Agilent Technologies 6300 Ion Trap Mass Spectrometer.

The ESI-MS analysis in edible marine algae was performed with an Agilent Technologies 6330 Ion Trap Mass Spectrometer as presented in Fig 3.6. The ESI Ion Trap MS/MS conditions used are described in the Section 5.5. This system can perform up to 5 stages (MS^n , $n = 1, 2, 3, 4$, and 5). In order to achieve optimum CID fragmentation, collision-energy ramping is performed and controlled by the ‘SmartFrag’ software.

3.5 MOLECULAR DETECTION (2): ELECTROSPRAY IONIZATION – ORBITRAP – MS/MS

In order to obtain reliable identification of real-world samples with multiple co-eluting compounds, a mass spectrometer capable of highly resolving power with excellent accurate mass measurement and offering good ion transmission is required. Considered as a potential alternative for Fourier Transform-Ion Cyclotron Resonance (FT-ICR), the Orbitrap mass analyzer provides high resolution and good mass accuracy. The ExactiveTM Bench-Top LC-MS, which has an electrospray ionization (ESI) source, has been used in the accumulating plant work described in the section 4.5. The schematic layout of the instrument and its features are illustrated in Fig 3.7.

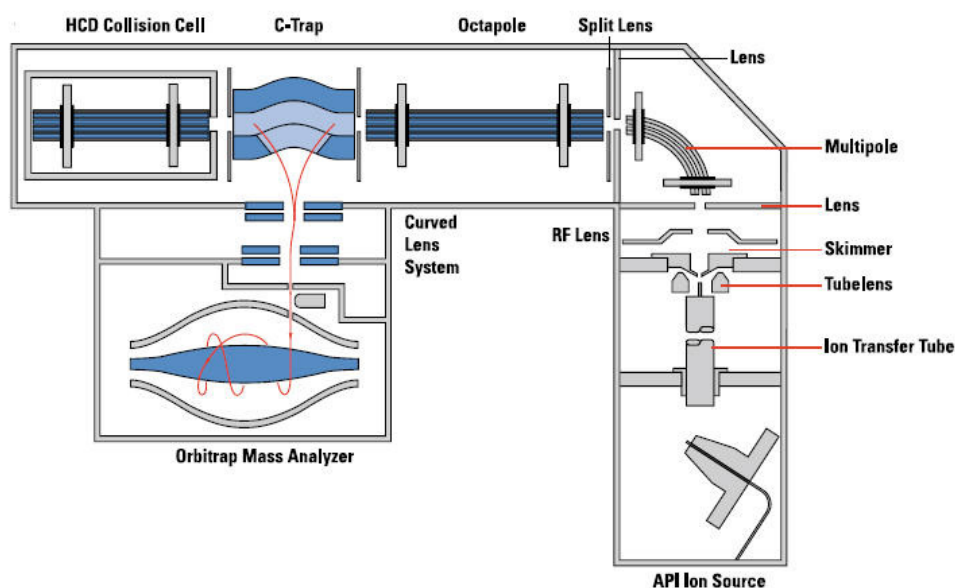


Figure 3.7 Schematic layout and its feature of the Exactive™ Bench-Top LC-MS.

In brief, sample solutions can be introduced into the API (or ESI) source *via* either a direct infusion or an Accela HPLC system. Positive or negative ions produced by API (or ESI) source are transferred through four stages of differential pumping using RF-only multipoles into a curved RF-only quadrupole, so called “the C-trap”. In the C-trap, ions are stored, accelerated and collisionally cooled using a bath gas (e.g. nitrogen) before injection into the Orbitrap. Pulse ion beams are subsequently passed through three additional stages of differential pumping using a curved lens system into the Orbitrap analyzer, where they are captured due to their electrostatic attraction to the inner electrode. The vacuum inside the Orbitrap mass analyzer is maintained below 1×10^{-9} mbar. Ions then gradually cycle around the inner electrode in rings. Ions of a specific m/z ratio move in rings, oscillating along the central spindle.

Independent of the ion velocity, their oscillation frequencies are inversely proportional to the square root of the m/z ratio. By sensing the ion oscillation, the Orbitrap can be used as a mass analyzer. Image current detection from coherent ion packets subsequently occurs after the electrostatic field is stabilized. By fast Fourier transformation, the amplified signals from each of the outer electrodes are transformed into a frequency spectrum, which is in turn converted into a mass spectrum using Xcalibur[®] software.

Furthermore, the Exactive[™] Bench-Top LC-MS can be used to perform a higher energy collision induced dissociation (HCD) experiments. Ion beams are passed through the C-trap into the gas filled HCD collision cell. The HCD cell voltages are then ramped and all fragmented ions and parent ions remaining are delivered back into the C-trap.

The main performances of the Exactive[™] Bench Top LC-MS system are described as follows:

- Mass resolution (100,000 at 1 scan s^{-1} , 10,000 at 10 scans s^{-1})
- Mass accuracy (better than 2 ppm in full scan and HCD mode)
- Sensitivity (500 fg Buspirone with signal to noise ratio > 10:1)
- Dynamic range (>4000 within a spectrum)
- Scan speed (Up to 10 scans sec^{-1})
- Mass range (m/z 50-4,000)
- Polarity switching, full cycle of 1 positive and 1 negative scan < 1 s (25 k resolution)

3.6 HYPHENATED TECHNIQUES

3.6.1 Liquid Chromatography coupled with Inductively Coupled Plasma-Mass Spectrometry (LC-ICP-MS)

Combining advantages of both techniques, the hyphenated LC-ICP-MS technique, capable of operating in a continuous mode for on-line, real time, element-specific detection following LC separation, provides useful information on the distribution of elemental species. Considered to be straightforward, the physical coupling of LC to ICP-MS is performed by connecting the outlet of the HPLC column directly to the inlet of the nebulizer using a short length of narrow-bore polymeric tubing such as polytetrafluoroethylene (PTFE) and polyether ether ketone (PEEK). The length of the tubing should be kept minimal to reduce the dead volume of the transfer line and therefore minimize peak broadening. Typical flow rates of the mobile phase ($\sim 1 \text{ mL min}^{-1}$) are compatible with those used in a conventional pneumatic nebulizer. It is known that this nebulizer gives poor sample transport efficiency. In order to improve the efficiency of nebulization, micro-nebulizers such as direct injection nebulizer (DIN), high efficiency nebulizer (HEN), direct injection high efficiency nebulizer (DIHEN), MicroMist (MM), and plastic (PFA) pneumatic concentric micronebulizer can be used. Moreover, the combined use with a narrow bore column for minimizing the introduction of the mobile phase into the plasma is also an attractive approach to obtain both high sample transport efficiency and enhanced resolution.

The challenges of interfacing HPLC with ICP-MS are associated with the introduction of HPLC effluents into the plasma. Mobile phases generally contain salts in the buffer solution, organic solvents or ion-pairing reagents at high concentration, which can cause cone blockage. The use of non-volatile buffer salts or high salt load for analyte elution can result in poor sensitivity and precision of the analysis. In order to prevent these problems, the quantity of dissolved salts should be kept below 2% and it is advised to clean the system with 1-2% nitric acid regularly. Although a small amount of organic solvent can enhance the ionization of certain metalloids such as As, Se, Sb and Te, the use of organic solvent in excess not only decreases the sensitivity, but also causes plasma instability. Several practical strategies to overcome these problems are the uses of introduction systems equipped with a desolvation unit and water-cooled spray chamber to help condense a large portion of the solvent vapor. Furthermore, the addition of (5-10%) oxygen to the nebulizer gas flow to facilitate the

combustion and operating the plasma at higher powers can help maintain plasma stability.

3.6.2 Liquid Chromatography combined with Electrospray Ionization - Tandem Mass Spectrometry (LC-ESI-MS/MS)

Since it provides the soft ionization process, enabling labile complexes to be transferred intact in the gas phase, the LC-ESI-MS/MS has gained popularity for structural elucidation of metal(loid) complexes and characterization of small organometallic compounds. The application of a tandem mass spectrometric system providing structural fragmentation patterns from CID MS/MS experiments makes it possible to minimize the risk of misidentification of arsenic compounds from obtaining only chromatographic retention times and molecular masses. However, due to its inferior detection limit typically 2-3 orders higher than ICP-MS, the use of LC-ESI-MS/MS is occasionally challenging for characterizing low-abundant peaks observed in the LC-ICP-MS profiles. Some compounds in mobile phases such as ion-pairing reagents or ionic surfactants, known as ionization suppressors, should be avoided. Moreover, this technique is vulnerable to the matrix composition (such as non-volatile salts, exceeding 10 mM). The matrix effects occur when concomitant ions from matrix components are co-eluted with the analyte of interest, resulting in ionization suppression of the target analyte and therefore poor sensitivity. Therefore, the purification of crude extracts by either chromatography or sample extraction methodology (desalting process) of the fractions of interest are often required. An alternative to compensate for the matrix effect is to use matrix-matched standards. The addition of solvents such as methanol and acetonitrile can facilitate the ionization by electrospray source.

CHAPTER 4

DEVELOPMENT OF METHODOLOGIES FOR ARSENIC SPECIATION IN PHYTOREMEDIATING PLANTS

Due to high levels of toxicants, especially heavy metals, and economic pressures driving the need for “land recycling”[167], remediation of pollutants in contaminated soil needs to be considered. Phytoremediation has gained a significant interest for coping with hazardous consequences since it is thought of as a cost-effective and green method. This involves the use of living plants to remove, degrade, immobilize and retain contaminants *in situ*. In general, a wide variety of trace elements in plant tissues can be managed by processes within the organism. To control the homeostasis in living cells, plants create biomolecules to deal with essential and toxic elements. Metal-binding ligands occurring from a bio-induction process are synthesized to respond to environmental stress and to deal with heavy metals [168]. The functional groups in bio-induced ligands serve as coordination sites capable of sequestering metals taken up by the organic tissues. The two groups of metal complexes of interest are metallothionein (MTs) and phytochelatin (PCs) complexes. Phytochelatins (PCs), small peptides of general formula $(\gamma\text{-Glu-Cys})_n\text{Gly}$, where n can range from 2-11, are believed to be responsible for the metabolism and translocation of metal(loid)s such as arsenic contaminants in plants.

There is evidence to suggest that arsenic and selenium species can interact in biological systems and cause mutual detoxification [169, 170]. Also, selenium has been known for decades to antagonize mercury toxicity in mammals, presumably attributed to the formation of biologically inert Se-Hg compounds [171]. However, the presence of Se may influence the As and Hg detoxification by phytochelatins in plants, which could be a beneficial or harmful nature. To gain a better grasp of factors affecting the mechanisms controlling the uptake of species by plants exposed to metals/non metals, metabolism and detoxification processes, not only individual metallospecies, but also other metal species which might be involved should be included for study. Although methods for total metal determination in plants have been developed, metallomics approaches to study element speciation in plants are still scarce and, therefore, urgently needed. Such methods are essential tools to gain a better grasp of factors involved in metal uptake and translocation by plants, which may be useful to produce a better phytoremediating plant.

4.1 PHYTOREMEDIATING PLANTS

The majority of work conducted to date has focused on the accumulation of single metals by *Brassica juncea* and *Arabidopsis thaliana*. Initial studies on the interaction of species of Se and Hg in plant tissues suggest that the presence of Se has an effect of the uptake of the toxic Hg [172]. As such, further investigations of the effects of the presence of Se upon the accumulation of toxic As and Hg by these plants need to be addressed. Very few papers have investigated the interactions of As and Se in plants [173] and, to the authors' knowledge, no information is available on the simultaneous speciation of these elements in *Arabidopsis thaliana*, which is one of the most investigated metal accumulating plant for trace-element speciation [174]. Hydroponics, a method for growing plants without soil but using mineral nutrient solutions instead, was utilized for this study. Using hydroponics, the nutrition levels can be controlled for all samples under investigation and more reproducible results are obtained.

4.1.1 Growing conditions

Arabidopsis thaliana seeds from Herbiseed (West End, Twyford, UK) were germinated on bedding compost in a seed tray. Following germination, seedlings were carefully transferred to 200 mL pots filled with Hoaglands solutions from Sigma-Aldrich (Gillingham, Dorset, UK), spiked with sodium selenite, sodium arsenite, mercury nitrate, sodium selenite-sodium arsenite and sodium arsenite-mercury nitrate (5 plants per pot, 5 pots per metal concentration) in the following levels: 1, 3 and 5 mg L⁻¹ in order to investigate the inter-elemental effect of Se on the uptake of As and Hg. Plants were grown hydroponically for 7 weeks at temperature set to 20/15°C, with white light (photon flux, above 400 µmol PAR m⁻²s⁻¹) on a 16/8 h photoperiod cycle. Control plants, nourished with Hoaglands solution alone, were grown in the same condition as metal(loid) supplemented plants.

4.1.2 Harvesting and storage condition

Each plant was removed from the perlite, washed carefully with deionized water and roots separated from the plants and the leaves from the shoot. Samples were finally immersed in the 5-litre cryogenic tank filled with liquid nitrogen for a few minutes. The protocol for growing and harvesting the plants in details is described in appendix 1 and 2.

4.2 SAMPLE PREPARATION

Due to very limited amount of roots and stems acquired, the study emphasized the plant leaves. The leaves of *A. thaliana* were kept in closed vessels, transported into LGC Ltd, and stored in a freezer at -20°C to minimize bacterial activities affecting original properties of samples. Prior to freeze drying, fresh samples were weighed accurately and a piece of parafilm was placed on the vessels, which were pierced to create pores for facilitating evaporation of plant water. In a freeze-dry process, fresh samples were frozen at -40°C for at least 3 h and then left overnight to be dried at 5°C. Dried samples were weighed accurately again in order to keep a record of the moisture content in samples. Dried leaves were then ground in an electrical blender to obtain fine powder. Immediately when ground homogeneously, they were transferred into plastic bags and maintained in the freezer at the temperature of -20°C prior to the analysis.

4.2.1 Closed-vessel microwave digestion (for total measurement)

A 0.03 gram of homogenized samples was weighed accurately into a glass vial designed for the microwave oven (The CEM Focused MicrowaveTM Synthesis System, Model Discover) and then 1 mL of a mixture of 1:1 concentrated HNO₃ (Ultrapurity) and H₂O₂ (Suprapure) (Romil/Cambridge, UK), was added. Nitric acid and hydrogen peroxide have occasionally been used for sample digestion for ICP-MS analysis, because other acids such as sulfuric acid and hydrochloric acid lead to more spectral interferences [175]. Hydrogen peroxide was chosen not only because its composition is similar to water, but also as it reduces the occurrence of remaining black particles such as ash (oxidized) arising from digestion.

For extracts, a 0.5 mL of conc. HNO₃ is sufficient for obtaining complete digestion. CRMs (NIST 1575 pine needles and NIST 1568a rice flour) were used to check the suitability and accuracy of digestion method along with the measurement method used. A single step was used with temperature of 150°C, 150 W power supply and 10 min digestion time were employed to obtain a clear complete digestion. The digest samples were then transferred into centrifuge tubes and weight was adjusted to 5.0 g with deionized water (18 MΩ cm) obtained from an Elga water purification unit (Marlow, Buckinghamshire, UK) and recorded the actual weight of the samples. All of samples and CRMs were digested in duplicate.

4.2.2 Extraction procedures (for speciation analysis)

Ammonium acetate buffer

A 0.03 gram of homogenized sample was accurately weighed in an eppendorf tube, followed by the addition of 0.5 g of 20 mM ammonium acetate buffer, pH 7.5, which was adjusted by either ammonium hydroxide or acetic acid. Ammonium acetate was purchased from Fluka (Steinheim, Switzerland). Leaching with buffer, which was carried out by ultrasonication [94], was aimed at investigating the extraction efficiency of arsenic by varying extraction times to 2, 5, and 12 h (each in duplicate).

Formic acid

A 0.5 gram of 1% (v/v) formic acid obtained from Fisher Scientific (Loughborough, UK) was transferred into an eppendorf tube containing 0.03 g of dried sample. The tubes were placed in an ice bath (1°C) for 1 h as described in Bluemlein *et al* (2008) [28]. The extraction with diluted formic acid (in duplicate) was used to compare total As extractable amounts obtained from 3 different levels of As exposed samples, and to investigate As speciation in the leaves.

Before use, de-ionized water was always sonicated to eliminate oxygen gas that might cause oxidation of certain As species during extraction. Following the extraction, samples were ultracentrifuged at 3,000 rpm, at 4°C for 30 min. Supernatants were separated from residues by centrifugal force and subsequently transferred to eppendorf tubes. Extracts were analyzed as freshly as possible or within 24 h (should be stored at a -80°C freezer to preserve the entity of compounds).

4.3 DETERMINATION OF TOTAL ARSENIC, SELENIUM AND MERCURY USING C/RC ICP-MS

4.3.1 Instrumental setup

The ICP-MS used was an Agilent Model 7500ce (Agilent Technologies, UK) equipped with collision cell. It was fitted with Pt/Ni sampler and skimmer cones due to the corrosive nature of the digests. The system has two rotary pumps and an ASX-510 autosampler (Cetac Technologies, USA). The samples and standards were all introduced using the autosampler system with a peristaltic pump and a mixer block. The solution from the mixer block passed to a micro flow concentric nebulizer with a double flow chilled (2°C) spray chamber.

A 10 µg g⁻¹ stock solution comprising a mixture of As, Se and Hg standards was prepared from standards purchased from Romil for arsenic, Ultra Scientific for

selenium and Assurance for mercury. All working solutions as following 0.01, 0.10, 0.50, 1.0, 5.0, 10, 50, 100, and 200 ng g⁻¹ were prepared in 10% HNO₃ on a weight basis. A mixture consisting of 60 ng g⁻¹ of Ge and 10 ng g⁻¹ of Rh and Tl used as internal standards for As, Se and Hg in the presence of 5 µg g⁻¹ of Au were prepared in 10% HNO₃.

The determination of total As, Se and Hg was performed using three different tuning conditions. Standard mode was used for the mercury analysis, whereas the determination of As and Se was carried out using reaction cell mode. The only use of He mode at the flow rate of 4.2 mL/min was used for arsenic analysis, while both He and H₂ gases at the flow rate of 0.7 and 3.6 mL/min, respectively were used for selenium analysis. In order to obtain the maximum sensitivity of As and Se analysis, a tuning solution with 1% HCl was used with m/z at 75 and 78 monitored. The operating conditions of ICP-MS used were illustrated in Table 4.1. For Hg analysis, a solution containing 5 µg g⁻¹ of Au in 5% HNO₃ were employed as a rinse solution to alleviate the memory effect. It was believed that gold chloride functions as a strong oxidizing agent can convert or maintain mercury as mercuric ion in HNO₃ solution. This effect can reduce adsorption/desorption of mercury in the sample introduction system.

Table 4.1 Operating parameters of ICP-MS measurement for As, Se and Hg in three different modes.

Parameters	Modes in operation		
	Standard mode	He mode	He and H ₂ mode
RF power (W)	1520	1520	1520
RF matching (W)	1.61	1.61	1.61
Sampling depth (mm)	7.4	7.4	7.4
Carrier gas (mLmin ⁻¹)	0.86	0.86	0.86
Makeup gas (mLmin ⁻¹)	0.26	0.26	0.26
Spray chamber temp. (°C)	2	2	2
Extract1(V)	2.2	2.2	2.2
Extract2 (V)	-129	-129	-129
Cell entrance (V)	-28	-28	-28
Cell exit (V)	-40	-48	-48
Octopole RF (V)	165	165	165
Octopole bias (V)	-6	-18	-18
Quadrupole bias (V)	-3	-16	-16
H ₂ gas (mL.min ⁻¹)	0	0	3.6
He gas (mL.min ⁻¹)	0	4.2	0.7

To determine all three elements simultaneously, eleven isotopes: ⁷³Ge, ⁷⁵As, ⁷⁷Se, ⁷⁸Se, ⁸²Se, ¹⁰³Rh, ¹⁹⁹Hg, ²⁰⁰Hg, ²⁰¹Hg, ²⁰²Hg and ²⁰⁵Tl were monitored. The calculation of sample concentrations was processed by the Fileview32 software. The reported concentrations were automatically calculated following correction with the relevant standards.

4.3.2 Total arsenic, selenium and mercury analysis in digested samples

For quality control of both procedures for microwave digestion and ICP-MS measurements/external calibration quantification, certified reference materials (NIST 1575, pine needles and NIST 1568a, rice flour) was analyzed (as QC samples) together with the leaves of *A. thaliana*. The results of As, Se and Hg in the CRMs were in a good agreement with their certified values. The total concentration of arsenic as determined was 0.22 ± 0.00 and $0.30 \pm 0.01 \mu\text{g g}^{-1}$ dry sample, for NIST 1575 (cert. 0.21 ± 0.04) and NIST 1568a (cert. 0.29 ± 0.03), respectively. The value of $0.38 \pm 0.02 \mu\text{g.g}^{-1}$ dry sample for NIST 1568a (cert. 0.38 ± 0.04), and that of $0.15 \pm 0.01 \mu\text{g.g}^{-1}$ dry sample for NIST 1575 (cert. 0.15 ± 0.05) were reported for the analysis of selenium and mercury, respectively. The recoveries of As, Se and Hg from CRMs analysis are summarized in Table 4.2. The external calibration with internal standards utilized in this work to compensate signal fluctuations enabled good linearity ($r^2 = 1.0000$ for As and Se and 0.999 for Hg) throughout the concentration range. Limits of detection for ^{75}As , ^{78}Se and ^{202}Hg by external calibration with ^{73}Ge , ^{103}Rh , and ^{205}Tl as internal standards were 0.025, 0.040 and 0.010 ng g⁻¹ respectively. Table 4.3 illustrates the results of total As, Se and Hg determination in the *A. thaliana* leaves, which were exposed to such elements individually or co-exposed to Se & As or Se & Hg.

Table 4.2 Arsenic, selenium and mercury recoveries from CRMs analysis.

Code	CRM	% recovery		
		Arsenic (As)	Selenium (Se)	Mercury (Hg)
NIST 1568a	Rice flour	103 ± 3	100 ± 5	-
NIST 1575	Pine needles	105 ± 0	-	100 ± 7

Table 4.3 The determination of total arsenic selenium and mercury in the leaves of *Arabidopsis thaliana*.

Sample No.	Exposure (mg.L ⁻¹)	Amount of analytes of interest in µg g ⁻¹ dry sample, n=2						Dried weight per replicate (g)
		Arsenic (As)		Selenium (Se)		Mercury (Hg)		
1	Control (unexposed)	0.250	0.439	0.223	0.227	0.068	0.084	0.2950
2	Se (1 mg.L ⁻¹)	0.222	0.224	78.9	79.7	0.146	0.162	0.1902
3	Se (3 mg.L ⁻¹)	0.418	0.430	222	230	0.123	0.131	0.1743
4	Se (5 mg.L ⁻¹)	-	-	-	-	-	-	Non growth
5	As (1 mg.L ⁻¹)	18.4	18.6	0.422	0.478	0.108	0.112	0.2018
6	As (3 mg.L ⁻¹)	57.4	57.7	0.634	0.754	0.206	0.286	0.2111
7	As (5 mg.L ⁻¹)	141	145	0.445	0.469	0.024	0.030	0.1103
8	Hg (1 mg.L ⁻¹)	0.75	1.43	0.267	0.287	9.13	9.23	0.2480
9	Hg (3 mg.L ⁻¹)	0.343	0.471	0.272	0.280	19.0	19.3	0.2311
10	Hg (5 mg.L ⁻¹)	0.287	0.297	0.371	0.373	41.5	41.8	0.1340
11	Se+As (1 mg.L ⁻¹)	11.8	13.0	50.7	50.8	1.52	1.54	0.2515
12	Se+As (3 mg.L ⁻¹)	-	-	-	-	-	-	Non growth
13	Se+As (5 mg.L ⁻¹)	-	-	-	-	-	-	Non growth
14	Se+Hg (1 mg.L ⁻¹)	14.6	14.6	57.2	57.4	3.33	3.57	0.1792
15	Se+Hg (3 mg.L ⁻¹)	0.327	0.341	152	152	12.2	12.5	0.1696
16	Se+Hg (5 mg.L ⁻¹)	-	-	-	-	-	-	Non growth

With the exception of hyperaccumulators, the translocation of metal(loid)s in most plants from roots to shoots or even fronds are generally restricted [176]. This might be due to the defensive mechanism of plants to prevent the excessive amounts of elements to the aerial parts. In this study, the results indicate that *A. thaliana* is a potential accumulating plant especially for three elements investigated. The capability of appreciably accumulating As, Se and Hg stored in the leaves is very impressive for potentially remediating growing media. The amounts of these elements incorporated into the leaves were found to exponentially rise with regard to their concentration

supplemented. The highest value of selenium accumulated in the leaves was $200 \mu\text{g g}^{-1}$, even though only 3 mg L^{-1} of selenium was added to the growing medium. Although the highest level of selenium exposure (5 mg L^{-1}) seriously affected the plant growth, thereby resulting in plant death, a similar exposure concentration of As or Hg did not have the same effect; plants remained alive after exposure to 5 mg L^{-1} As or Hg, but the total metal uptake was found much lower than that observed for selenium. The reason why plants were dead may be ascribed to overdose of exposed elements taken up. Interestingly, plants co-exposed with As and Se at 3 and 5 mg L^{-1} and those with Se and Hg at 5 mg L^{-1} were found dead, while plants individually supplemented with 5 mg L^{-1} As or Hg were alive. It is hypothesized that selenium might promote the uptake of As and Hg into the roots and the levels acquired might over their tolerant limits. In this study, selenite co-exposure seems to cause toxicity in *Arabidopsis* plants.

Bluemlein *et al* (2009) reported the enhancement in As toxicity when *Thunbergia alata*, a non-hyperaccumulating plant, was co-administered with selenite [173]. Their study showed the significant reduction in EC_{50} (effective concentration that inhibits growth by 50%) values for selenite and arsenate when both elemental species were supplemented simultaneously. It was indicated that phytochelatin synthesis in the plant was not induced by selenite administration. The results revealed that the co-exposure to selenite resulted in less healthy appearance of the plants due to the lack of phytochelatin induction. One of the reasons behind this was thought to be the competition between As and Se for PCs as binding partners. With no arsenic complex of a seleno-peptide, the presence of the $\text{Se}^{\text{II}}\text{-PC}_2$ complex and Se-cysteinylserine glutathione found suggested the competition of Se^{II} and As^{III} for sulfhydryl group. This led to the depletion of S pool in plants, which in turn affects the ability of *T. alata* to detoxify cellular arsenite. In addition, the enhanced arsenate uptake into the roots with selenite co-exposure was observed. It is probable that the change in the uptake behaviour of arsenate when simultaneously administered with selenite might be partly responsible for the increased As toxicity in *T. alata*.

Due to the limitation of materials in this study, only information of these elements in the leaves of *A. thaliana* is given. Therefore, it is unlikely to compare quantitatively in terms of the distribution of arsenic from roots to other parts following the exposure. It is believed that accumulators capable of detoxifying metal(loid)s especially in roots by forming glutathione or phytochelatin complexes,

which are being stored and sequestered in their root vacuoles [29, 176, 177]. Very recently, Liu *et al* (2010) reported that the arsenic phytochelatin complexes play an important role in translocating arsenic to above-ground, through the investigation of the distributions of arsenic and its complexes from root to shoot of the wild-type and two GSH-deficient and PC-deficient mutant plants [30]. In the wild type of *A. thaliana*, they found that approximately two thirds of total arsenic in the roots were bound to phytochelatins, while in the both mutants plants, a substantially smaller proportion of arsenic was stored as the arsenic complexes of glutathione and PCs in the roots and a significant increase of As(III) concentrations (4.5-12 folds) was accumulated in the plant shoot. This study showed the increase in arsenic mobility when the capability of forming As-PCs complexes was reduced. It was therefore suggested that the extent of free As ions remaining from the complexation can be used to be indicative of the mobility in roots and above-ground translocation. The transportation of metal(loid)s from the roots to leaves is thought to occur through xylem or phloem of plants.

Carey *et al* (2010) investigated how arsenic species are unloaded into grain rice, whose arsenic is dominated by the inorganic As species and DMA [178]. The roles of phloem and xylem transport, using stem-girdling experiment, which restricts phloem transport to the grain in panicles pulsed with arsenite and DMA, were investigated. The results revealed that the majority of arsenite (90%) was taken up to the rice grain through phloem transport. Through both the phloem and xylem, DMA was reported to have greater translocation efficiency to the grain than inorganic species and more mobile than arsenite. Therefore, there is the possibility that the phloem transport is mainly responsible for the large majority of inorganic As found in the *Arabidopsis* leaves.

4.3.3 The inter-elemental effect of selenium on arsenic and mercury incorporation into the leaves

The effect of selenium on the uptake of arsenic and mercury incorporated into the leaves during co-exposure was also investigated. The comparative concentrations of As or Hg in leaves of *A. thaliana* with and without the presence of Se in the growing medium are represented in Fig 4.1. Selenium was found not to affect the levels of As and Hg incorporated into the leaves of *A. thaliana* if administered simultaneously in comparison with individual exposure.

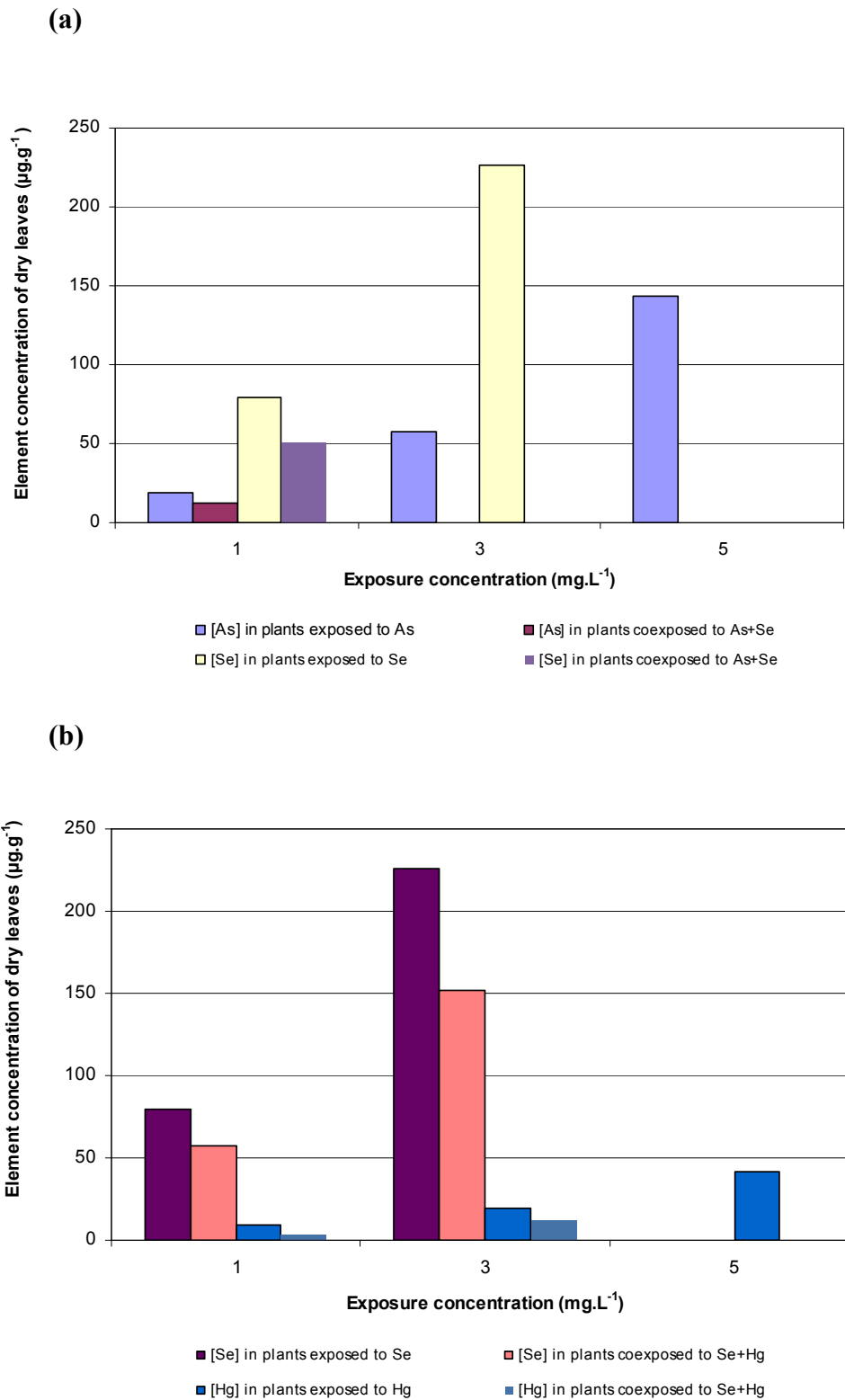


Figure 4.1 Effect of the presence of Se on the incorporation of (a) As and (b) Hg in the leaves of *A. thaliana* following co-exposure to both elements (n=2).

However, it is interesting to note that the levels of As, Se and Hg found in the leaves of co-exposed plants (As+Se and Se+Hg) seemed to be lower than those in the leaves of plant individually supplemented. It is probable that there is the presence of inhibition effect of elemental translocation to above-ground. Monicou *et al* (2006) studied the Se-Hg antagonism by co-exposure of these elements in hydroponically grown *Brassica juncea* [172]. They used sequential extraction approach and found that a substantial portion of Hg and Se-containing protein complexes were formed in the plant roots, whereas no compounds containing both Hg and Se were observed above-ground. In the root exudate solution, it appeared the co-elution of Se and Hg which interact with the same high MW biomolecules (≥ 70 kDa). It was thought that the translocation of the metals to the aerial parts of the plants might be precluded by their sufficiently stable complexes. It seems like there is a defensive mechanism of plants for preventing translocation of these toxic metal(loid)s to above-ground. However, there is still further evidence needed to elucidate the translocation mechanism of metal(loid)s. The existence of As-Se and Se-Hg complexes in the plant root translocation following co-exposure needs further investigation.

On the basis of the results shown above, arsenic speciation in the leaves has received our main focus for further research. The total arsenic results revealed that considerable amounts of arsenic can be accumulated in the leaves. Little is known of the arsenic uptake of *A. thaliana*, and which forms are predominantly present in the leaves how the plant deals with such a tremendous arsenic content. Consequently, the leaf sample, which was exposed to 3 mg L^{-1} of arsenic was chosen for the optimization of sample preparation.

4.3.4 Comparison of arsenic extraction efficiency between two different extraction conditions

Formic acid (1%) was used to extract As-PCs in *Holcus lanatus* [95], *Pteris cretica* [95] and *Thunbergia alata* [28]. The presence of As-PC₃ and GS-As-PC₂ were identified as predominant species found in *Holcus lanatus* and *Pteris cretica*, respectively, whereas well above 50% of total extractable As in the root of *Thunbergia alata* were mostly As-(PC₂)₂, As-PC₃ and As-PC₄. By the virtues of direct analysis, X-ray absorption near-edge spectroscopy (XANES) and extended X-ray absorption fine structure (EXAFS) were used to confirm the original As species

present, following the use of 1% formic acid as an extracting solution and the separation on a reversed-phase column for the As-PCs analysis [28]. Apart from formic acid used for As-PCs extraction, an ammonium acetate buffer with different pH (4.4-7.8) as an extractant and mobile phase was investigated in *Brassica juncea* by Montes-Bayon *et al* (2004) [94]. Using size-exclusion chromatography, the As-PCs region (2 kDa) was differentiated from non-specific retained As (< 1 kDa). It is interesting to investigate arsenic speciation in the extracts obtained from two different extraction methods

Leaching of the solid leaves with ammonium acetate buffer, pH 7.5 and 1% formic acid was compared for speciation purposes. As a first step, the total As content of such extracts was determined for mass balance purposes. The results of the total arsenic in the extracts are presented in the Table 4.4 and Fig 4.2. They show that the extraction efficiency in terms of total As for the different extraction times with ammonium acetate buffer are very similar. It is also interesting to note that only 2 h extraction with ammonium acetate buffer is enough to extract approximately 70% (higher than 60% obtained with formic acid) of total As in the solid. This is a new finding since conventional extraction methods occasionally require at least 12 h [80, 92, 119, 126, 128, 129] and to our knowledge, the influence of extraction time on the extraction efficiency of As from the plant leaves has not been reported before.

Table 4.4 Comparison of the amount of extracted As obtained from leaching with 20 mM NH₄OAc, pH 7.5 (varying extraction time as following: 2, 5 and 12 h, respectively) and extraction with 1% formic acid (1 h, 1°C).

Extractants	Extraction time	Condition	Amount of extracted As (µg g ⁻¹), n=4	% extraction efficiency
NH ₄ OAc buffer, pH 7.5	2 h	Sonication	39.0 ± 0.6	68
NH ₄ OAc buffer, pH 7.5	5 h	Sonication	40.1 ± 1.2	70
NH ₄ OAc buffer, pH 7.5	12 h	Sonication	43.6 ± 1.0	76
1% formic acid	1 h	1°C, ice bath	33.4 ± 1.4	58

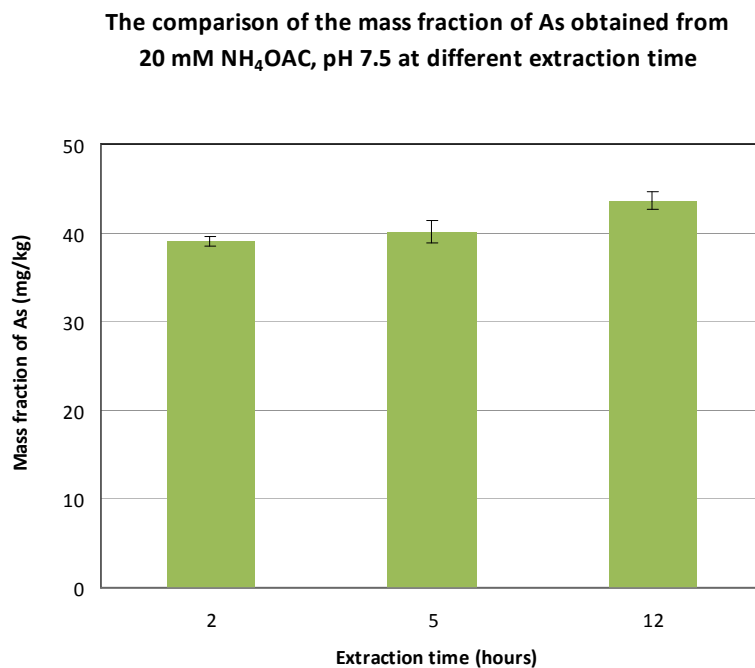


Figure 4.2 Comparison of the mass fraction of As obtained from 20 mM NH₄OAc, pH 7.5 at different extraction time. *Error bars are standard deviations for $n = 4$.*

Moreover, the use of 1% formic acid as an extractant was found to provide proportional extraction of As with approximately 60% efficiencies when the leaves of plant exposed to 3-different levels of As were extracted as illustrated in Fig 4.3. However, the rest of unextractable As species (40%) were thought to be strongly bound to cell wall constituents or some proteins.

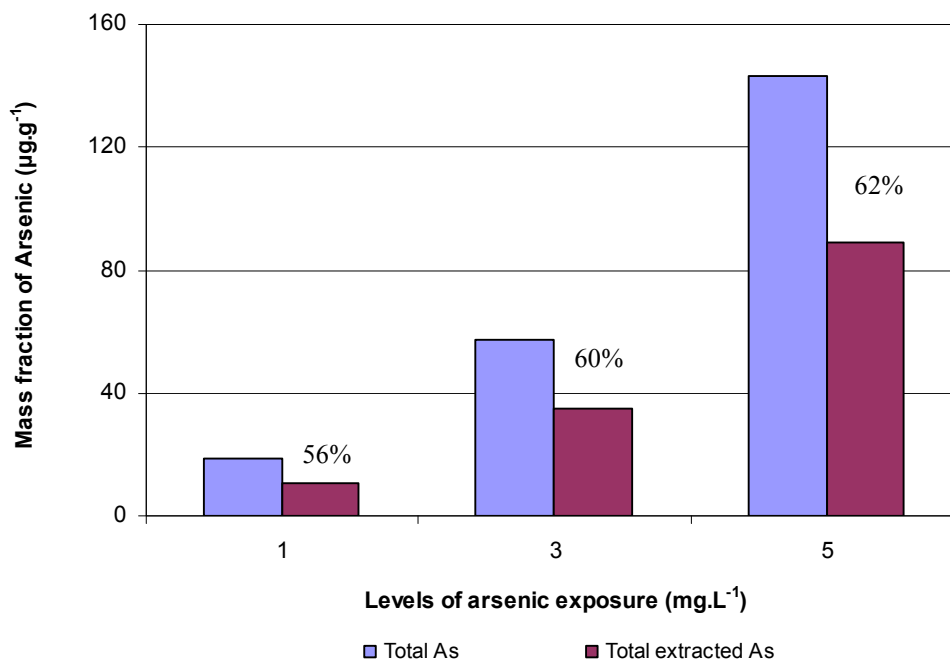


Figure 4.3 Proportional As extraction with 1% formic acid in the leaves of *A. thaliana* exposed to different levels of As^{III} exposure: 1, 3 and 5 mg L⁻¹ (n=2).

4.4 DEVELOPMENT OF HPLC COUPLED TO ICP-MS FOR ARSENIC SPECIATION IN PHYTOREMEDIATING PLANTS

In order to investigate on the capability of metal(loid) accumulation, individual analysis of total PCs and metal(loid) concentrations in plant samples is not sufficient. Methods for metal(loid) complexes with PCs are therefore required. The use of a combination of elemental and molecular mass spectrometric techniques has been proved to be a promising method for detecting and characterizing essential chemical structures of metallocompounds [3, 27, 94, 174, 179-181]. However, the As-PCs are known to be unstable during pretreatment and chromatographic separation. Consequently, erroneous results might be possibly ascribed to either degradation of compounds during analysis, or *de-novo* formation in the course of extraction and detection. The disintegration of glutathione complexes was reported when conventional speciation methods for organoarsenicals using ion chromatography and formic acid or carbonate buffer were performed, while the use of reversed phase chromatography with formic acid/acetonitrile gradient system can maintain their stability [93]. The selection of pretreatment and chromatographic procedures for As-PCs analysis is of paramount importance. Milder separation mechanisms such as size

exclusion and reversed phase are occasionally considered [93-95]. Moreover, cooled storage in an acidic environment to preserve the integrity of the arsenic species and quick measurement are strongly recommended.

4.4.1 Size exclusion chromatography-inductively coupled plasma mass spectrometry (SEC-ICP-MS)

Size exclusion chromatography is known to be used for separating molecules according to their effective size: molecules larger than the pore size of the stationary phase are not retained on the column. This technique is especially useful for speciation studies to screen for metal(loid)s bound to biomolecules [182], since its interactions in size exclusion are the weakest. SEC does not require organic solvents that might denature the biological compounds, so it is thought to provide best preservation of species integrity. The selection of the column is occasionally considered as the separation range and size of organoarsenic species. In order to see the distribution of metal(loid) containing biomolecules, the size exclusion column with the minimum separation range, initially designed for the small peptides (10 kDa) was chosen.

HPLC-ICP-MS measurements were performed using an Agilent Technologies 1200 HPLC system (Palo Alto, CA USA) for chromatographic separation and an Agilent 7500ce ICP-MS for element-specific detector. The HPLC is equipped with a dual pump, a vacuum de-gasser, an autosampler and a heated column compartment. The HPLC column was directly connected to a 100 $\mu\text{L}\cdot\text{min}^{-1}$ PFA microflow concentric nebulizer, part of the ICP-MS, *via* PEEK tubing (30 cm x 0.1 mm id). Integration of the chromatographic signal was performed using Agilent Technologies ICP-MS chromatographic software (G1824 Version C.01.00).

Extracts obtained using two different extractants (NH_4OAc buffer and formic acid) were diluted 4-fold with the counterparts in reverse to obtain the same pH, followed by filtration through a 0.45 μm cellulose membrane to minimize matrix affecting a column. Filtrates were kept in amber vials and cooled down in the autosampler controlled at 4°C before injection. This experiment was performed by means of SEC-ICP-MS and 10 mM NH_4OAc buffer, pH 8.5 acted as a mobile phase with isocratic system at the flow rate of 0.500 mL min^{-1} throughout a 30-min chromatographic run. A size-exclusion column utilized in this study was TSK gel 3000PW_{XL} (Supelco, 30 cm length x 7.8 mm id x 6 μm particle size), which was

calibrated with a gel filtration standard solution containing bovine albumin ($M_r \sim 66$ kDa), superoxide dismutase or SOD ($M_r \sim 32$ KDa), rabbit liver metallothionein I or MT ($M_r \sim 10$ kDa), vitamin B12 ($M_r \sim 1.35$ kDa) and glutathione ($M_r \sim 307$ Da). This column was cleaned up using 100 mM NH_4OAc prior to conditioning the column with the mobile phase. This study was performed using a 40 μL of each injection (at least twice for each extract). The ICP-MS functioned as a detector was set up for monitoring m/z ^{75}As , ^{63}Cu (for albumin), ^{64}Zn (for SOD), ^{111}Cd (for MT), ^{59}Co (for vitamin B12) and ^{34}S (for glutathione) in a He mode. The arsenic and sulfur profiles obtained from SEC-ICP-MS in different extracts were recorded. Such preliminary information like retention time and peak areas could be used for planning further speciation studies.

4.4.1.1 Arsenic species distribution in the leaf extracts using SEC-ICP-MS

Arsenic standards: sodium (meta) arsenite (NaAsO_2 , $\geq 99\%$), di-sodium hydrogen arsenate heptahydrate ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$, $\geq 98.5\%$), and cacodylic acid (dimethylarsinic acid or DMA, $\text{C}_2\text{H}_7\text{AsO}_2$ $\geq 99\%$), were all purchased from Fluka (Sigma-Aldrich Co., Gillingham, UK), whereas BCR-626 arsenobetaine (certified value revised 1.03 ± 0.07 g kg^{-1} as compound) was obtained from EC-JRC-IRMM and monomethylarsonic acid di-sodium salt (MMA, $\text{Na}_2\text{CH}_3\text{AsO}_3$ $\geq 98\%$) was purchased from Argus Chemicals (Vernio, Italy). Stock standard solutions were prepared by dissolving an appropriate amount of the solid standard in ultra pure water. These solutions were stored at 4°C in the dark.

Reduced glutathione (GSH) and four phytochelatin standards were purchased from Sigma-Aldrich (St. Louise, MO, USA) and CloneStar Peptide Services (Praha, Czech Republic), respectively. Complexes of $\text{As}^{\text{III}}(\text{GS})_3$ and $\text{As}^{\text{III}}\text{-PCs}$, used for spiking experiments and confirmation of compound identities, were synthesized by incubating an aqueous solution under nitrogen atmosphere to prevent oxidation, as described in Scott *et al* (1993) [183] and Bluemlein *et al* (2008) [28], respectively.

Prior to As speciation analysis of the leaf extracts, standard solutions of As species were prepared to investigate the peak profiles and retention times of individual standard relevant. The SEC-ICP-MS As profiles showing retention time of As standards: As(V) 14.1 min, MMA 14.9 min, DMA 15.0 min, AsB 18.6 min, and

As(III) 21.6 min (t_r of standard calibrants: albumin 10.5 min, SOD 11.6 min and MT 13 min) were presented in Fig 4.4.

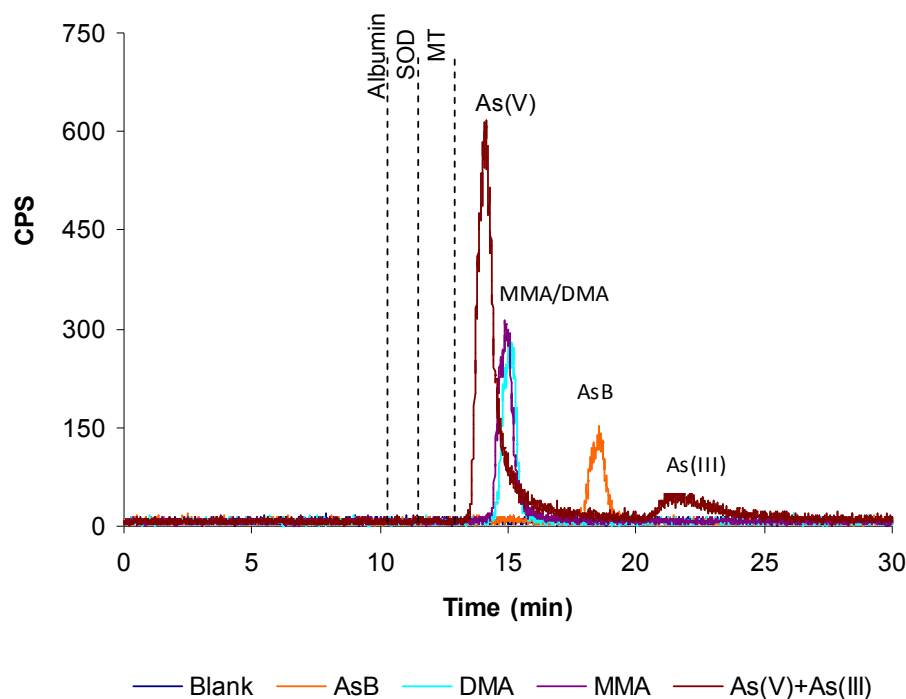


Figure 4.4 The SEC-ICP-MS As profiles of As standards: As(V), MMA, DMA, AsB, and As(III) in the order of retention time.

In the plant leaf extract, there are two main As regions, one of which appeared at the retention time (t_r) 14 min and the other showed at t_r 21 min. According to the retention time matching with As standards, it is likely that the former might mostly belong to As(V) and the latter might be As(III). However, it was found that, in Fig 4.5, the ^{34}S peak was co-eluted with the main As peak in the 20-fold diluted formic extract. It is probable that the As is located in molecules which also contain sulfur. In the preliminary study, individual synthesized standards of arsenic complexed with GSH, PC₂, PC₃, PC₄ and PC₅ were injected into the SEC-ICP-MS and found in the vicinity of the region in which the main peak of formic extract was present.

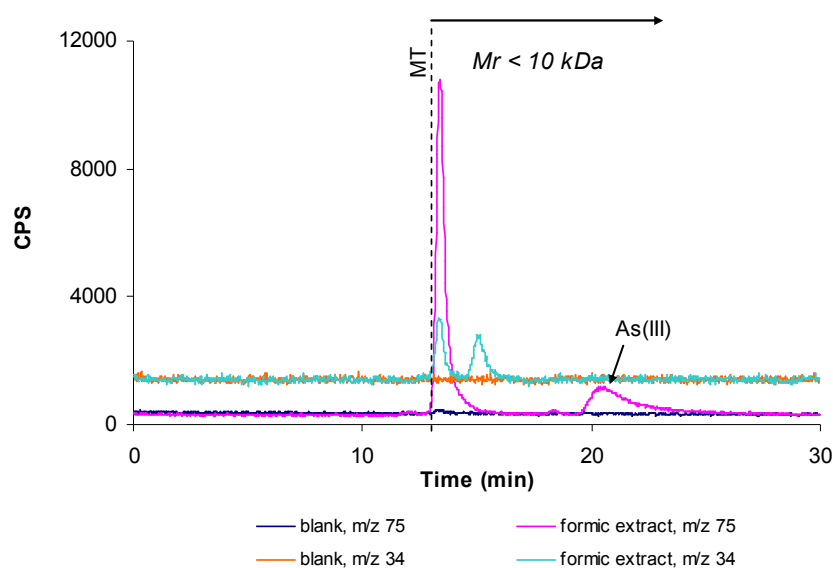


Figure 4.5 The SEC-ICP-MS As profiles showing the simultaneous elution of As species with S at 14.2 min.

4.4.1.2 Comparison between different extraction conditions

Speciation analysis of As in the ammonium acetate buffer was undertaken by SEC-ICP-MS. Comparative profiles obtained after different extraction times are presented in Fig 4.6. Similar arsenic profiles at varying extraction times (5 in total) were obtained. Two major As peaks can be detected by HPLC-ICP-MS, eluting at 14.2 min, and 20.9 min. It is also interesting to note that at large extraction times (e.g. 12 h) the peak area of the species previously identified as inorganic As seems to slightly increase, probably due to degradation of higher molecular weight As species. Although increasing the sonication time can slightly improve the extraction efficiency, the As species obtained were found to be inorganic As, in particular As(III). This might be due to the fact that the more sonication time, the weaker bonding As is bound to biomolecules. Consequently, arsenic (as inorganic As) can be gradually detached from biomolecules over the sonication time. Extraction with ammonium acetate buffer during 2 h was selected as optimal, to be compared with the 1% (v/v) formic acid extraction in terms of As profiles.

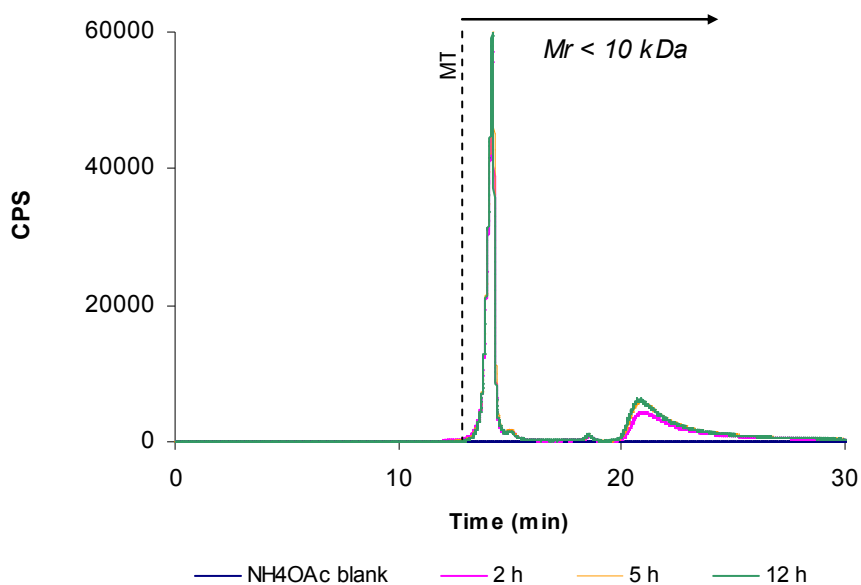


Figure 4.6 The SEC-ICP-MS As profiles obtained from NH₄OAc extracts with varying extraction times.

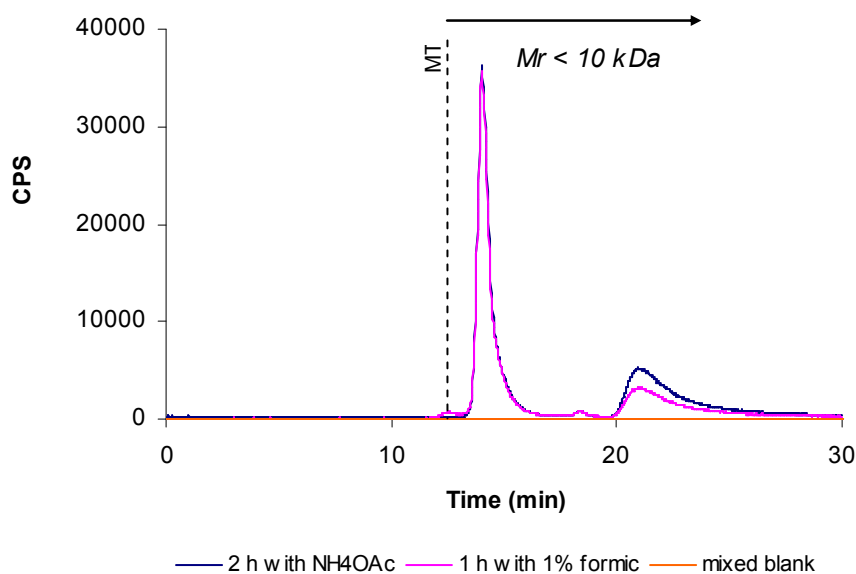


Figure 4.7 The SEC-ICP-MS As profiles obtained from two different extracts.

Comparative As profiles obtained by leaching with ammonium-acetate buffer (2 h) and 1% formic extract are illustrated in Fig 4.7. It was noted that the small peak at 12.6 min eluted before the main peak was found more evident for 1% formic extract. It appears that the less harsh extraction with formic acid in an ice bath seems

to be the choice as a compromise between extraction efficiency and preservation of the compound entity. Therefore, 1% formic acid was selected for use in the further study.

With 1% formic acid, the extraction of the plant leaves, which were exposed to 3 different levels of As exposure (1, 3 and 5 mg As L⁻¹), provided different total extractable As: 10.3, 34.6 and 89.2 µg As g⁻¹, respectively. As presented in Fig 4.8, it was found that plants with different As exposure gave the constant ratio of extractable As species present in the main peak (mostly As(V)) and the last eluted peak (As(III)) by the factor of 2.8.

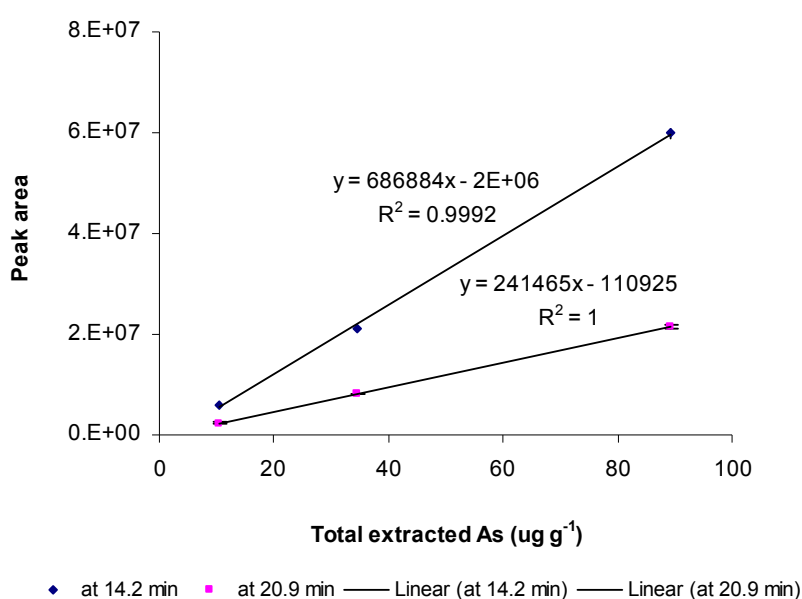


Figure 4.8 The relationship between the peak area obtained from the main peak (t_r 14.2 min) and the last peak (t_r 20.9 min), and total As in extracts from plants exposed to 3 different levels of As exposure.

4.4.2 Anion exchange liquid chromatography-inductively coupled plasma mass spectrometry (AE-LC-ICP-MS)

To gain a deeper insight into the As species distribution in the plant leaf, anion exchange HPLC was used in combination with ICP-MS detection. A PRP-X100 (250 mm x 4.1 mm id x 10 µm) column was used in order to determine the total inorganic arsenic (As^{III}+As^V) and see the distribution of organoarsenicals such as AsB, MMA, and DMA. This study was performed in 10-fold diluted extracts using the recently

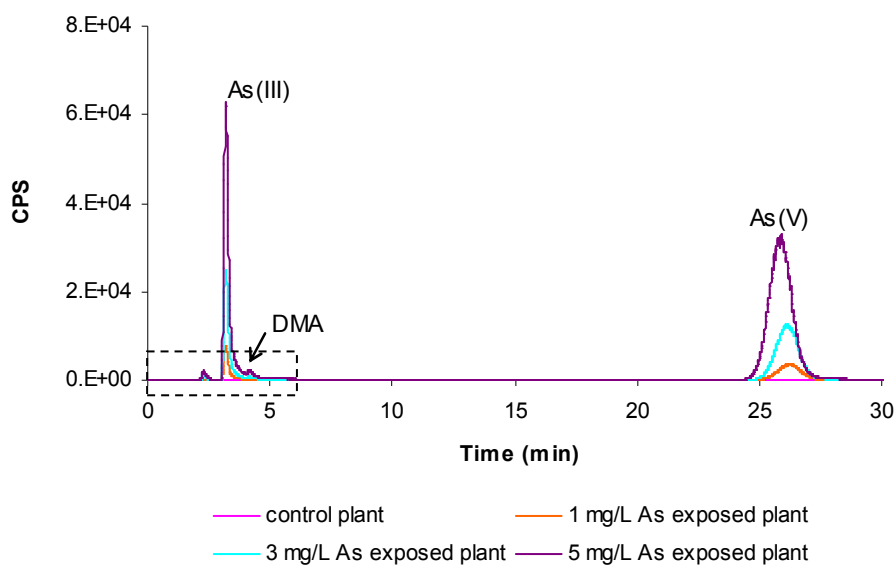
developed chromatography, 20 mM NH_4HCO_3 in 1% MeOH, pH 9.0 as a mobile phase with isocratic elution system at the flow rate of 1.0 mL min^{-1} and 50- μL injection volume (Section 5.4.2.1). ICP-MS measurement was performed in He mode with integration time of 300 msec. To check the accuracy of extraction method and column recovery for inorganic arsenic, the control plant was spiked with $50 \mu\text{g g}^{-1}$ of As^{V} , and was then extracted in the same manner as sample extraction described earlier.

4.4.2.1 Arsenic species distribution in the leaf extracts

The distribution of water-soluble As species of the plant leaves in the formic extracts are depicted in Fig 4.9, showing the retention time at: 2.2, 3.2, 4.2, and 26 min. These peaks were preliminarily identified as AsB, As(III), DMA, and As(V), respectively. The presence of a majority of inorganic As (>95% of the total PA) in the formic extract was found. There was a peak in vicinity of AsB (0.8% of PA) based on spiking experiment. However, the assignment of peak near a void peak should be considered carefully. Cation-exchange chromatography, which allows the species near the void volume in AE column to be more retained, and the use of organic MS are needed to confirm the species identity. Due to the lack of evidence in the presence of AsB and AsC in terrestrial plants, there is a possibility that the peak near the void volume is TMAO rather than AsB. Reported to elute very close to the void volume in anion-exchange column [184], TMAO has been identified in some plants [185] and detected as the dominant organoarsenical species in atmospheric particulate matters [184].

It is not surprising that more than half of inorganic As were As(V) despite the high level of As(III) exposure to the plants, as it is commonly known the nature of interconversion for inorganic arsenic: As(III) can be readily changed into As(V) when coming into contact with oxidizing agents like oxygen gases.

(a)



(b)

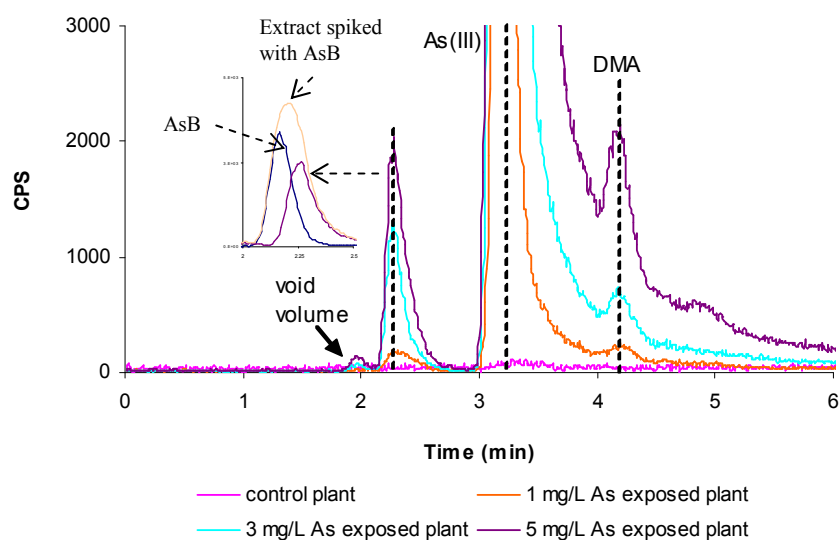


Figure 4.9 The AE-LC-ICP-MS As profiles shows (a) the presence of the majority of As species in the *A. thaliana* leaves being inorganic As (b) the presence of the minority of (1) As species, which has the retention time in the region of AsB (0.8% of total peak area) and (2) DMA (0.4% of total peak area) on the basis of spiking experiment.

4.4.2.2 Quantitative analysis of inorganic arsenic

To quantify As concentration obtained, individual standard of 50 ng g⁻¹ inorganic As prepared from solids were subjected to the total ICP-MS measurement following microwave digestion. Insignificant differences between theoretical and experimental calculations 100±1% and 101±3% recoveries (n=2) were observed. Instrumental limits of detection (LODs, 3σ criterion) of As(III) and As(V) were 50 pg g⁻¹ and 10 pg g⁻¹, respectively, whereas a similar response of both species by ICP-MS was obtained. The result from spiking experiment with 50 µg g⁻¹ of As(V) into the controlled plant sample prior to the extraction shows the good recovery of As (96±2%), suggesting the extraction method used and the column recovery were satisfactory for inorganic As determination. The content of inorganic As and its percentage found in the leaves are shown in Table 4.5. It presents the amounts of inorganic As measured accountable for almost 90% of the total As extractable content in the extracts.

Table 4.5 The amount of total extractable As, inorganic arsenic (As^{III}+As^V) as well as the percent of inorganic arsenic in the formic extract.

<i>A. thaliana</i> leaves (treatment)	Total extractable As µg g ⁻¹ (n=4)	Total inorganic As (As ^{III} +As ^V), µg g ⁻¹ (n=2)		% Inorganic As of the total As in the extract
Control plant	0.4 ± 0.1	0.4	0.4	
1 mg L ⁻¹ As exposure	10.3 ± 0.2	9.0	9.0	87%
3 mg L ⁻¹ As exposure	34.6 ± 0.4	30.1	30.5	88%
5 mg L ⁻¹ As exposure	89.2 ± 5.4	78.0	78.0	87%

4.4.3 Reversed phase liquid chromatography-inductively coupled plasma mass spectrometry (RP-LC-ICP-MS)

Due to the nature of less polar As-PCs, the separation of these species can be achieved by eluting with the mobile phase containing methanol and reversed-phase column used. A number of publications have reported the presence of As-PCs in plant roots and shoots [27, 28, 99, 182]. A few reports has shown the evidence of As-PCs in plant leaves [27]. The complementary use of RP-HPLC-ICP-MS and ESI Orbitrap MS were selected to identify and characterize As species in the leaves of *A. thaliana*.

The As-PC complexes were separated by a reversed phase column, Dionex Acclaim[®] organic acid OA (250 x 4.0 mm id) with a particle size of 5 μ m, together with gradient elution. The HPLC column was connected to a 100 μ L min⁻¹ PFA microflow concentric nebulizer of the ICP-MS *via* PEEK tubing (30 cm x 0.1 mm id). The effluent was diluted with de-ionized water (1:1) on the way *via* a T piece for a post column line controlled by an external pump. Since As-PC complexes can be stabilized in a slightly acidic condition, mobile phase (A and B) were added with a small amount of 0.5% (v/v) formic acid. The optimum chromatographic separation conditions and instrumental parameters for on-line measurements with ICP-MS are summarized in Table 4.6.

Table 4.6 ICP-MS instrumental settings and HPLC separation conditions.

ICP-MS settings						
7500ce ICP-MS with a narrow torch						
RF power: 1550 W						
Nebulizer Ar flow rate: 0.82 L min ⁻¹						
Makeup Ar flow rate: 0.15 L min ⁻¹						
Optional gas (O ₂) 5%						
Sampler/ skimmer cones: Pt/Ni						
Spray chamber temperature: -5°C						
C/RC gases: He+H ₂ : 0.6 + 2.8 mL min ⁻¹						
Data acquisition:						
Points per spectral peak: 1						
Isotopes monitored: ⁷⁵ As						
Integration time per mass: 300 msec						
HPLC conditions						
Analytical column: Dionex acclaim [®] organic acid (4 x 250 mm)						
Injection volume: 40 μ L						
Column flow rate: 0.4 mL min ⁻¹						
Post-column flow rate: 0.4 mL min ⁻¹ (only with ICP-MS)						
Mobile phase: concentration gradient of MeOH in 0.5% formic acid						
Mobile phase A: (2 + 98) MeOH-H ₂ O						
Mobile phase B: (80 + 20) MeOH-H ₂ O						
Time/min	0	10	50	55	60	80
Mobile phase B (%)	0	0	50	50	0	0

The analysis of ultra-trace metal(loid) species in complex samples by HPLC-ICP-MS is not straightforward. The problems encountered with HPLC-ICP-MS mainly involve organic solvents. HPLC techniques often use an organic modifier in the mobile phase and sometimes large volumes of organic solvent. When the analyzing solution reaches the ICP-MS, this leads to carbon-build up on the sampling cone orifice. Some strategies need to be used to tackle the problem of plasma instability, such as the use of narrow torch rather than wide torch and the use of oxygen gas, cooling spray chamber as well as high RF power. The use of narrow torch allows analyzing solution containing organic solvent to be analysed because the shorter diameter of the torch decreased the rate of sample volume to the ICP-MS. Utilizing a double-pass spray chamber cooled to -5°C can reduce the solvent loading on the plasma. Oxygen gas added post nebulization can be used to convert organic carbon to carbon dioxide in the plasma and avoid carbon build up on the cone. Post-column with water dilution can also be used to alleviate this problem. These tactics above-mentioned were applied to this work.

4.4.3.1 Arsenic species distribution in the leaf extracts

In a common work flow, SEC is used as a first separation (pre-cleaning step) followed by fraction collection and lyophilization and the subsequent analysis using RP-LC-ICP-MS/ ESI-MS. There has been some concern that this procedure, consisting of a number of steps, may result in alteration of the species of interest and maintaining species integrity is of paramount importance. Raab *et al* (2009) demonstrated the instability of these As species in the root extract of *Thunbergia alata* exposed to As(V). They found that greater than 90% of As-PC₃ complex and apo PCs can be degraded during a freeze-drying process. Similarly, the preliminary results found no As containing phytochelatins in the leaf extract. As a result, arsenic speciation in fresh leaf extracts was investigated by direct on-line method.

The RP-LC-ICP-MS As profiles of 2-fold diluted formic extracts in the plant leaves with different levels of As exposure were compared in Fig 4.10. The distinctive peaks on the chromatogram were found to be inorganic As, which were less retained on the reversed phase column. A cluster of As peaks in the range of 30-45 min, were also observed with relatively much lower signal than the former, which were eluted with 20-35% MeOH. This As region is believed to contain As-phytochelatins. Attempts to identify by injecting house-hold prepared As-PCs into the reversed phase

column were made. It was found that individual As-PCs prepared gave a cluster of peaks in this region as well. Since the synthesis was not selective for individual As-PC_n species, it was found to be difficult to identify the type of phytochelatin complexes in the leaf extracts using ICP-MS alone.

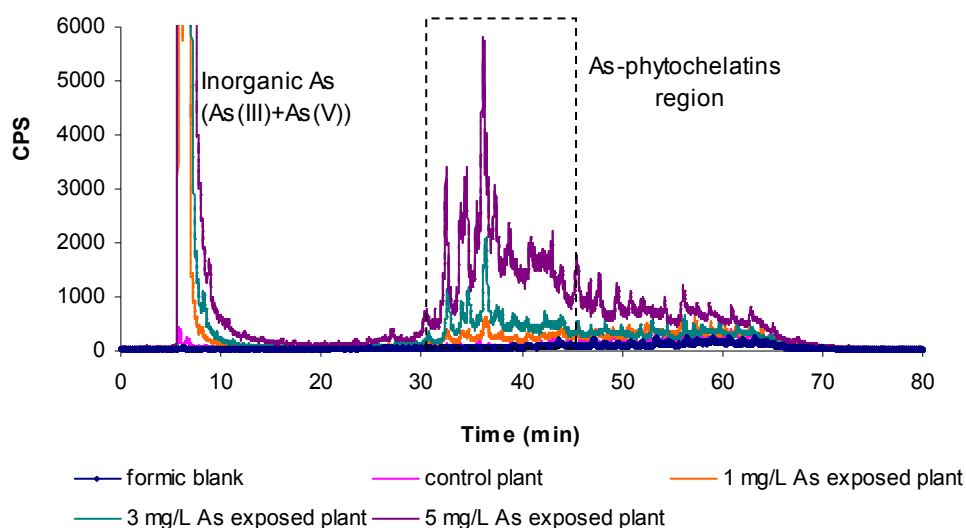


Figure 4.10 The RP-LC-ICP-MS As profiles showing the retention time of As-PCs region, corresponding to those observed in RP-LC-ESI Orbitrap MS/MS.

In order to characterize this type of As species, ESI-MS/MS was used for structural elucidation of metal complexes. It is ideal for detecting high MW metallocompounds and able to investigate metal accumulation in plant tissues.

4.5 DEVELOPMENT OF HPLC COMBINED WITH ESI ORBITRAP MS/MS FOR IDENTIFICATION OF ARSENIC-PHYTOCHELATIN COMPLEXES

Due to the presence of a range of biomolecules of similar composition and relative low stability, accurate mass measurements of the precursor and fragment ions is important to minimize ambiguity in plant species identification. HPLC-ESI MS/MS were equipped with an Exactive MS system incorporating OrbitrapTM Technology and an Accela (ThermoFisher Scientific, Hemel Hempsted, UK) HPLC system. The ExactiveTM BenchTop LC-MS can provide high resolution and accurate mass measurement. Reversed phase HPLC separation was performed on a Dionex acclaim[®] organic acid OA (4.0 x 250 mm i.d.) with a particle size of 5 μm . The effluent of the RP-HPLC column (0.4 mL min⁻¹) was fed into the electrospray source *via* a PEEK connecting tube. Mass calibration was performed daily by direct infusion of a solution containing caffeine (mass 195.0877), MRFA (mass 524.2650) and ultramark (mass 1121.9970, 1321.9842, 1621.9651, 1721.9587) in 50/25/25 MeCN:MeOH:H₂O containing 0.1% acetic acid. Data acquisition and processing were performed using the Thermo Xcalibur software version 2.1.

The Orbitrap conditions were used in positive mode with an ESI source as following (spray voltage: 3978 V; spray current: 39.91 μA ; capillary temperature: 325°C; sheath gas flow rate: 50.02; auxiliary gas flow rate: 19.96; HCD collision energy (for MS/MS): 40 eV; collision gas: N₂. Mass spectra were recorded over an m/z range of 150-2000 with a resolution of 100,000 scan s⁻¹. Product ion spectra were acquired in the HCD Scan mode, throughout the entire chromatogram, alternating (~ 0.02 s) with MS scan mode.

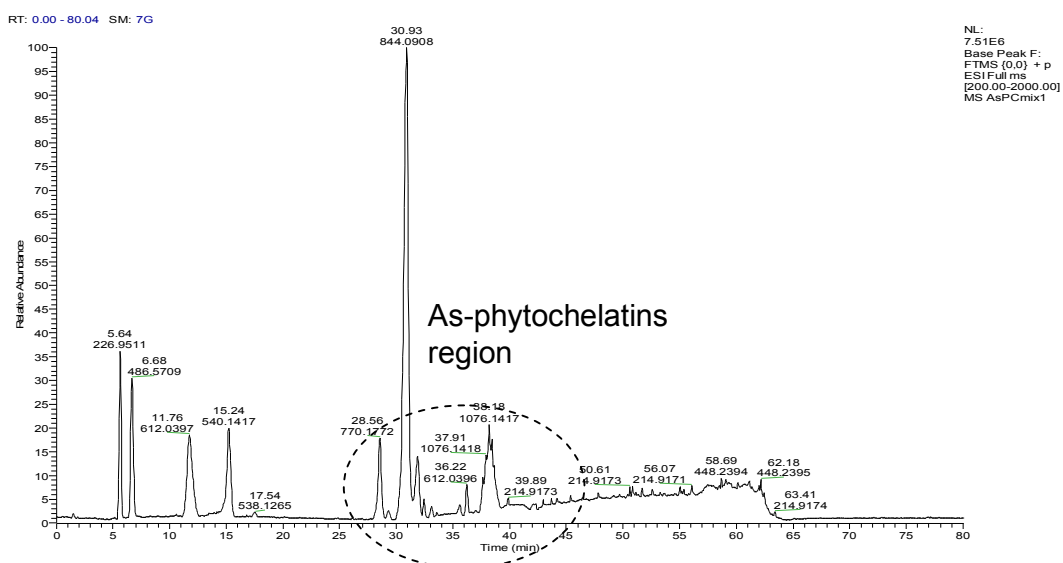


Figure 4.11 Total Ion Chromatogram for matrix-matched As-PC_{2.5} standards from RP-LC-ESI-Orbitrap MS.

The tuning solution, for optimizing the sensitivity of the As-phytochelatins obtained from organic mass spectrometry, was comprised a mixture of at least 200 ng g⁻¹ of As-PC_{2.5} standards in the mobile-phase media. Matrix-matched standards of As-PC_{2.5}, prepared by spiking a mixture of freshly synthesized As-PC_{2.5} into the control plant extract, were injected into the reversed-phase column for giving an idea of the possible retention times of each As-PC in the real matrix. The total ion chromatogram for the matrix-matched standards is shown in Fig 4.11. In order to know the peak profiles and retention times of individual As-PC standard, extracting the total ion chromatogram of the m/z range covering individual As-PC were performed: m/z 612.034-612.046, 844.082-844.099, 1076.131-1076.153, and 1308.181-1308.207 for As-PC₂, As-PC₃, As-PC₄, and As-PC₅, respectively. The extracted ion chromatograms of As-PC mixture are presented in Fig 4.12. The chromatograms show the retention times of As-PC_{2.5} peaks at 11.7, 30.9, 38.2, and 42.1 min, respectively. It is interesting to note that the retention times found on the RP-LC-ICP-MS As profile of the leaf extract correspond well with those obtained from matrix-matched standards. Consequently, the complementary use of both techniques was found to be useful for locating the retention time of As-PC_n in the real samples.

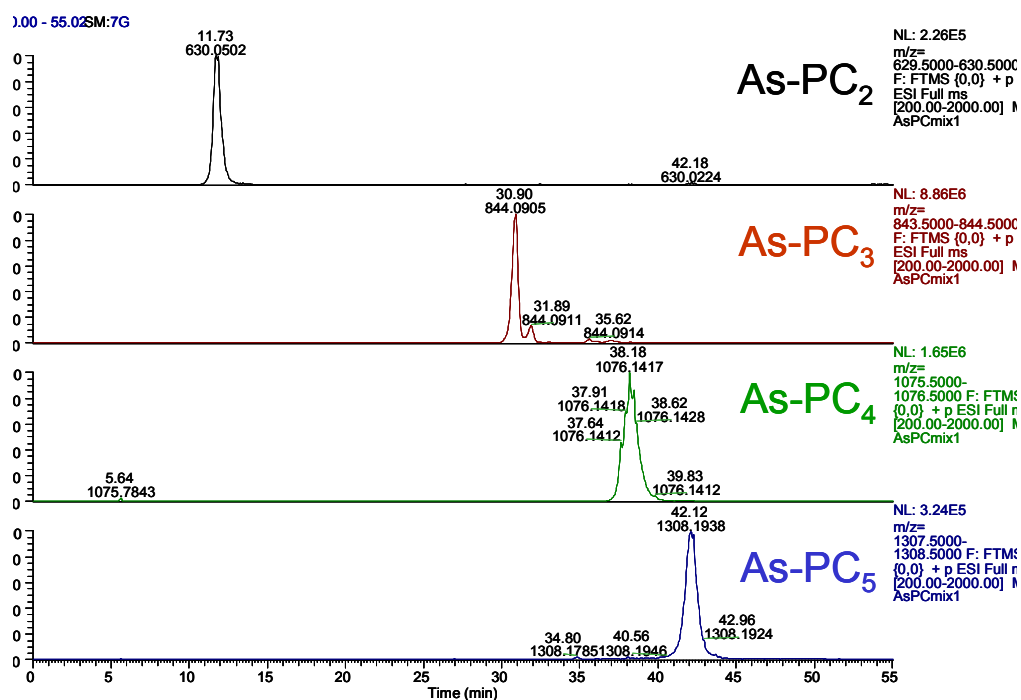


Figure 4.12 Extracted Ion Chromatograms for individual As-PC_n, n = 2-5 from RP-LC-ESI-Orbitrap MS.

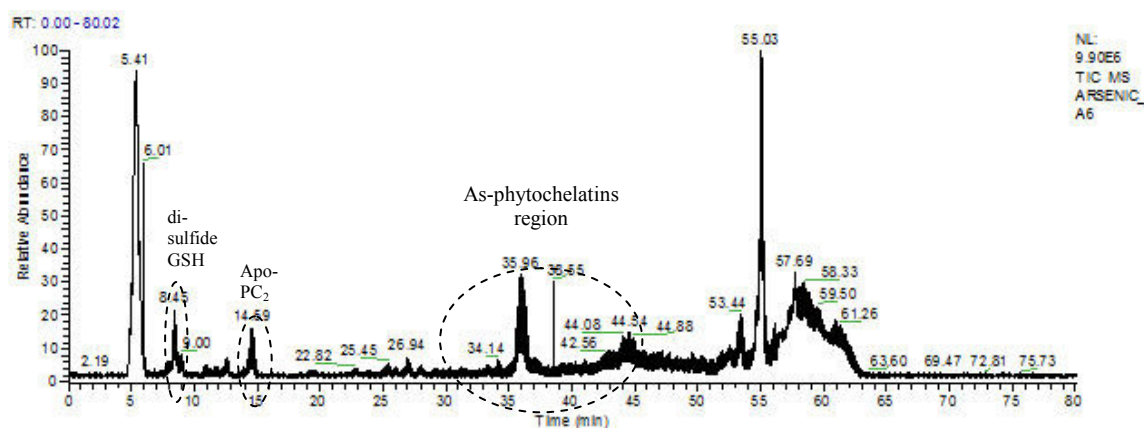
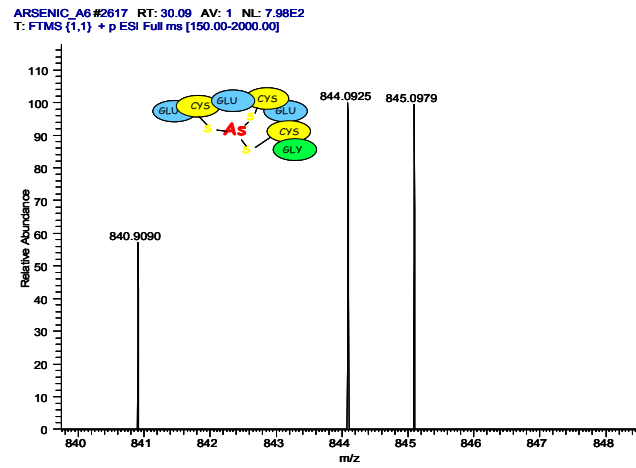


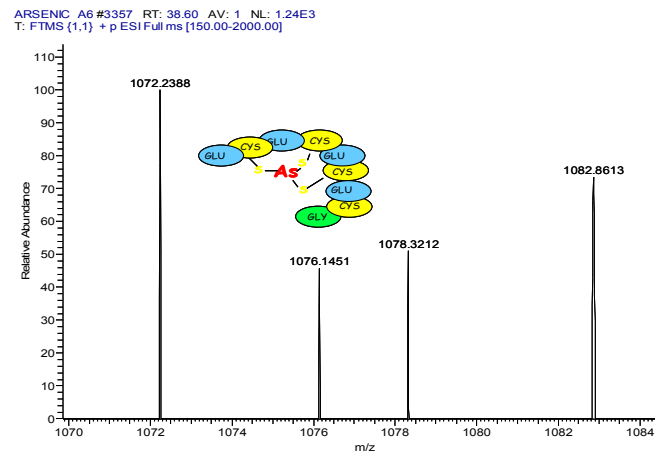
Figure 4.13 The RP-LC-ESI Orbitrap MS total ion chromatogram obtained for an extract of *A. thaliana* leaves with 5 mg L⁻¹ As exposure.

The total ion chromatogram of RP-LC-ESI Orbitrap MS obtained for the leaf extract is shown in Fig 4.13. The presence of As-PC₃, As-PC₄, and As-PC₅ in the leaves of *A. thaliana*, supplemented with 1-5 mg As L⁻¹ was revealed using RP-LC-ESI Orbitrap MS. The retention times of these As-PCs complexes in the plant extracts correspond to those of matrix-matched standards; 30, 38, and 42 mins for As-PC₃, As-PC₄, and As-PC₅, respectively. The longer the peptide chain coordinated with arsenic, the higher the degree of hydrophobicity of the mobile phase used. For this reason, As-PC₅ was eluted later than As-PC₃ and As-PC₄, respectively. The mass spectra showing the presence of As-PC₃, As-PC₄ and As-PC₅ are presented in Fig 4.14. The comparison between the observed and theoretical protonated molecular masses of these As phytochelatin complexes are presented in Table 4.7. The mass accuracy between both masses within the range of 5 ppm enables the identification of arsenic phytochelatin complexes present in the plant leaves. However, the product ions of the phytochelatins were not detected by RP-LC-ESI Orbitrap MS. These might be due to the presence of low concentrations of analytes and high background signals from sample matrix.

(a)



(b)



(c)

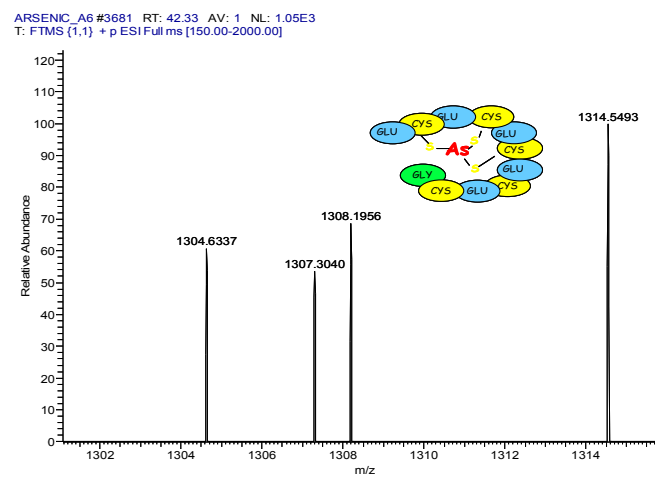


Figure 4.14 The RP-LC-ESI Orbitrap MS spectra showing the presence of (a) As-PC₃ (b) As-PC₄ (c) As-PC₅.

Table 4.7 The RP-LC-ESI Orbitrap MS results showing the comparison between the measured and exact protonated molecular masses of the As-PC₃, As-PC₄ and As-PC₅ compounds found in the plant leaves, exposed to 5 mg As L⁻¹.

Standard					
Standard	Retention Time (min)	Proposed Compound (formula), [M+H] ⁺	Theoretical m/z [M+H] ⁺	Observed m/z [M+H] ⁺	Mass accuracy (ppm)
As-PC ₂	10.01	C ₁₈ H ₂₉ O ₁₁ N ₅ AsS ₂	630.0515	630.0538	3.52
As-PC ₃	29.72	C ₂₆ H ₃₉ O ₁₄ N ₇ AsS ₃	844.0928	844.0942	1.75
As-PC ₄	38.16	C ₃₄ H ₅₁ O ₁₈ N ₉ AsS ₄	1076.1445	1076.1466	1.93
As-PC ₅	42.24	C ₄₂ H ₆₃ O ₂₂ N ₁₁ AsS ₅	1308.1968	1308.1981	1.38
Sample					
Sample	Retention Time (min)	Proposed Compound (formula), [M+H] ⁺	Theoretical m/z [M+H] ⁺	Observed m/z [M+H] ⁺	Mass accuracy (ppm)
<i>A. thaliana</i> (5 µg g ⁻¹)	42.19	As-PC ₅ (C ₄₂ H ₆₃ O ₂₂ N ₁₁ AsS ₅)	1308.1963	1308.1987	1.85
	42.33			1308.1956	-0.58
	42.63			1308.1974	0.82
	38.28	As-PC ₄ (C ₃₄ H ₅₁ O ₁₈ N ₉ AsS ₄)	1076.1445	1076.1494	4.53
	38.60			1076.1451	0.56
	29.68	As-PC ₃ (C ₂₆ H ₃₉ O ₁₄ N ₇ AsS ₃)	844.0928	844.0951	2.83
	29.72			844.0950	2.69
	30.09			844.0925	0.34

Apart from As-PC₃₋₅ found in the formic extract, protonated precursors were observed at $t_r \sim 8.4$ min (m/z 613.1609) for oxidized glutathione (2.7 ppm error), with its fragment ions at m/z 484.1177, 355.0746, 231.0439, shown in Fig 4.15. Furthermore, at $t_r \sim 14.6$ min (m/z 538.1281), apo-PC₂ (forming a disulfide bridge, 0.7 ppm error) with its fragment ions at m/z 409.0850 (loss of Glu), 334.0528 (loss of

Glu-Gly) and 306.0580 (loss of Glu-Cys) were also found in the extracts. These compounds have been previously reported [28, 94, 131, 181, 186].

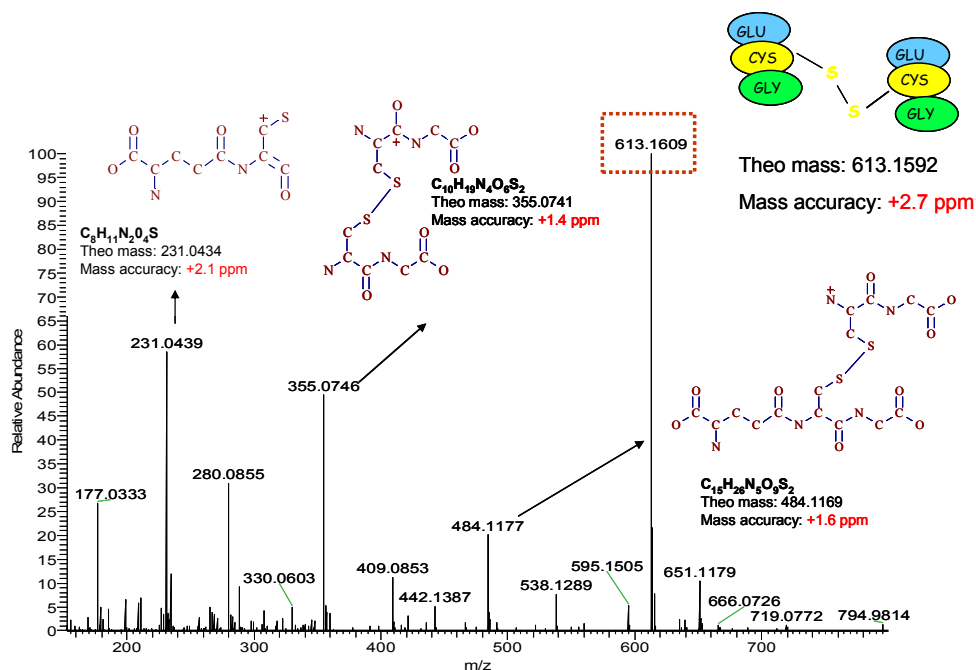


Figure 4.15 The RP-LC-ESI Orbitrap MS/MS chromatogram showing typical product ion spectrum of oxidized glutathione (GSSG).

This study shows the presence of a tiny amount of As-PC₃, AsPC₄, and As-PC₅ complexes in the leaf extracts of *A. thaliana* following the exposure to 1-5 mg L⁻¹ As. Moreover, there is availability of free inorganic As ions, oxidized glutathione and apo-PC₂. The question of how the arsenic phytochelatins in the leaves occur is still unclear. There have been no reports on the presence As-PCs and arsenic glutathione species detected in xylem or phloem saps. However, several studies have shown some interesting findings; trace levels of PCs and oxidized glutathione from As-exposed sunflower [27] and tiny amounts of PCs from Cd-exposed *Brassica napus* [187] were found in the xylem sap, whereas high levels of GSH, PCs and Cd were detected in the phloem sap of *B. napus* [187]. The presence of trace levels of As-PCs in the leaves might be derived from the translocation of a small proportion of As-PCs complexes, which were not sequestered from root vacuoles. A large proportion of total arsenic (almost 70%) in the root of *A. thaliana* (wild type) was recently reported in the forms of As-(PC₂)₂, As-PC₃ and As-PC₄ complexes [30]. However, the presence of small levels of As-PCs complexes in the plant leaves might imply their stability of the As-

PCs in the roots, which preclude from the translocation. The formation of a tiny amount of As-PCs complexes might occur following the uptake of free As(III) and apo-PCs *via* phloem and xylem into the plant leaves.

The presence of a small proportion of As-phytochelatin complexes is not surprising because it is known that most of inorganic As taken up is stored in plant roots as GSH/ phytochelatin complexes [28]. Raab *et al* (2005) reported the presence of GS-As-PC₂ and As-PC₃ in roots, stems and leaves of sunflower (*Helianthus annuus*) [27]. A diversity of As species and large amounts were found more evident in the root.

Despite the fact that As^{III}-PC complexes have a small peptide chain of Glu-Cys-Gly in common, there was no evidence for arsenic glutathione species present. It might be due to the more favorable binding of As with vicinal sulfhydryl group than that with monodentate sulfhydryl compound. Therefore, it seems that the complexation or detoxification of As is ascribed to PCs rather than GSH.

4.6 SUMMARY

Phytoremediation is an attractive alternative to eliminate toxic metal(loid)s from contaminated soils. Hydroponically grown and supplemented with different levels of elements under investigation, *Arabidopsis thaliana*, a well known accumulator, was used to study not only the capability of accumulating As, Se, and Hg, but also the effect of Se on As and Hg incorporated into the leaves. The capability of appreciably accumulating As, Se and Hg stored in the leaves is very impressive for potentially remediating media. The co-exposures of Se-As and Se-Hg (1-5 mg L⁻¹) in plants for 7 weeks were indicated that selenium has no positive influence on the arsenic incorporated into the leaves. To the authors' knowledge, no one reported arsenic speciation in *A. thaliana* before, so this study was focused on development of metallomics approaches for arsenic speciation in the leaves.

The selection of extracting solutions (20 mM ammonium acetate, pH 7.5 and 1% formic acid) and extraction time for the plant leaves were investigated. It was found that there were no differences in As extraction efficiencies (approx. 70% of total As) for 2, 5, and 12-h sonication time using the ammonium buffer. Despite the fact that a lower extraction efficiency of As (approx. 60% of total As) was achieved using extraction with 1% formic acid in an ice bath for 1 h, the comparative SEC-ICP-MS As profiles obtained by leaching with both extracting solutions indicated the less

harsh extraction with formic acid. Due to the choice as a compromise between extraction efficiency and preservation of the compound entity, the use of 1% formic acid in a cooled condition makes it possible to reduce the conventional extraction time from 12 h [92] to only 1 h.

Water-soluble As species in the plant leaves can be separated and detected by AE-LC-ICP-MS. Preliminary study on species identification revealed that a majority of As species were found to be inorganic As, accounting for almost 90% of the total As in the extracts, whereas a small amount of DMA and the other As species (found in the vicinity region of AsB) were observed.

Reversed phase chromatography performed on Dionex acclaim[®] organic acid OA was successfully used to separate As-phytochelatin complexes, which were eluted as a cluster of peaks (t_r 30-45 min) with 20-35% MeOH content in the mobile phase. The use of RP-LC-ICP-MS alone was found to be difficult to identify individual As-PC peak due to a lack of discrete As-PCs availability. In this case, not only ICP-MS offering selective elemental information, but ESI-MS providing molecular information is also needed. The complementary use of reversed phase HPLC with ICP-MS and ESI Orbitrap MS/MS proved to be a powerful tool to obtain essential information on the presence of As-phytochelatin complexes (As-PC₃₋₅) in the leaves of *A. thaliana* following As exposure. The importance of accurate mass measurements (with Orbitrap) of precursor and fragment ions (obtained by collision induced dissociation) to minimize ambiguity in plant species identification has been demonstrated due to the possibility of a diversity of biomolecules of similar composition and relative low stability present.

Although small amounts of arsenic phytochelatin complexes in the leaves were found in this study, the presence of a majority of As species in wild type of *Arabidopsis* roots (69%) being complexed as As-(PC₂)₂, As-PC₃ and As-PC₄ was reported [30]. This suggests the stability of As-PCs in roots, which precludes their root-to-shoot translocation. It is known that the restriction in As translocation from roots to shoots is ascribed to the sequestration of As-PCs in the root vacuoles. Liu *et al* (2010) indicated that phytochelatin complexes influence arsenic mobility in plants [30]. They found that the reduction in As-PCs complexes in *Arabidopsis* roots resulted in the increasing As mobility. Therefore, it is probable that enhancing PC-synthesis in roots may be an effective means to decrease As concentrations in the edible part of food crops. In addition, phytochelatin plays a role in As detoxification.

Enhancing the induction of phytochelatin synthesis in plants might help increasing their tolerance.

CHAPTER 5

DEVELOPMENT OF METHODOLOGIES FOR ARSENIC SPECIATION IN EDIBLE MARINE ALGAE

Edible marine seaweed is consumed worldwide either raw or in cooked foods, and also as an ingredient in ice cream, mayonnaise, cheese and chocolates [188]. Despite the fact that seaweed is regarded as being of high nutritional value with an abundance of carbohydrates, proteins, fibers, vitamins as well as minerals, the presence of high As content ($> 1 \text{ mg kg}^{-1}$) raises a considerable concern for human health. By means of detoxification, algae can transform inorganic arsenic incorporated from surrounding seawater to various arsenic species, mainly arsenosugars. Occurring at high concentrations (up to 100 mg kg^{-1} wet weight) in algae [20], arsenosugars are believed to originate from alkylation of arsenate in the organism. The consumption of edible marine algae is deemed to be a significant route of arsenic exposure. Due to the fact that metabolism pathways of arsenosugars are still not established, questions in association with possible toxic aspects of the algae consumption arise.

Because the bioavailability of As depends not only on the matrix, but also on its chemical species, the speciation of bioaccessible As in algae is required to precisely identify and specify the different As species involved in the gastrointestinal tract. Ideally, bioavailability should be evaluated in human studies. However, *in vivo* studies in both human and animals are considered to be costly and ethnically controversial. Many studies have preferred and evaluated the bioavailability of minerals using a simulated *in vitro* gastrointestinal approach, since it is not only simple, rapid, inexpensive, but also gives more reproducible results. Information on arsenic speciation in gastrointestinal digestion might contribute to the occurrence of DMA(V) found in urinary excretion by degradation of arsenosugars consumed [51, 53]. Taking advantage of this approach, it is also worthwhile investigating arsenic species occurring in the digestion to assess potential health risks for algae consumers.

5.1 ALGAE SOURCE

Provided by a local manufacturer, edible marine algae used in this study were harvested in the Galician coast (Northwestern, Spain). Four marine algae comprise two brown algae: *Undaria pinnatifida* (Wakame) and *Laminaria ochroleuca* (Kombu), a red algae: *Porphyra umbilicalis* (Nori), and a green algae: *Ulva rigida*

(Sea lettuce) were investigated. These algae samples are commercially dehydrated products. Approximately 100 g of each sample were dried in an oven at 40°C to eliminate water traces prior to pulverization in an agate mortar. The four homogenized algae samples were subsequently preserved in polyethylene bottles at -20°C.

5.2 SAMPLE PREPARATION

5.2.1 Closed-vessel microwave digestion

Using a mini CEM unit and the microwave conditions used in section 4.2.1, the total As digestion in all four homogenized algae samples were carried out using 0.05 g of sample and 1 mL of a mixture of 1:1 (v/v) HNO₃ and H₂O₂ added to the digestion vessels. The reason why small amounts of samples were used in the digestion is due to the limitation of the microwave vessel size and the digestion condition used. Following the digestion, all digests were made up to 5 g with de-ionized water. There are currently several marine algae CRMs available (BCR279 *Ulva lactuca* and IAEA-140 *Fucus sp.* plant homogenate) for the total arsenic measurement; however, there were unavailable in the laboratory. As QC samples, rice flour (NIST1568a) and pine needles (NIST1575) were used to check the suitability of digestion and measurement methods used. It is important to note that the use of similar matrix is strongly recommended for QC samples. In addition, the similar digestion method was applied to the algae extracts and dialyzates, the dialyzable solutions following the dialysis process used to predict the arsenic bioaccessibility (Section 5.2.3). This was performed using 0.2 g of algae extracts obtained from Wakame, Kombu, Nori, and 0.4 g of Sea lettuce extract, while for all algae dialyzates, 0.5 g was used for the digestion. Triplicate of all sample digests were performed for total As measurement.

5.2.2 Arsenic extraction methods

Despite the fact that water is the most recommended extractant for polar or ionic arsenic species, mixtures of MeOH and water are reported to be the most extensively used for the As extraction. In this study, it is worthwhile investigating the effect of extracting solutions on the As extraction efficiency and its speciation.

Two extracting solutions: 20 mM NH₄OAc, pH 7.4 and 1:1 (v/v) of a mixture of MeOH and water were compared in terms of As extraction efficiencies and the distribution of As species in the extracts. The extraction procedures was performed in

triplicate by sonicating the mixtures of samples and both extractants for 1 h, where 0.05 g of Kombu, 0.15 g of Wakame and Nori, and 0.20 g of Sea lettuce were added with 5 mL of degassed extractants in centrifuge tubes. The sample size used is dependent on their As concentrations in the sample. Following extraction, samples were centrifuged at 3,000 rpm for 30 min. Extracts obtained from 50% MeOH were evaporated in order to eliminate MeOH at 40°C by a TurboVap LV concentration and evaporation workstation (Zymark Corporation, Hopkinton, MA, USA), which was operated under an inert nitrogen atmosphere, and subsequently diluted with 5 mL of de-ionized water. Supernatants were decanted and filtered through 0.45 µm cellulose membranes prior to analysis. The study on the effect of sonication time on As extraction at 1, 2, 4 and 6 h was investigated using NH₄OAc as an extractant.

5.2.3 Arsenic bioaccessibility by a simulated *in vitro* gastrointestinal method

One of the most extensively used, the *in vitro* dialysis method developed by Miller *et al* (1981) [62] has proved to be a promising means of providing bioavailability measurement, correlating well with results from *in vivo* methods [189-191]. However, in order to obtain a final digest/dialyzate pH of 6.5-7.5 in digest/dialyzate systems, corresponding to a physiological pH of intestine, a modification of the *in vitro* Miller's method, introduced by Haro-Vicente *et al* (2006) [192], was used for estimation of the As bioavailability in algae samples. This can be achieved by adjusting the pH of the 0.15 M PIPES buffer (piperazine-N,N-bis(2-ethane-sulfonic acid) disodium salt) to 7.5 with HCl as a dialyzing solution to obtain the desirable final pH of digest/dialyzate. The optimization of pH of 0.15 N PIPES for the studies in algae samples was reported (Appendix 3) [70].

The details of the chemicals and enzymes used are as follows: Porcine pepsin, P-7000, porcine pancreatin, P-1750, bile salts (approx. 50% sodium cholate and 50% sodium deoxycholate) and PIPES were obtained from Sigma Chemicals (St Louis, MO, USA). Hydrochloric acid from Panreac (Barcelona, Spain) and sodium hydrogen carbonate (NaHCO₃) from Merck (Darmstadt, Germany) were used to prepare the gastric solution and intestinal solution, respectively.

The bioavailability of arsenic in all raw marine algae was determined by *in vitro* dialysis method, consisted of two parts based on the simulation of the gastrointestinal digestion of food with pepsin enzyme during the peptic digestion and pancreatin-biliary salts during pancreatic digestion. Peptic digestion was carried out

by the addition of 20 mL of ultrapure water into 0.5 g of seaweed powder contained in a 100 mL Erlenmeyer flask and left for 15 min. A 6 M HCl solution was added to the mixture to obtain pH 2.0, followed by the addition of 0.15 g of freshly prepared gastric solution prepared by 6.0% (w/v) pepsin dissolved in 0.1 M HCl. The reaction flasks were covered with lids and allowed to incubate at 37°C in a Boxcult incubator situated on a Rotabit orbital-rocking platform shaker (J.P. Selecta, Barcelona, Spain) set up at 150 rpm for 120 min. When the incubation time was terminated, it was placed in an ice bath to stop the enzymatic reaction. In the next step, the simulated pancreatic digestion was initiated by the addition of 5 mL of intestinal solution, prepared by dissolving a mixture of 0.4 g of pancreatin and 2.5 g of bile salts in 100 mL of 0.1 M NaHCO₃. At this point, a dialysis membrane (Cellu Sep[®] H1 high grade regenerated cellulose tubular membrane with molecular weight cut off 10 kDa, 50 cm length, dry diameter 25.5 mm and 5.10 mL cm⁻¹) from Membrane Filtration Products Inc (Texas, USA), filled with 20 mL of a 0.15 N PIPES solution adjusted pH to 7.5 with HCl, was immersed into the reacting mixture with 150 rpm shake at 37°C and allowed As species to diffuse for 120 min dialysis, which is believed to be lifetime for the absorption in small intestine. Once finished, the enzymatic reaction was again terminated by immersing the flask in an ice bath. The membrane was removed and its outer surface was rinsed with ultrapure water. The membrane containing dialyzable solution (dialyzate) and the residual or non-dialyzable slurry remaining in the flask were later transferred to polyethylene vials and weighed separately. The schematic diagram of the simulated *in vitro* gastrointestinal method is presented in Fig 5.1. Both dialyzable and residual fractions were stored in a freezer at -20°C before measurements. The dialyzable fractions of arsenic diffusing through the dialysis membranes during pancreatic digestion were measured to predict the arsenic dialyzability and to investigate arsenic speciation following the digestion.

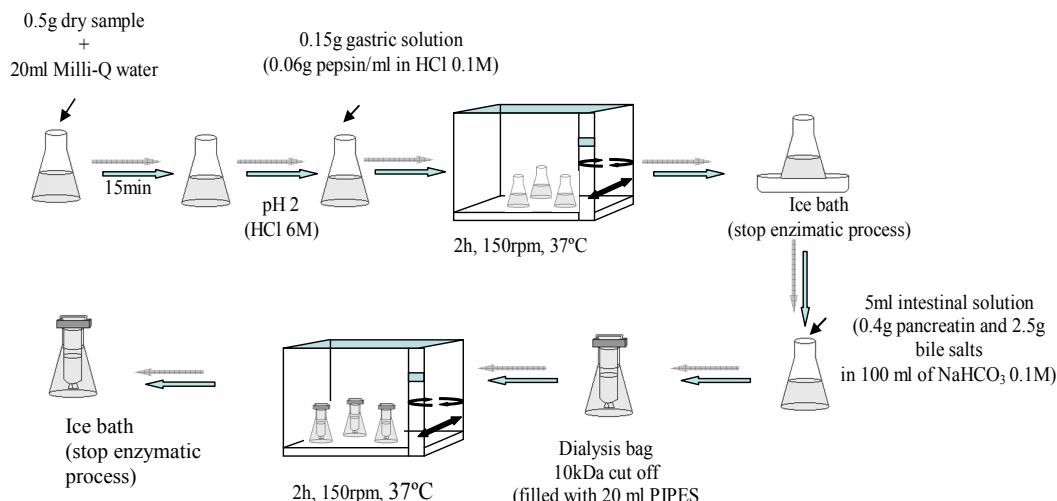


Figure 5.1 Diagram of the simulated *in vitro* enzymatic digestion for arsenic bioaccessibility study.

5.3 DETERMINATION OF TOTAL ARSENIC USING HE-MODE ICP-MS

5.3.1 Instrumental setup

The ICP-MS used was equipped with collision cell. It was fitted with Pt/Ni sampler and skimmer cones due to the corrosive nature of the digests. The system has two rotary pumps and an ASX-510 autosampler (Cetac Technologies, USA). The sample digests and standards were all introduced using the autosampler system with a peristaltic pump and a mixer block. The solution from the mixer block passed through a micro flow concentric nebulizer with a double flow chilled (2°C) spray chamber.

A 10 $\mu\text{g g}^{-1}$ arsenic stock solution was prepared from standards purchased from Romil. Working As standards for external calibration were prepared in 10% HNO₃ in the As concentration range from 0-200 ng g^{-1} , with 10 ng Rh g^{-1} as an internal standard. The determination of total As was performed using He mode at the flow rate of 4.2 mL/min. In order to minimize the ArCl^+ interferences, a tuning solution with 1% HCl was used with m/z at 75 monitored. A 10% HNO₃ solution was employed as a rinsing solution to alleviate the memory effect from As. To determine As concentration: ^{75}As , and ^{103}Rh , were monitored. The calculation of sample concentrations was processed by the Fileview32 software. The reported concentrations were automatically calculated following correction using ^{103}Rh internal standard. For quality assurance of both procedures for microwave digestion and ICP-

MS measurements/external calibration quantification with ^{103}Rh as an internal standard, certified reference materials (NIST 1575, pine needles and NIST 1568a, rice flour) were analyzed (as QC samples) together with the four edible marine algae. The results of total As in the CRMs were in a good agreement with their certified values. The total concentration of arsenic as determined was 0.23 ± 0.02 and $0.27 \pm 0.01 \mu\text{g g}^{-1}$ dry sample, for NIST 1575 (cert. 0.21 ± 0.04) and NIST 1568a (cert. 0.29 ± 0.03), respectively.

5.3.2 Optimization of arsenic extraction

The comparison of As extraction efficiencies obtained from 20 mM NH_4OAc and 50% MeOH solutions in all four algae by 1-h sonication were performed. As shown in Table 5.1, it was found that the use of ammonium acetate solution can provide higher As extraction efficiencies than that of 50% MeOH. This means As species existing in the selected marine algae seem to be extracted in the more polar extractant. In other words, the capability of extracting As species using 50% MeOH alone was found to be limited.

Table 5.1 The comparison of the extraction efficiencies using 20 mM NH_4OAc , pH 7.4 with those obtained by 1:1 MeOH/water in four marine algae (Kombu, Wakame, Nori and Sea lettuce).

Sample	% As extraction efficiencies (n=3)	
	NH_4OAc extracts	1:1 MeOH/water extracts
Kombu	55.9 ± 1.9	19.5 ± 4.8
Wakame	43.3 ± 1.9	8.1 ± 1.4
Nori	61.4 ± 3.3	45.3 ± 2.4
Sea lettuce	40.0 ± 0.8	32.0 ± 0.9

For 1-h sonication, the efficiencies of As extraction were found in the range between 40% to 60%. In order to improve the As extraction efficiency, the effect of sonication time was investigated. Two marine algae: Kombu and Wakame were chosen for this study. Results shown in Table 5.2 revealed that no significant differences of As extraction were found when different sonication times at 1, 2, 4, and

6 h were applied. Using a sonication bath, the As extraction for 1 h in the algae was found to be optimal. It is probable that some of unextractable As species (50%) might strongly bind with cell wall components/ or proteins. Differences between algae are thought to be responsible for the variation of arsenic extraction efficiencies, due to morphological variability and structural complexity. Rubio *et al* (2010) suggested that differences in chemistry between three types of algae (green, brown, and red) are associated with the chemical composition of cell walls [108]. It is worthwhile developing such well-defined protocol appropriate for extracting specific species of algae. Moreover, there is possibility of the presence of a wide variety of arsenic species in marine algae (from non-polar to polar species). Morita and Shibata (1988) purified the chloroform fraction of a brown algae (Wakame) and identified the presence of a phosphatidyl-arsenosugar, which is a class of arsenolipids following alkaline digestion [112]. In their subsequent comprehensive study, it was indicated that arsenic bound to lipid can contribute up to 50% of the total arsenic in algae [193]. The use of sequential arsenic extraction with at least two extractants having different polarity such as water and chloroform might help to improve the arsenic extraction efficiency in the samples.

Table 5.2 Optimization of sonication time using the NH_4OAc buffer as extractant.

Sample	Sonication time/h	Total extractable As ($\mu\text{g g}^{-1}$, n=3)	% As extraction from the solid (n=3)
Kombu	1	31.0 ± 1.2	55.0 ± 2.5
Kombu	2	31.9 ± 1.8	56.5 ± 3.5
Kombu	4	31.2 ± 1.6	55.4 ± 3.1
Kombu	6	34.4 ± 1.1	61.1 ± 2.5
Wakame	1	17.7 ± 0.3	46.2 ± 2.2
Wakame	2	17.2 ± 1.9	45.0 ± 5.4
Wakame	4	18.0 ± 0.1	47.1 ± 2.1
Wakame	6	18.0 ± 0.2	47.0 ± 2.2

5.3.3 Comparison of quantitative analysis between total arsenic in solids, extracts and dialyzates

The total arsenic determination in acid digests of algae (solid), NH₄OAc extracts and dialyzates were measured by He-mode ICP-MS. As presented in Table 5.3, results show the total arsenic levels range from 4-57 $\mu\text{g g}^{-1}$, in which the highest concentration was found to be Kombu, whereas the lowest appeared to be Sea lettuce. Using the ammonium acetate solution as an extractant, arsenic extraction efficiencies were overall found to be more or less 50%. The bioaccessibility of As in algae can be estimated by the *in vitro* gastrointestinal digestion using the percent dialyzability. This method is believed to be useful for implication of toxic effect from consumption of algae. It was found that less than 20% of dialyzable amounts of As were detected, suggesting a majority of As-species in algae samples were impermeable through the dialysis membranes. There are two possible reasons for these observations. Firstly, some of non-dialyzable As might still bind with algae matrix following the pancreatic digestion. In other word, the use of simulated *in vitro* gastrointestinal digestion did not improve releasing small arsenic species bound to algae matrix. Another reason is that although some As species were soluble but the permeability was restricted to their molecular sizes and the dialysis process. It is reasonable that sizes of arsenic molecules larger than the cut-off membrane pore sizes (10 kDa) are not allowed to pass through the membrane. Despite the fact that the molecular sizes are smaller than the membrane pore, the diffusion transport of arsenic species is limited by the dialysis between the two liquid layers (inside and outside the membrane).

Using the solubility method, the study of arsenic bioaccessibility in seaweed was reported [61]. For raw samples, the bioavailability of arsenic was varied in this order: *Porphyra sp.* (87%) > *H. fusiforme* (53%) > kelp powder (45%) > *U. pinnatifida* (38%). A slight increase in the bioaccessibility was found in boiled *H. fusiforme* (57%), and baked *Porphyra sp.* (106%). In addition, the results of arsenic extraction efficiencies in raw algae, obtained using 50% MeOH as an extraction with 15-min shaking, were presented: *Porphyra sp.* (73%) > *H. fusiforme* (14%) > kelp powder (63%) > *U. pinnatifida* (49%). These results suggested that the use of *in vitro* gastrointestinal digestion can help arsenic being released from algae matrix for *Porphyra sp* and *H. fusiforme*, but not for kelp powder and *U. pinnatifida*. It is not surprising that the bioaccessibility results obtained by the solubility method are higher than those obtained by the dialyzability method, with the reason of the permeable

limitation. The bioaccessibility results of raw *U. pinnatifida* (Wakame) obtained by the dialyzability method were found to be merely one-thirds of those obtained by the solubility method. Considering the fact that the proportion of the volume of dialyzing solution filled in the dialysis membrane to that of the reacting mixture outside the membrane is 20 mL/ 25mL (see Fig 5.1), small soluble arsenic species in the reacting mixture should be able to diffuse into the solution within the dialysis membrane, when reaching 2-h time set for lifetime in small intestine, with the ratio of 44% of As dissolved in the dialyzable fraction, and 56% of As remaining in the residue fraction. One of the most critical parameters for determining the permeability of metal(loid)s into dialysis membrane, the matrix effect, possibly from alginate gel compounds [194], may prevent the diffusion of arsenic species during the time set.

Using *in vivo* studies in seaweed-eating sheep under a controlled feeding trial, it was found that large amounts (>86%) of arsenic were retained and bioavailable and only 13% of the total ingested was excreted, following consuming their diets containing a major arsenic species being arsenoribosides [56]. It was indicated that significant amounts of arsenic ingested were absorbed and preferably accumulated in the lipid tissues such as muscle fat, liver fat and kidney fat (see Section 2.2.4). The high bioavailability of arsenic in seaweed, which was studied in herbivores, might be ascribed to the presence of specific digestive enzymes, capable of effectively cleaving main plant components strongly bound to arsenic such as cellulose, to release from the matrix.

Although the use of *in vitro* dialysis method has been proven to be a promising means of providing availability measurement in minerals such as iron, zinc, selenium and calcium, correlating well with results from *in vivo* methods [189-191], comparative studies of the As bioavailability obtained by human *in vivo* and *in vitro* dialysis methods have not been proved. Although most of human *in vivo* experiments have been investigated in synthetic arsenosugars [52, 58, 60, 195], there is currently less information available on the bioavailability of As in real seaweed samples. It is known that arsenosugars are bio-accessible, readily taken up and metabolized when ingested. However, the bioavailability of As in algae matrix is largely dependent on the matrix, which strongly influences the absorption by intestinal mucosa. Furthermore, it is possible that the absorption mechanism of such a toxic element in human body is different from other essential minerals. The digestion process in human, which is considered to be dynamic, might provide different results from the

use of the *in vitro* dialysis method, which is performed in a batch system. Therefore, these results obtained by the *in vitro* dialysis method need to be proved with results obtained by human *in vivo* studies to assess the accuracy of the method.

Table 5.3 Results of total determination of As in four marine algae (solids), NH₄OAc extracts and dialyzates.

Algae	Total As ($\mu\text{g g}^{-1}$, n=2)	NH ₄ OAc extraction		Dialyzability	
		Extractable	% Extraction	Dialyzable	%
		As ($\mu\text{g g}^{-1}$, n=3)	efficiency (from solid)	As ($\mu\text{g g}^{-1}$, n=3)	Dialyzability (from solid)
Kombu	56.4 ± 1.4	31.5 ± 0.7	55.9 ± 1.3	7.6 ± 1.1	13.5 ± 1.9
Wakame	38.3 ± 1.7	16.6 ± 0.0	43.3 ± 0.1	4.4 ± 0.8	11.4 ± 2.1
Nori	48.8 ± 2.5	30.0 ± 0.4	61.4 ± 0.7	8.0 ± 1.3	16.4 ± 2.8
Sea lettuce	4.82 ± 0.05	1.93 ± 0.03	40.0 ± 0.7	0.56 ± 0.07	11.6 ± 1.4

5.4 DEVELOPMENT OF HPLC COUPLED TO ICP-MS FOR ARSENIC SPECIATION IN EDIBLE MARINE ALGAE

5.4.1 Size exclusion chromatography-inductively coupled plasma mass spectrometry (SEC-ICP-MS)

A study of arsenic speciation in algae extracts using SEC-ICP-MS was performed as described in the section 4.4.1. The overlaid SEC-ICP-MS As profiles of NH₄OAc and 50% MeOH extracts for all 4 types of algae, which were diluted 3 times, were compared in Fig 5.2. The SEC-ICP-MS As profiles of both extracts were found to be similar. Due to the fact that NH₄OAc extraction gave better As extraction efficiencies in algae observed, the more intense As peaks were observed in the NH₄OAc extracts of Kombu and Wakame. However, for Nori, the use of 50% MeOH shows slightly higher intensity of SEC-ICP-MS As profiles than that of NH₄OAc, while comparable responses were found in Sea lettuce. It is probable that methanol used for As extraction remained following the evaporation of MeOH; therefore it positively affected the ionization of As in the plasma due to charge transfer effect. Under investigation, there were two main As peaks appeared at the retention time 16.0 and 17.7 min in all algae extracts, which are corresponding to the peaks found in arsenosugar standards. The former As peaks in Kombu, Wakame, and Nori were

found to be dominant over the latter, whereas in Sea lettuce the latter became significant.

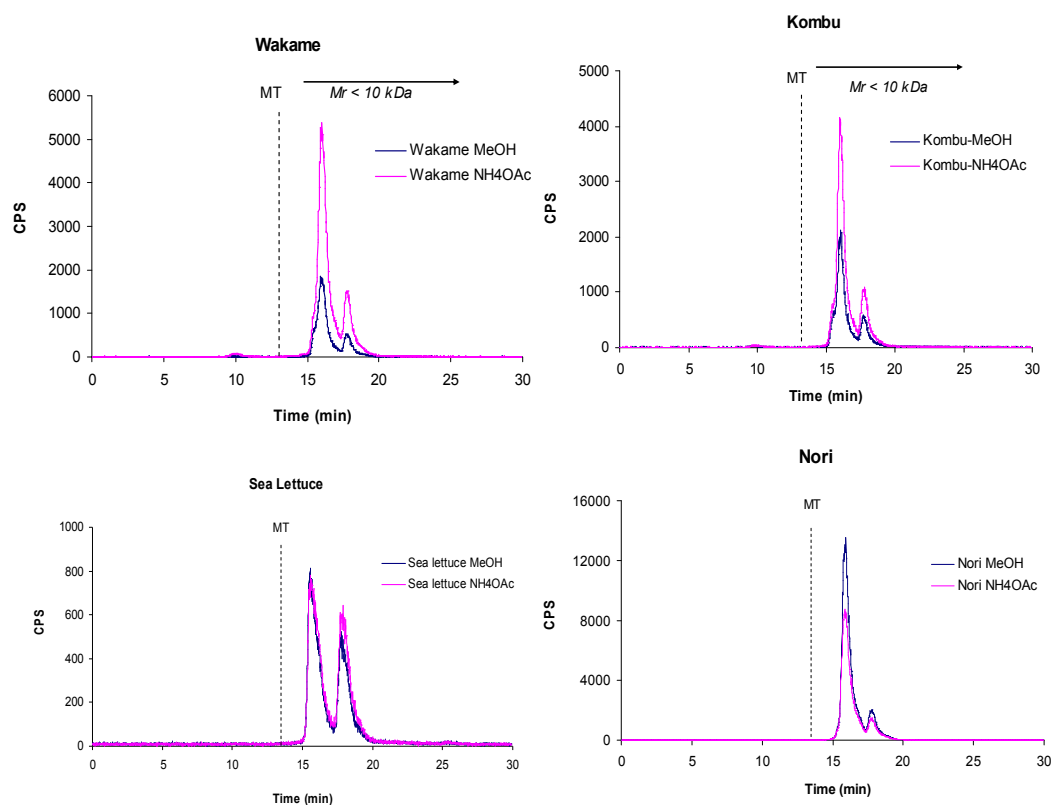


Figure 5.2 The comparison of SEC-ICP-MS As profiles of 20 mM NH₄OAc extracts with 50% MeOH extracts for Wakame, Kombu, Sea lettuce and Nori.

5.4.2 Anion exchange liquid chromatography-inductively coupled plasma mass spectrometry (AE-LC-ICP-MS)

Most of arsenic speciation studies in algae samples rely on the ion exchange chromatographic separation using anionic and/or cationic columns due to the ionic character of As species present. Anion-exchange mechanisms have occasionally been employed not only for organoarsenicals and inorganic arsenic, but also for oxo-arsenosugars [61, 118, 120, 133, 142, 196, 197].

Wahlen *et al* (2004) developed an anion exchange LC-ICP-MS method using a Hamilton PRP-X100 column and the mobile phase consisted of 2.2 mM NH₄HCO₃ and 2.5 mM tartaric acid in 1% MeOH at pH 8.2 [135]. As illustrated in Fig 5.3, this method can successfully separate AsB, As(III), DMA, and As(V) in marine animals.

However, the resolution between AsB and As(III) separation is limited and problems can occur if samples contain high levels of AsB or As(III) species. It is therefore necessary to develop a chromatographic method offering better resolution for the separation. A chromatographic condition compatible with both ICP-MS and ESI-MS/MS is required to use for unambiguous species identification and molecular and structural verification of the compounds.

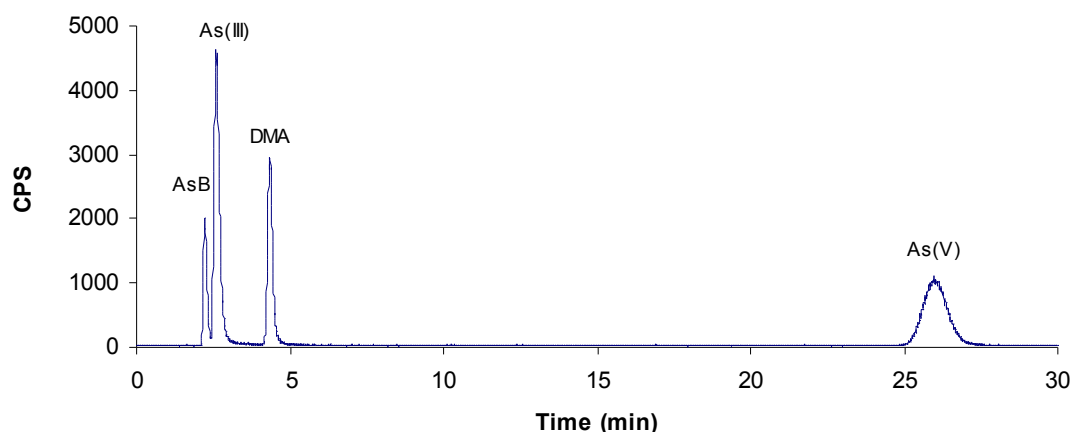


Figure 5.3 Chromatogram of a mixture of arsenic standards using chromatography: 2.2 mM NH_4HCO_3 and 2.5 mM tartaric acid with 1% MeOH at pH 8.2, Hamilton PRP X-100 column showing the separation of As species in the order of retention times: AsB 2.22 min; As(III) 2.59 min; DMA 4.34 min; and As(V) 26.0 min.

5.4.2.1 Mobile phase optimization

Hirata *et al* (2007) successfully separated four oxo-arsenosugars in marine algae using a Hamilton PRP X-100 using 20 mM NH_4HCO_3 solution (pH 8.4) as a mobile phase [118]. The volatile mobile phase containing ammonium hydrogen carbonate solution is considered to be compatible for sample introduction and on-line detection of arsenic species by both ICP-MS and ESI-MS/MS. It was reported not to cause signal drift due to physical clogging of the sampler or skimmer cones of the ICP-MS after prolonged use [139]. The use of alkaline pH of carbonate buffer as mobile phase was required for the control of chromatographic selectivity due to it can convert As(III) to its corresponding anion (pK_a 9.2), allowing As(III) to retain in the anion exchange column [198]. Furthermore, the chromatographic retention behaviour of the arsenic species in the column is also dependent on the concentration of the carbonate buffer

[199], due to ionic strength effect. Therefore, there is a potential to modify this condition to allow the proper separation of a wide variety of As species present in algae samples. Results of the optimization of mobile phase conditions such as pH and NH_4HCO_3 concentration performed on the separation of AsB, As(III), DMA and As(V) species using a 50 μL injection volume and isocratic elution at the flow rate of 1 mL min^{-1} are depicted in Fig 5.4-5.5.

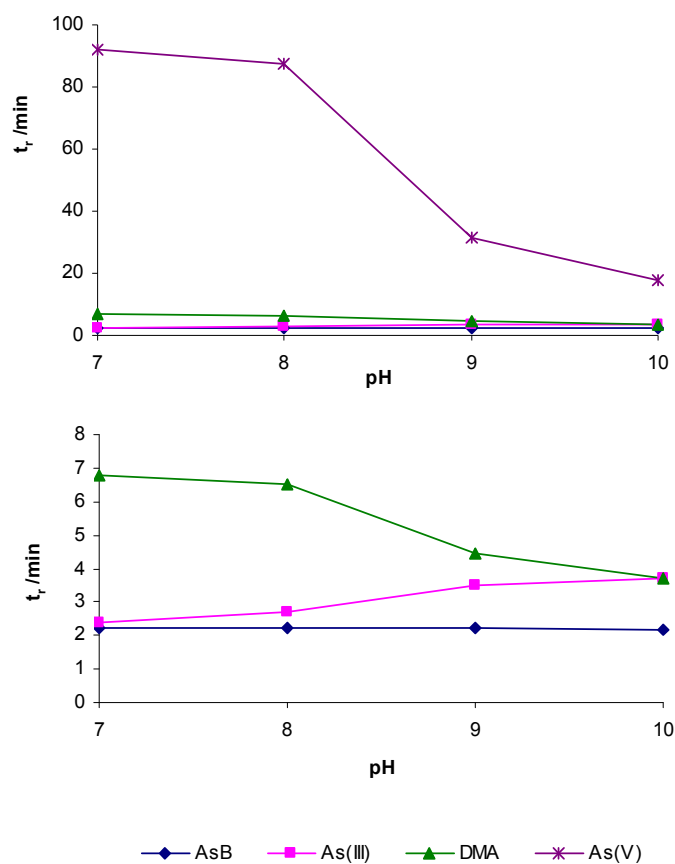


Figure 5.4 Optimization of pH of mobile phase (20 mM NH_4HCO_3 solution with isocratic elution 1 mL min^{-1} at pH 7, 8, 9 and 10).

It was found that at $\text{pH} < 8$, not only was As(V) eluted very late at $t_r > 80$ min, but also the resolution of AsB and As(III) seemed to be degraded, while at pH10, As(III) and DMA were not separated. It clearly show that at pH 9, AsB, As(III), DMA and As(V) could be separated with the acceptable analysis time (t_r for As(V) ~31 min). Thus, pH 9 was found to be optimal for further study.

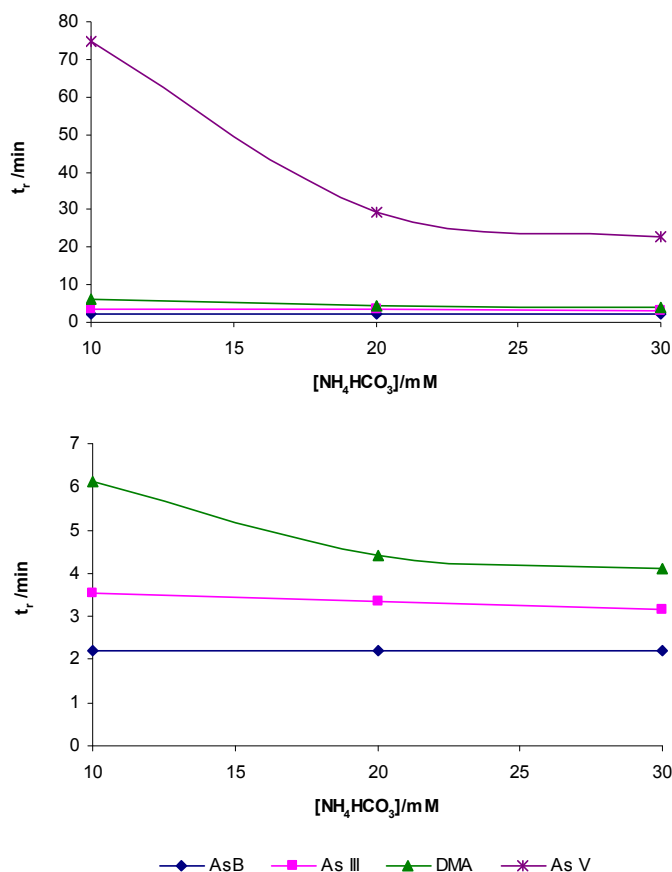


Figure 5.5 Optimization of mobile phase concentration at 10 mM, 20 mM and 30 mM NH_4HCO_3 , pH 9.

The use of 10 mM NH_4HCO_3 required long analysis times (> 70 min for As(V) to be eluted), which was inappropriate for the routine analysis. Moreover, long chromatographic separation might lead to the degradation of labile arsenic species in the column. Despite the fact that the mobile phase concentration at 30 mM provided good separation of AsB, As(III) and DMA and considerably shorter analysis time (t_r for As(V) ~ 23 min), to ensure adequate resolution of As-species separation in algae, 20 mM NH_4HCO_3 was chosen even at the expense of slightly longer analysis time.

In order to enhance the ionization of As species, the addition of 1% MeOH to the mobile phase was found to significantly improve the sensitivity for all As species investigated (2-3 fold increase in peak area). The chromatographic separation could be degraded for base-line resolution between AsB and As(III), when the MeOH concentration above 1% is used.

Naturally occurring As species in marine algae comprise not only organoarsenicals, but also arsenosugars, a main group of arsenic compounds present. As arsinoyl-ribose standards are not commercially available, their principal source is occasionally acquired by isolation and characterization using the natural product approach [117, 200]. Madsen *et al* (2000) prepared a large batch of brown algae extract from *Fucus serratus*, mainly containing four arsenosugars (1-4), by steeping the stripped algae in methanol, followed by the evaporation of MeOH to dryness [117]. The subsequent process involves with the pre-purification by washing with acetone and diethyl ether; the resulting aqueous solution was subsampled and then freeze-dried and stored at -18°C . Kindly donated by professor K. V. Francesconi, the freeze-dried *Fucus* extract, prepared as described [117], was used as check samples for arsenic speciation studies in marine algae. As illustrated in Fig 5.6, the four arsenosugars: arsenosugar1 (OH-ribose), arsenosugar2 (PO_4 -ribose), arsenosugar3 (SO_3 -ribose) and arsenosugar4 (OSO_3 -ribose) were obtained in the extract. The reported results of the quantification of four oxo-arsenosugars using LC-ICP-MS and LC-ESI-MS along with those using independent different techniques are presented in Table 2.9.

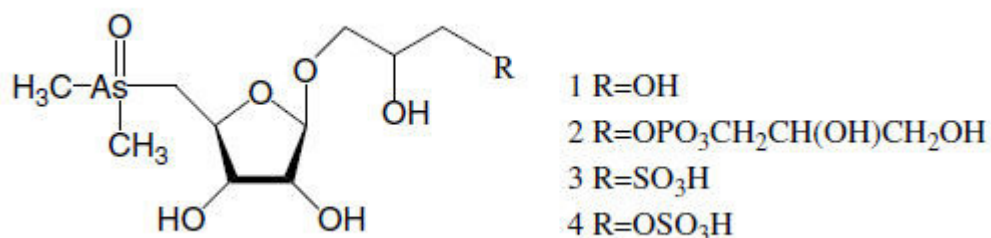


Figure 5.6 Structures of the four oxo-arsenosugars [118].

In order to test the separation of As species on the improved chromatography, two sets of the mixture of arsenic standards: (1) $\sim 10 \text{ ng g}^{-1}$ AsB, As(III), DMA, MMA and As(V) ; and (2) arsenosugars 1, 2, 3 and 4, prepared by 10-time dilution of the original *Fucus* extract, were investigated. A $50\text{-}\mu\text{L}$ injection volume of each As standard mixture was introduced to the column using $20 \text{ mM NH}_4\text{HCO}_3$, pH 9.0 in 1% MeOH as a mobile phase and the He-mode ICP-MS was used with the integration

time of ^{75}As for 300 msec. The overlaid chromatograms of the two mixtures of As standards are shown in Fig 5.7. It was found that the separation of nine As species was adequate for As-species identification.

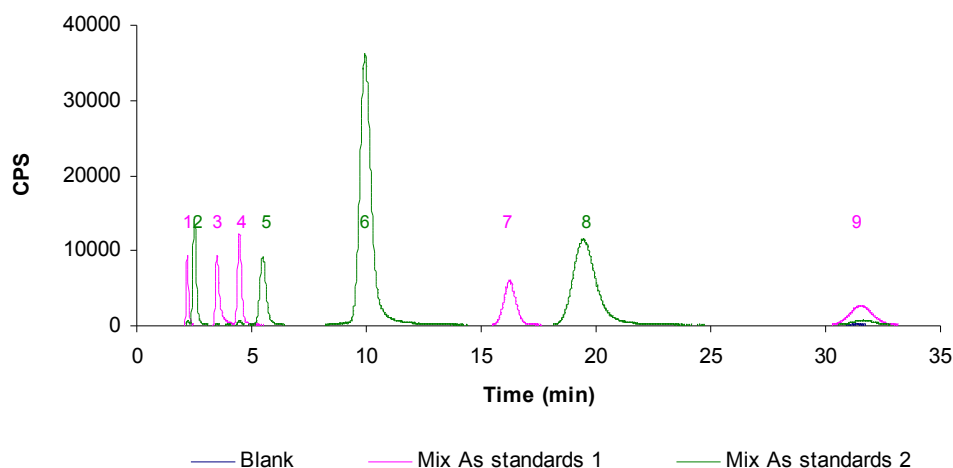


Figure 5.7 The overlaid AE-LC-ICP-MS As chromatograms of two mixtures of As standards consisted of (1) AsB, t_r 2.20 min, (2) Arsenosugar1, t_r 2.52 min, (3) As^{III} , t_r 3.48 min, (4) DMA, t_r 4.46 min, (5) arsenosugar2, t_r 5.48 min, (6) arsenosugar3, t_r 9.96 min, (7) MMA, t_r 16.3 min, (8) arsenosugar4, t_r 19.4 min, and (9) As^{V} , t_r 31.3 min.

5.4.2.2 Identification of arsenic species in the extracts and dialyzates of edible marine algae

The distribution of arsenic species for four algae extracts and their dialyzates, according to AE-LC-ICP-MS As profiles, were compared in Fig 5.8. It is found that the As profiles obtained for both extracts and dialyzates show similar distribution patterns. This is because most of arsenic species found in the algae extracts were much smaller than the membrane pore size (< 10 kDa), which can be observed from the SEC-ICP-MS As profiles (Fig 5.2). Consequently, they were readily permeable into the dialysis membrane. In addition, the similar profiles of both extracts and dialyzates of algae also suggest that there was no transformation of arsenic speciation in marine algae occurring following the *in vitro* gastrointestinal digestion.

The slight changes of the retention times of arsenic species were observed, since the algae matrix might contain constituents that modify the stationary phase or interact with the arsenic species. To prevent wrong assignment of signals, matrix-effected retention shifts needed to be monitored by spiking algae extracts with arsenic

standards. More than 90% of peak area obtained by AE-LC-ICP-MS As profiles in Kombu, Wakame and Nori and 70% of the peak area were preliminarily identified as arsenosugars. The presence of arsenosugars in marine algae as predominant As species (>50% of total extracted As) were found in most studies [61, 142, 197, 201].

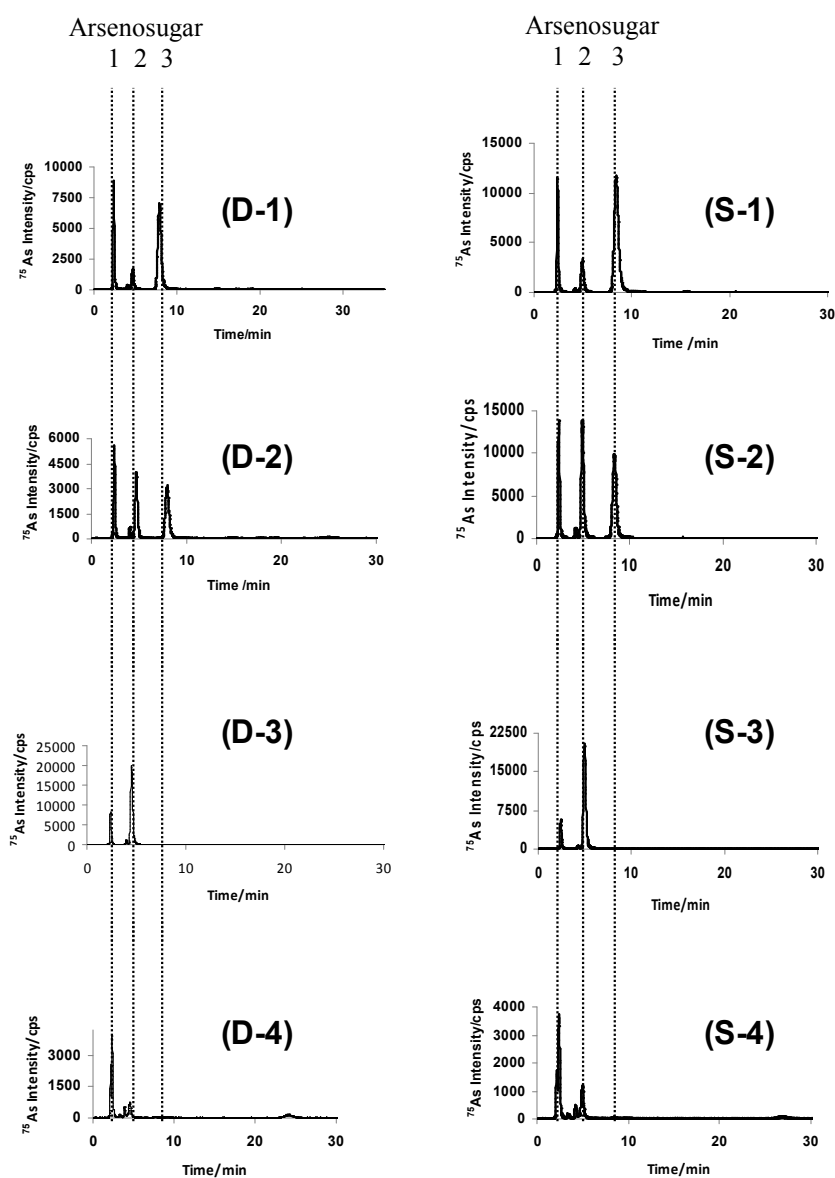


Figure 5.8 The comparison of AE-LC-ICP-MS As profiles in the NH_4OAc extracts and dialyzates for four types of algae, 1-Kombu, 2-Wakame, 3-Nori and 4-Sea lettuce showing the preliminary species identification of the presence of arsenosugars by spiking experiment. D and S stand for dialyzates and sample extracts.

Differences in the peak area of individual arsenosugar were found in all extracts (Table 5.4), which two arsenosugars (arsenosugar 1 and 2) were found in common. These arsenosugars were the most abundant As species in Sea lettuce, and Nori, respectively, while arsenosugar 3 was found to be dominant in both Kombu and Wakame. However, there were negligible levels of inorganic As in all extracts, with the absence of arsenosugar 4 and MMA. Although commonly found in marine algae, the presence of DMA was obtained in low abundance, which was similar to other studies [62, 83, 133, 135, 141, 200]. Results show that the percent of peak area of DMA in Sea lettuce (~7%) was found to be slightly higher than those in others (0.7-2.1%).

It is also interesting to note that the presence of lower amounts of AsB in Sea lettuce, Kombu and Wakame was found when compared with arsenosugar contents. Among the algae investigated, the largest proportion of AsB (~14%) was found in Sea lettuce (green algae), whereas negligible levels (lower than 1%) were observed in others. Despite the fact that high concentrations of arsenobetaine were reported in a variety of marine animals, there have been a limited number of reports on identification of AsB in marine algae [79, 80]. It is possible that AsB often found in low concentrations was overlapped by peaks of highly abundant species such as arsenosugars. In this case, identification of the trace species by spiking experiment is often equivocal. Using the improved chromatographic method, sufficient separation of AsB from arsenosugar1 can allow us to preliminarily identify, as depicted in Fig 5.9. The detection and quantification of AsB in marine algae (*Ascophyllum nodosum*, *Laminaria digitata*, *Fucus vesiculosus*, *Ulva lactuca*, *Padina pavonica* and red algae) using HPLC-ESI-MS/MS was first reported by Nischwitz *et al* (2005) [79]. They suggested that the presence of arsenobetaine in some algae could be attributed to the artifacts in arsenic speciation resulting from epifauna. Therefore, extra care has to be taken in order to eliminate contaminations that are attributable erroneously to algae. The assignment of AsB peak near a void peak should be considered carefully. It is necessary to confirm the species using organic MS. The presence of AsB is believed to be derived from transformation of arsenosugars (Fig 2.5). In literature reports, it was found that some algae species can contain a moderate level of arsenobetaine. In green algae, Nischwitz *et al* (2005) reported the presence of AsB accounting for 7.5% of the extracted arsenic in *Ulva lactuca*, whereas other types such as brown algae (*Ascophyllum nodosum*, *Laminaria digitata*, *Padina pavonica*, and *Fucus vesiculosus*)

and a species of red algae contained in the range 0.25% to 1.3% [79]. Despite the same type of red algae (but different location), *Phyllophora antarctica* were found to contain AsB by approximately 17% of total extracted As, but the peak signal of AsB was not detectable in *Iridaea cordata* [80]. The presence of AsB in some algae is found to be a significant link to explain the source of AsB to marine animals, supporting the assumption of the occurrence of this compound, which derives from the transformation of arsenosugars.

These results were in good agreement with the study of Almela *et al* (2005) [61], which indicated no degradation of arsenosugar in both cooked and raw seaweed following *in vitro* digestion by the solubility method. However, using human *in vivo* method, Francesconi *et al* (2002) reported that no intact arsenosugar was found in human urine during the 4 days of the experiment (~80% of total ingested As found), following the ingestion of a synthetic arsenosugar [60]. In addition, negligible levels of arsenosugars were detected in human urine either, when the algae, *Laminaria*, was ingested [59]. Human *in vivo* studies have shown the urinary excretion of a number of arsenic metabolites, primarily DMA(V) following ingestion of arsenosugars [52, 123]. Using excretion time profiles of the arsenic species found in human urine, Raml *et al* (2005) reported that oxo-arsenosugar was first degraded to oxo-DMAE (dimethylarsenoethanol), oxo-DMAA (dimethylarsenoacetate), and their thio-analogues [52]. These metabolites were then further degraded to DMA(V) and thio-DMA. In a recent report, the great variability in human metabolism of a synthesized oxo-arsenosugar, ingested by 6 volunteers, was revealed [195]. Results showed the considerable difference in the urinary recovery of As within 4 days from 4-95%. Interestingly, the presence of thio-DMA and thio-DMAA detected in blood serum suggested that liver might be responsible for the formation of these species [195]. The thio-arsenic species formed are believed to be intermediates in the metabolism of arsenosugar to DMA (see Fig 2.8).

The presence of arsenosugars in seaweeds still raises a significant concern for human consumption, since the conversion of DMA(V) into its reduced form DMA(III) in human metabolism, which was reported to be much more toxic, is possible [12]. In human *in vivo* studies, the considerable individual variability in arsenosugar metabolism has implications for risk assessment of seaweed consumption [40, 195]. If low excretors do absorb the ingested As and hardly excrete it, they may more vulnerable to adverse health effect of the long-term accumulation, in

comparison with, high excretors. However, at present, the exact mechanism of arsenosugar degradation in human is still unclear. The elucidation of their metabolic pathways is therefore urgently required.

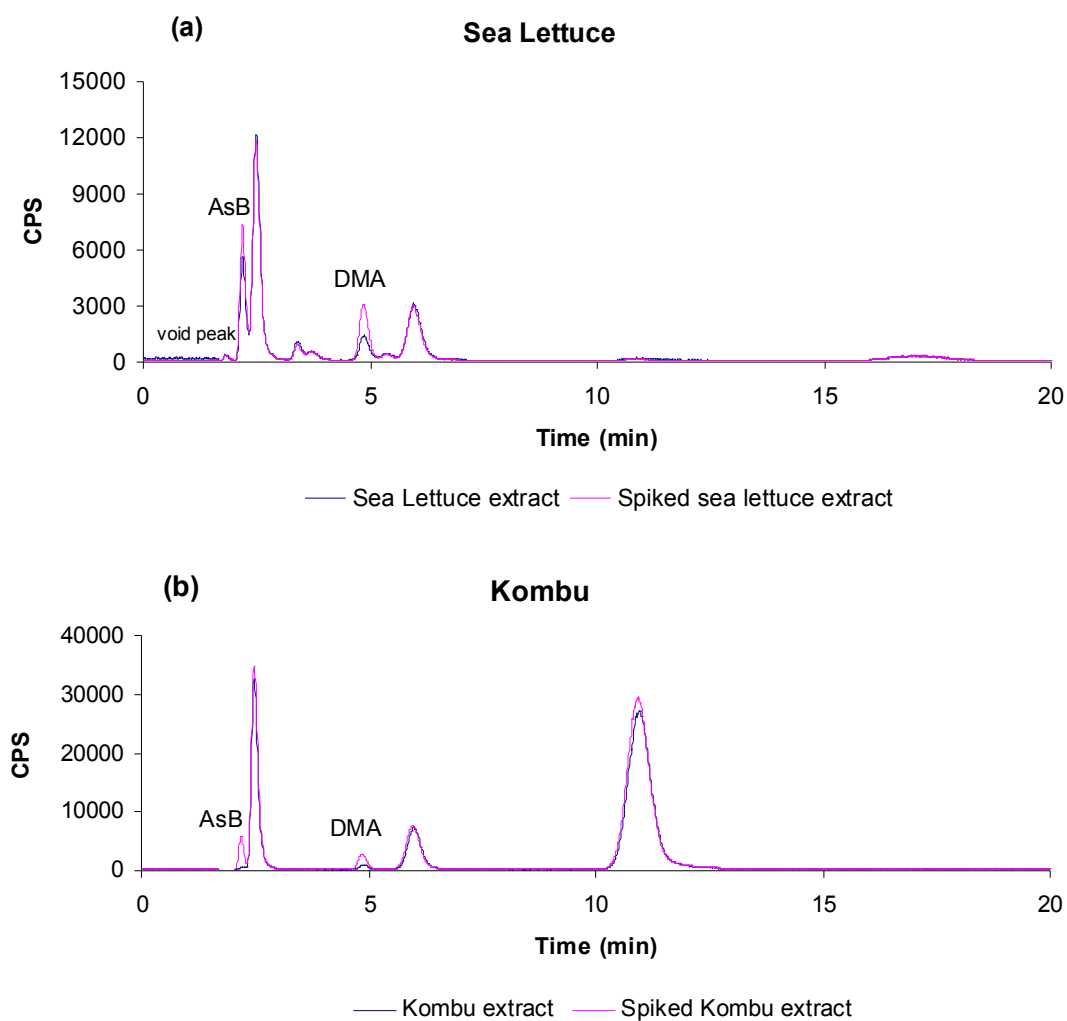


Figure 5.9 Preliminary As-species identification by spiking AsB and DMA in the extracts of (a) Sea lettuce and (b) Kombu.

Table 5.4 As-species distribution in NH₄OAc extracts of marine algae according to the percent of peak areas obtained from AE-LC-ICP-MS As profiles.

Algae	% Peak areas of AE-LC-ICP-MS As profiles (n =2)												% Arsenosugars	
	AsB		DMA		Arsenosugar 1 (HO-ribose)		Arsenosugar 2 (PO ₄ -ribose)		Arsenosugar 3 (SO ₃ -ribose)		Arsenosugar 4 (OSO ₃ -ribose)	Others		
Kombu	0.1	0.1	0.7	0.6	22.3	22.2	10.3	10.3	66.6	66.7	ND	0.3	0.2	99
Wakame	0.2	0.2	2.1	2.2	20.7	20.6	35.3	35.7	38.1	38.3	ND	3.6	3.0	94
Nori	0.1	0.1	1.5	1.6	13.4	13.3	83.5	83.6	ND		ND	1.5	1.7	97
Sea lettuce	13.5	13.9	7.2	6.2	47.5	47.1	23.5	25.1	ND		ND	8.2	7.8	72

5.4.2.3 Quantification of arsenosugars in edible marine algae

In the homogeneous extract from *Fucus serratus*, Madsen *et al* (2000) employed AE-LC-ICP-MS using a Hamilton PRP-X100 column for the quantification of arsenosugars 2-4 with the As(V) calibration curve [117]; however they employed cation exchange (CE)-LC-ICP-MS using a Zorbax 300 SCX cation exchange column for the quantification of arsenosugar 1 with the AsC calibration curve due to its different behavior as a cation with the chromatographic condition used. They found the ICP-MS responses for four arsenosugars and other arsenic species such as As^V, As^{III}, MMA, and DMA under the same AE-LC-ICP-MS condition were similar (within 5%), and those for the standard cationic species were comparable (within 7%) under the CE-LC-ICP-MS condition used. In addition, reported results showed complete column recoveries obtained for both columns, which suggested that no arsenic compounds were retained by either column. The arsenosugars of the *F. serratus* extract with independent different techniques have been quantified (Section 2.6.3). Since lengthy storage may influence the distribution patterns of arsenosugars in the *Fucus* extract, it is necessary to check the validity of the quantification of arsenosugars.

Efforts to quantify their actual concentrations in the marine algae extracts and dialyzates were made by acid digestion (as described in the section 4.2.1) of the *Fucus* extract donated by Prof Francesconi. A 1 mL extract was equally divided into 2 portions, one of which was used for total arsenic digestion, and the other was used for calibration standards for quantification of three arsenosugars. For total As measurement, digesting solutions of the *Fucus* extract and two CRMs (NIST1575 Pine Needles, and NIST1568a Rice Flour), which was used to assess the accuracy of digestion and measurement method, were quantified by external calibration method with Rh as an internal standard using a He-mode ICP-MS. When the total As content in the extract was known, the individual concentration of arsenosugar was determined, under the assumption that each arsenosugar gives similar signal responses, by multiplying its peak-area ratio with the total As value. The results of total As in the *Fucus* extract was determined as $1.3 \pm 0.05 \mu\text{g g}^{-1}$, $n=2$. Thus, the concentration of each arsenosugar was determined as follows: $74.2 \pm 3.0 \text{ ng As g}^{-1}$, $93.4 \pm 3.8 \text{ ng As g}^{-1}$, $671 \pm 27 \text{ ng As g}^{-1}$ and $444 \pm 17 \text{ ng As g}^{-1}$ for arsenosugar 1, 2, 3 and 4, respectively. Overall results obtained by this method were found to be in agreement with those reported (Section 2.6.3).

The instrumental LODs (3σ criterion) obtained by the method for individual arsenosugars were approximately 20 pg As g^{-1} . The sample mass (1 g) was taken into account to calculate the sample LODs. The lowest sample LODs obtained for arsenosugars in algae extracts were approximately 2 ng g^{-1} , the conversion of detection limits in ng As g^{-1} was based on 150 mg sample mass and the dilution of the algae extract by a factor of 100. In algae dialyzates, the lowest sample LODs obtained for arsenosugars were approximately 7 ng g^{-1} ; the conversion of detection limits in ng As g^{-1} was based on 500 mg sample mass and the dilution of the algae dialyrate by a factor of 360.

Table 5.5 Quantitative analysis of arsenosugars in NH₄OAc extracts of marine algae using AE-LC-ICP-MS.

Algae	Total As in NH ₄ OAc extracts (μg g ⁻¹ , n =3)	Arsenosugars (μg As g ⁻¹ , n =2)					Total Arsenosugars in the extracts (μg As g ⁻¹ , n =2)	% As as arsenosugars from total As in the solid	
		Arsenosugar 1 (HO-ribose)		Arsenosugar 2 (PO ₄ -ribose)		Arsenosugar 3 (SO ₃ -ribose)			
Kombu	31.5 ± 0.7	8.29	7.94	3.64	3.47	22.8	22.9	34.5	110
Wakame	16.6 ± 0.0	3.11	3.23	5.25	5.46	5.62	5.75	14.2	86
Nori	30.0 ± 0.4	4.24	4.36	25.9	26.5	ND		30.5	102
Sea lettuce	1.93 ±0.03	0.54	0.59	0.28	0.30	ND		0.85	44

Table 5.6 Quantitative analysis of arsenosugars in dialyzates of raw marine algae following the *in vitro* digestion using AE-LC-ICP-MS.

Algae	Total As in dialyzates ($\mu\text{g g}^{-1}$, n =3)	Arsenosugars ($\mu\text{g As g}^{-1}$, n =2)				Total Arsenosugars in the dialyzates ($\mu\text{g As g}^{-1}$, n =2)	% As as arsenosugars from total As in dialyzates		
		Arsenosugar 1 (HO-ribose)	Arsenosugar 2 (PO ₄ -ribose)	Arsenosugar 3 (SO ₃ -ribose)					
Kombu	7.6 ± 1.1	1.94	1.97	0.64	0.58	4.50	4.38	7.01	92
Wakame	4.4 ± 0.8	1.33	1.37	1.62	1.67	2.37	2.42	5.39	122
Nori	8.0 ± 1.3	1.76	1.80	7.41	7.65	ND		9.31	116
Sea lettuce	0.56 ± 0.07	0.15	0.15	0.08	0.07	ND		0.23	41

In order to quantify individual arsenosugars, algae extracts were diluted 3-fold, with the exception of Nori extract, which was diluted by a factor of 10 due to high levels of arsenosugars contained in it. For algae dialyzates, 4-fold dilution was made, except Sea lettuce, for which original dialyzate was used due to low concentration of arsenosugar present. External calibration curves for quantitative analysis were composed of four levels spanning the concentration range of arsenosugar 1 from 0 - 55 ng g⁻¹ As, $r^2 = 0.999$, arsenosugar 2 from 0-70 ng g⁻¹ As, $r^2 = 0.999$, and arsenosugar 3 from 0-500 ng g⁻¹ As, $r^2 = 0.999$. Determination of arsenosugars in algae extracts and dialyzates was achieved using AE-LC-ICP-MS with He as a collision gas.

Quantitative results of individual arsenosugar, total arsenosugars, with respect to the total As in the algae extracts and dialyzates are compared in Table 5.5-5.6. The majority of As species in both extracts and dialyzates were arsenosugars, showing that more than 80% were found in Kombu, Wakame, and Nori, and approximately 40% was determined in Sea lettuce. It shows that arsenosugars present in all four algae were not transformed following the *in vitro* gastrointestinal digestion. To gain understandings of the results, a simple model of the study of As bioaccessibility is given. Assuming that 1 g of algae containing 100 µg of As are ingested, only 10-20 µg of As are bioavailable (10-20% dialyzability), whose 8-16 µg of As are found to be arsenosugars.

This study shows although all four oxo-arsenosugars, which are readily soluble in water and having their molecular size (<500 Da), are significantly smaller than the pore size of the dialysis membrane (10 kDa cut off), approximately only 30% of total arsenosugars, which were extracted by the NH₄OAc buffer, are able to pass through the membrane. This is due to the use of membrane-based separation, performed in the batch system. Furthermore, algae matrix is thought to be a barrier of arsenic species being diffused through the dialysis membrane. Ouypornkochagorn and Feldmann (2010) investigated the effect of different arsenic species on the accumulation in human skin samples [194]. It was reported that dermal uptake of As *via* human skin is largely dependent on its speciation. The similar rate of penetration through the unbroken skin of arsenosugars and arsenate was found, whereas the uptake of arsenite and DMA(V) was reported to 26 and 59-time greater than that of arsenate. Interestingly, As species in the *Fucus serratus* extract hardly penetrated and accumulated through the unbroken skin. It was thought that gel-like alginates obtained

in the seaweed extract might prevent the absorption through skin. Therefore, it is probable that the low As dialyzability in the investigated marine algae is ascribed to the matrix effects from alginate gel compounds released in the course of *in vitro* digestion.

Almela *et al* (2005) employed the *in vitro* solubility method for the prediction of arsenic bioaccessibility [61]. They found that the concentration of arsenosugars following the *in vitro* digestion were similar to the value measured in the 50% methanol extract. By this means, they reported that the bioaccessibility of arsenosugars in the raw and cooked algae was >80%. The bioaccessibility of arsenic obtained by the solubility method is ignorant of the matrix effect, which is believed to influence the absorption by the gastrointestinal epithelium.

Due to the lack of the commercial standards, most of studies have quantified arsenosugars with regard to the response of a different As species such as As(V), and DMA(V). This can be justified due to the similar response of ICP-MS to all As species. In this study, efforts to measure the concentration of individual arsenosugar partially purified from the *Fucus* extract were made. Therefore, the accuracy of quantitative analysis of arsenosugar species in algae extracts and dialyzates to a certain extent relied on how accurately individual arsenosugars were determined, in which a number of sample preparation and measurement steps involved. Several possible errors for the quantification can occur. For instance, co-eluted compounds containing high carbon contents can result in signal enhancement due to the charge transfer effect [202, 203]. The partially purified arsenosugars from algae used as standards might be unreliable, since not all arsenic species possibly presented in complex biological materials are separable by a single chromatographic method. Some species might be irreversibly adsorbed to the column and then led to negative effect of the results. Moreover, quantification of arsenosugars by determining peak area manually is prone to error, depending on operator skill. According to the results, summations of arsenosugar contents in both extracts and dialyzates measured in Kombu, Wakame, and Nori show $100 \pm 20\%$ of the total As. In the case of top-end values, the effect of instrumental drift was thought to be responsible. Although the use of internal standard (such as Rh and Ge) *via* a T piece postcolumn for monitoring signal drifts can be performed [135], the mixing of flowing solutions in the joint can occur and base-line resolution between AsB and arsenosugar1 is then no longer achieved. Nevertheless, the addition of an internal standard such as 4-

hydroxyphenylarsonic acid as a species of interest into an analyzing solution prior to the analysis is thought to be an alternative for monitoring the sensitivity for speciation studies [132].

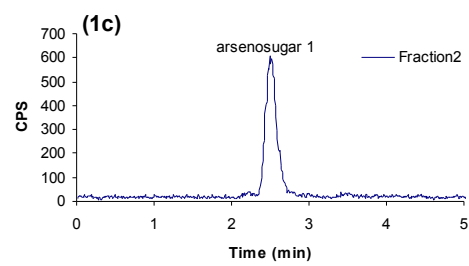
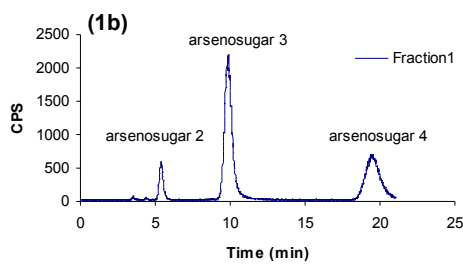
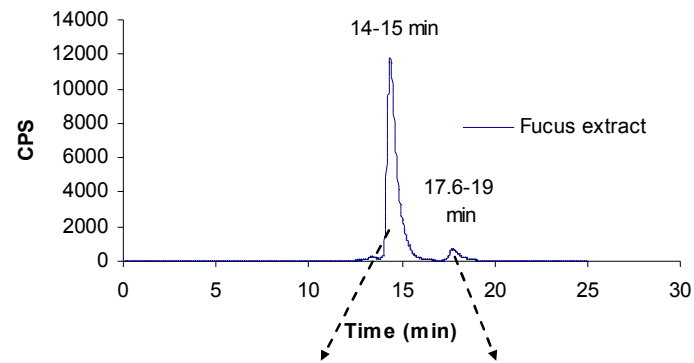
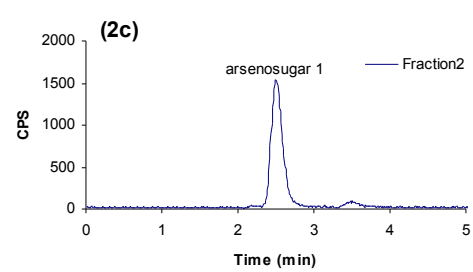
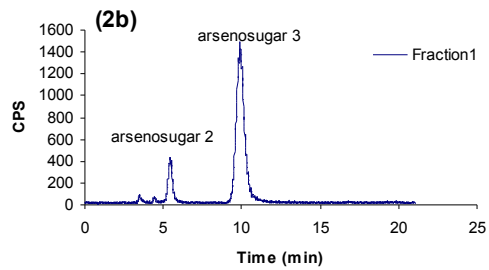
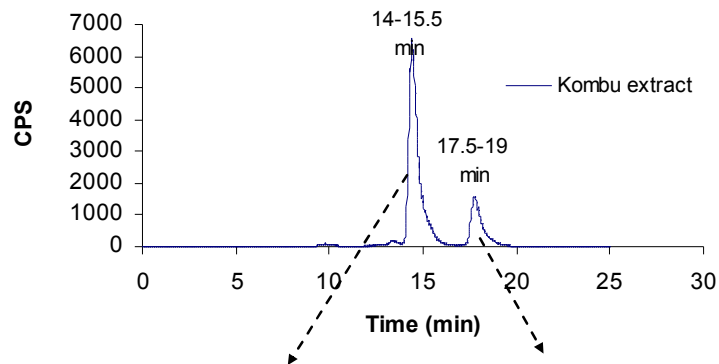
5.4.2 Two-dimensional (SEC-AE)LC-ICP-MS

In marine-life samples, the large number of naturally occurring As species make it highly likely that two or more arsenic compounds will be present which have the same retention times. Particularly, in the presence of a sample matrix, a number of problems rise such as retention time irreproducibility, and the degradation of chromatographic resolution. There is a possibility of the risk that the spike of one compound will match with the retention time of another one, resulting in misidentification. To avoid the wrong assignment due to the possible overlaps among organoarsenical species occurring in each of the techniques, the use of at least two chromatographic methods such as anion-exchange and cation-exchange in parallel was recommended for the analysis of samples containing significant amounts of arsenosugars [146, 204]. Introduced by McSheehy *et al* (2000), an alternative is the use of two-dimensional chromatography comprising two different separation mechanisms, not in parallel but in series, to achieve the chromatographic purity of eluted compounds in algae samples [133]. They indicated that signal identification by retention time matching is unreliable since this parameter is strongly affected by the current state of the column and the composition of the matrix. Although spiking experiments can correct for the shifts in the retention time of the As species, unambiguous positive signal identification on this basis remains elusive. In order to improve the resolution and minimize the risk of overlaps, they proposed the use of size-exclusion HPLC allowing us to fractionate organoarsenic species and to remove matrix prior to detection of arsenic species by AE-LC-ICP-MS.

In order to apply this approach, the limitations of the developed anion exchange method for speciation of As in algae were investigated. The possibility of improving the purity of analyte of interest was examined by preceding the AE separation by size-exclusion chromatography. A typical chromatogram obtained under the optimized conditions is presented in Fig 5.7. This shows the good baseline separation of nearly all As-species investigated, but a limited resolution was observed for AsB and arsenosugar 1. To see whether the signals of both species can be resolved

well, the retention times of the AsB and arsenosugar1 need to be investigated under the SEC condition used.

Using the previous SEC condition, two main As peaks were separated from the 15-fold diluted *Fucus* extract used for verification of arsenosugars. Effluents at two time interval from 14 to 15 min and from 17.6 to 19 min were separately collected. The SEC eluents acquired were separated onto the subsequent chromatography, AE-LC-ICP-MS. As illustrated in Fig 5.10, it was found that the first collected fraction from SEC contained arsenosugar 2-3, while arsenosugar 1 was found in the second fraction collected. To demonstrate the use of two-dimensional chromatography, Kombu and Sea lettuce extracts were selected. It is known that the first fraction collected from the SEC in Sea lettuce extract during 14-16 min contained not only arsenosugar 2, but also DMA (t_r 15.0 min). Following the anion-exchange separation, the presence of both arsenosugar 2 and DMA were still observed in the AE-LC-ICP-MS profiles. Likewise, in the second fraction collected from the SEC during 17.5-19 min, AsB (t_r 18.6 min) was co-eluted with arsenosugar1. Therefore, after the separation, both As species obviously appeared in the same AE-LC-ICP-MS profile. Although AsB and arsenosugar1 were not separable on the SEC, the sample matrix can be removed due to fractionation. This resulted in a better baseline resolution of the separation, thereby allowing species identification by spiking experiments and retention time matching.

(1a) Arsenosugar standards from *Fucus***(2a) Kombu**

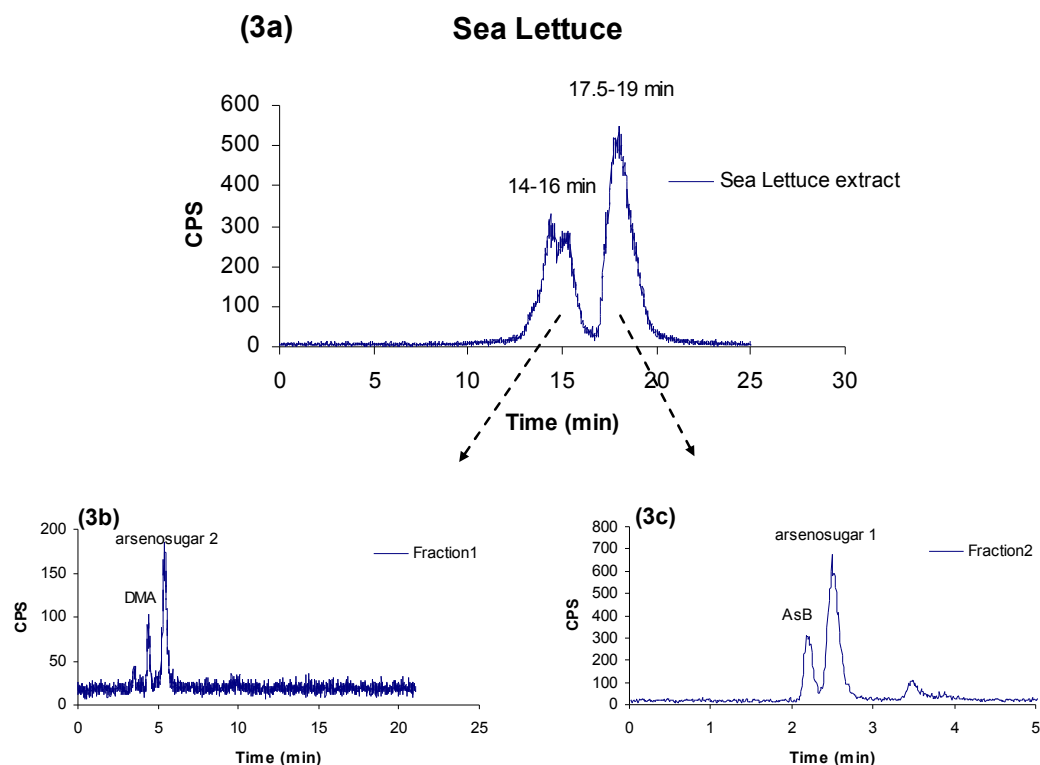


Figure 5.10 Demonstration of the use of two-dimensional chromatography (SEC-AE)LC-ICP-MS for (1) Arsenosugar standards from *Fucus*, (2) Kombu, and (3) Sea lettuce. (1a, 2a, 3a) show the SEC-ICP-MS As profiles of each algae and indicate two collected peaks at the specific time interval; (1b, 2b, 3b) show the AE-LC-ICP-MS As profiles for the first collected fraction of each algae; (1c, 2c, 3c) show the AE-LC-ICP-MS As profiles for the second collected fraction of each algae.

As matrix can modify the active sites of stationary phase, algae matrix in Kombu extract was found to slightly affect the retention time of As species present on the anion-exchange column. The use of two-dimensional chromatography, (SEC-AE)LC-ICP-MS can alleviate the matrix effect and enable the species identification by retention time matching (Table 5.7).

Table 5.7 The comparison of retention times of As-species standards in water and As-species present in Kombu extracts (following /without SEC) by AE-LC-ICP-MS.

As-species	Retention time (min) by AE-LC-ICP-MS		
	Standards in water	Kombu extract (following SEC)	Kombu extract (without SEC)
AsB	2.20	2.20	2.20
DMA	4.46	4.46	4.68
Arsenosugar1	2.52	2.51	2.48
Arsenosugar2	5.48	5.46	5.69
Arsenosugar3	9.96	9.89	10.3

5.5 FULL IDENTIFICATION OF ARSENIC SPECIES IN EDIBLE MARINE ALGAE USING AE-LC-ICP-MS IN PARALLEL WITH ESI-ION TRAP-MS/MS

With ICP-MS alone, preliminary identification (by spiking experiment) but no information on the structural composition can be obtained. Therefore, ESI-MS/MS or other organic MS techniques are needed for this purpose.

Characterization and confirmation of arsenic species in the algae extracts were performed using HPLC-ESI Ion Trap MS/MS. The system was operated using an Agilent Technologies 6330 Ion Trap MS coupled to an Agilent 1200 HPLC system. The mass spectrometer was equipped with an electrospray interface as the ionization source. The Bruker Daltonik 6300 series Ion Trap LC/MS software (version 6.2) incorporating Agilent Chemstation for LC 3D systems (for LC control) was used to control the LC-MS. Data analysis was performed using Bruker Daltonik Data Analysis for 6300 Series Ion Trap LC/MS (version 4). Full-scan mass spectra (80-550 m/z) were recorded every 18.077 msec in the positive-ion mode. For MS/MS experiments, the operating conditions were: isolation width: 4 m/z for arsenosugars1-3 and 2 m/z for AsB; ion trap drive or fragmentation voltage 82.3 V, and fragmentation amplitude varying from 50% to 80% applied to the protonated molecules and ions from the individual compounds isolated for m/z 179 and 161

(AsB); 329 and 237 (arsenosugar1); 483, 391 (arsenosugar2); 393, 237 (arsenosugar3).

The ESI Ion Trap MS detection system was employed for characterizing AsB and arsenosugars1-3 detected in the chromatographic eluates to confirm the results obtained by HPLC-ICP-MS. The optimization of the ESI-MS/MS conditions was performed by directly infusing a mixture of As standards containing AsB, DMA and arsenosugars extract (approximately 200 ng As g⁻¹ each) in the mobile phase used (20 mM NH₄HCO₃ in 1% MeOH, pH 9.0) into the ESI source. The coupling of the chromatographic system to the ESI-MS can be achieved using a PEEK tubing (30 cm x 0.1 mm id) connected directly from the outlet of the column to the inlet of the electrospray source. The flow rate of the chromatographic eluent (1 mL min⁻¹) was split equally (0.5 mL min⁻¹) by a T-piece connector before it delivered the ESI-MS and ICP-MS instruments. In this work, anion-exchange HPLC was employed with both ICP-MS and ESI Ion Trap MS/MS detection in an effort to obtain full identification of the arsenic speciation in the edible marine algae. The operating parameters for the use of AE-LC-ICP-MS in parallel with ESI Ion Trap MS/MS in edible marine algae are summarized in Table 5.8.

Table 5.8 Operating parameters for HPLC-ICP-MS in parallel with ESI Ion Trap MS/MS.

ICP-MS operating parameters (in He mode)

RF Power	1530 W
Carrier-gas flow rate	0.86 L min ⁻¹
Make-up gas flow rate	0.29 L min ⁻¹
Sample/Skimmer cones	Pt/ Ni
Spray chamber temperature	2°C
Optional gas (He) flow rate	4.0 mL min ⁻¹
Data acquisition	
Points per spectral peak	1
Isotopes monitored	⁷⁵ As
Integration time per mass	100 ms

ESI-MS/MS operating parameters

Drying temperature	350°C
Nebulizer gas pressure	35.0 psi
Drying gas flow rate	11.0 L min ⁻¹
Capillary voltage	3500 V

HPLC separation conditions

HPLC method	Anion-exchange
Analytical column	Hamilton PRP-X100 (250 mm x 4.1 mm id x 10 µm)
Injection volume	40 µL
Mobile phase	20 mM NH ₄ HCO ₃ in % (v/v) MeOH at pH 9.0
Flow rate	1.0 mL min ⁻¹

Despite the fact that negative ion detection is considered superior because more product ions are produced at greater intensity [205], positive ion mode has occasionally been used to obtain both elemental and molecular mass spectra in the one chromatographic run [125, 134, 143, 195, 196]. It also enables the detection of the fragment at m/z 237, which is a characteristic fragment for arsenosugar [196]. Moreover, mass spectra performed in the positive-ion mode provide strong signal from the protonated molecular ion at m/z 329, 483, and 393. However, the identification of arsenosugars may suffer from matrix components in real-world samples having a similar molecular mass to the analyte. A deeper insight into the arsenic compounds present can be gained by analyzing product ions resulting from the collision induced dissociation (CID) of the protonated molecule ions.

In this study, the characterization of arsenic species in algae extracts using ESI-MS/MS was carried out in the positive-ion mode by scanning from 80-550 m/z . Attempts to perform the characterization was made without any pretreatment step to reduce the preparation time, although the sample cleanup procedure is recommended for organic mass spectrometric detection. In order to achieve the full identification of As species, 40 µL of original algae extracts were introduced to the AE column with the flow rate of 1 mL min⁻¹, followed by equally splitting the column effluents using a

T piece connector, and then delivering to both (He-mode) ICP-MS and ESI-MS/MS. An example of the AE-LC-ICP-MS As profile of the Kombu extract, as well as the fragmentation patterns of daughter ions, believed to be arsenosugar 1-3 are shown in Fig 5.11. To ensure the species identity, a matrix-matched-standard solution, prepared by spiking AsB and DMA standards to the *Fucus* extract, were used for investigating individual retention time of the As species and daughter-ion fragmentation occurred.

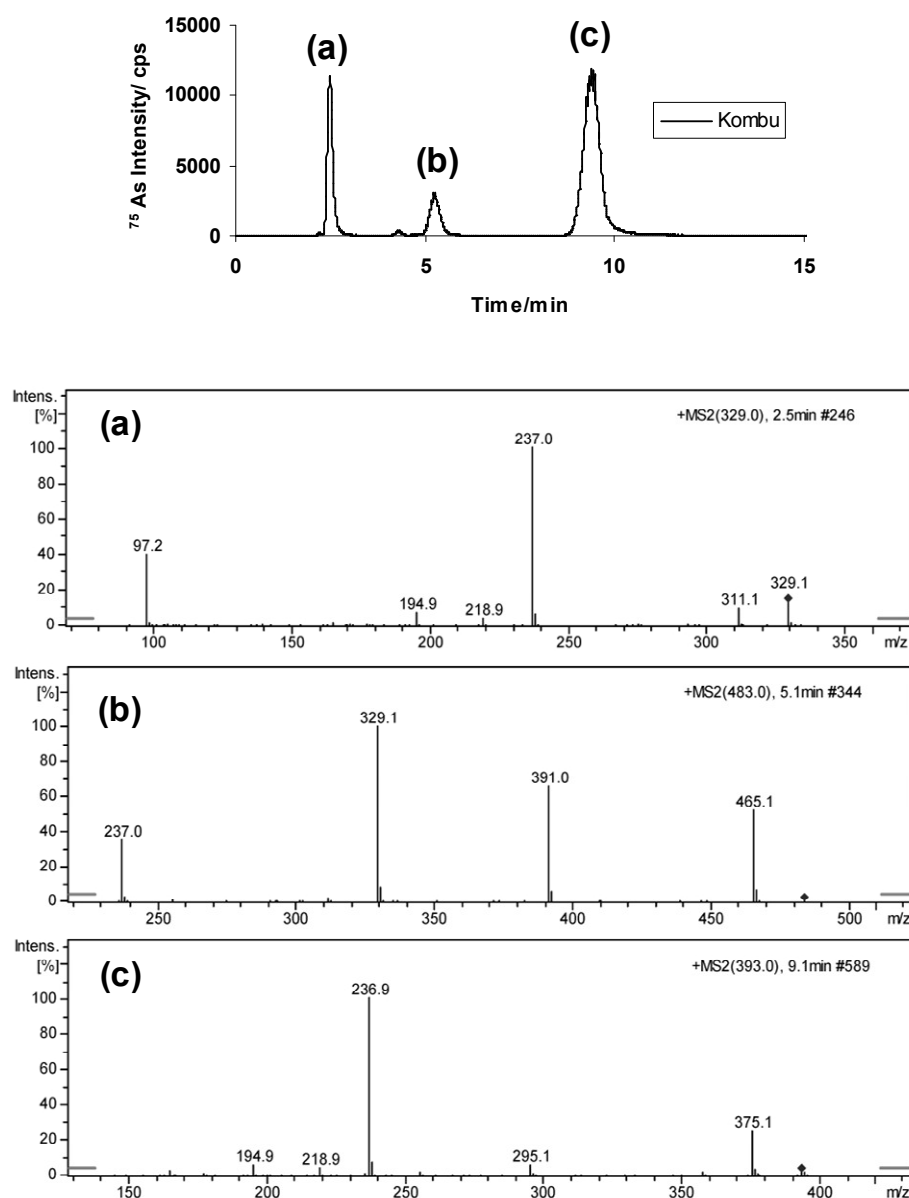


Figure 5.11 The AE-LC-ICP-MS As profile of Kombu extract showing the presence of (a) arsenosugar 1 (OH-ribose), (b) arsenosugar 2 (PO₄-ribose) and (c) arsenosugar 3 (SO₃-ribose), characterized by ESI Ion Trap MS/MS (Typical fragmentation patterns of the three arsenosugars presented).

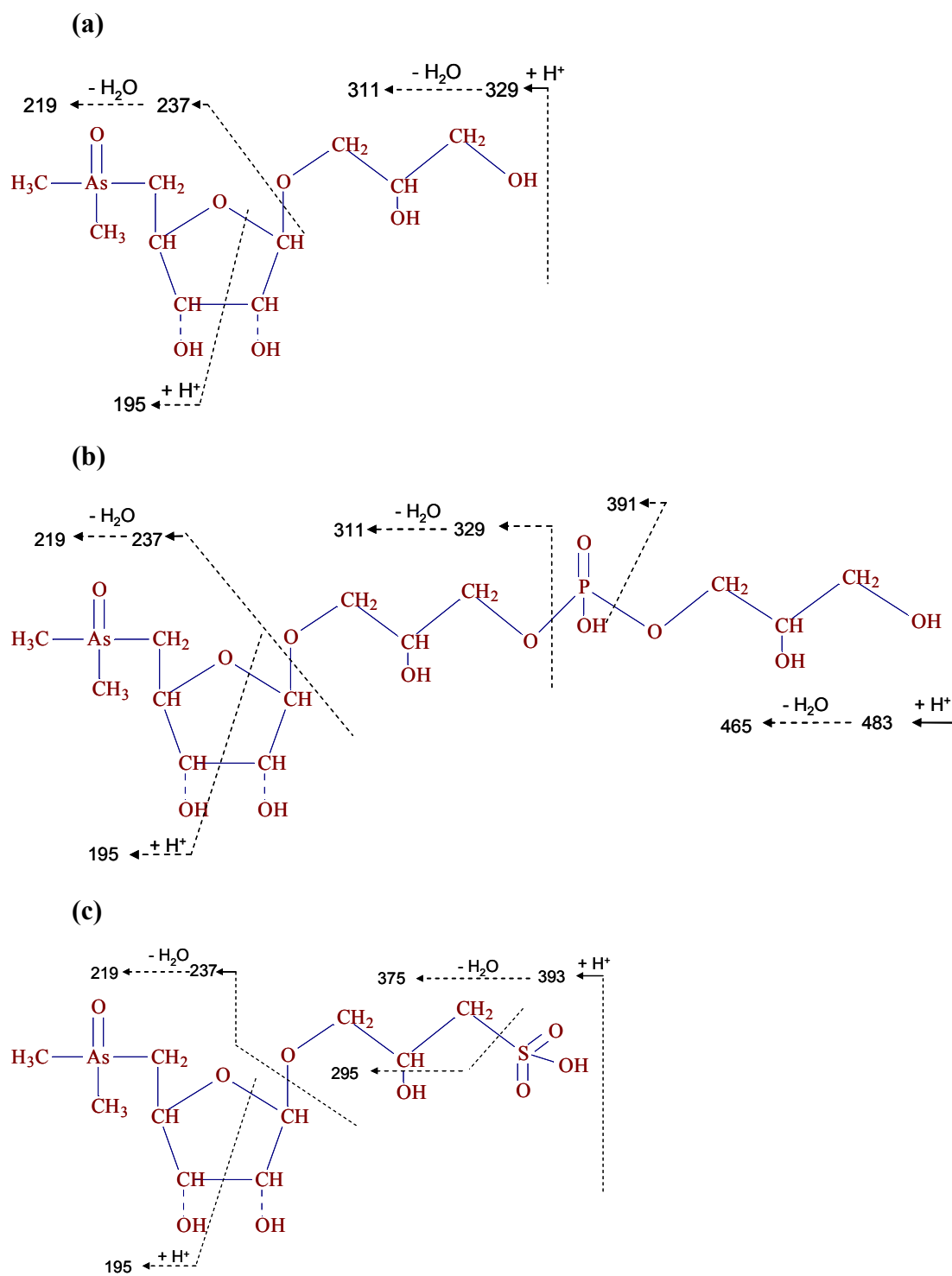


Figure 5.12 The fragmentation pathways of (a) arsenosugar 1 (OH-ribose), (b) arsenosugar 2 (PO_4 -ribose) and (c) arsenosugar 3 (SO_3 -ribose).

The presence of different arsenosugars and AsB found in the edible marine algae were confirmed by mass spectra using anion-exchange HPLC with ESI-Ion Trap-MS/MS. The anion-exchange chromatogram obtained for the Kombu extract, show the presence of three predominant arsenic-containing peaks observed at retention times of 2.5, 5.2, and 9.1 min (Table 5.9). It was found that arsenosugars 1, 2 and 3 can readily produce protonated molecular species $[M+H]^+$, although the chromatographic separation was done at high pH (pH 9.0). These observations were proposed to be a case of the phenomenon referred to as ‘wrong-way-round’ electrospray ionization, the precise mechanism of which is still unclear [206]. Confirmation of species identity can be performed by isolation of the respective protonated molecular ions at m/z 329, 483, and 393 and production of a fragmentation of these precursor ions under the MS instrumental condition described in Table 5.8. Typical MS/MS spectra of these arsenosugars are presented in Fig 5.11(a-c). The CID MS/MS spectra results obtained for these compounds are not only in agreement with those reported by other authors for the same arsenosugars [124, 133, 142, 196, 197], but also similar to those obtained from the matrix-matched standards. The daughter ions at m/z 237 corresponding to the oxonium ion of the dimethylarsinoylpentose moiety and at m/z 195 originating from the decomposition of the furane ring and indicating the attachments of the dimethylarsinoyl moiety to the 5'-position of the furane ring were identified as the characteristic of the presence of the arsenosugars. The proposed fragmentation pathways for individual arsenosugar are depicted in Fig 5.12. The characteristic fragmentation ions such as m/z 97, 195, 219, 237, 311 and the protonated molecular ion m/z 329, confirms that the As compound eluting at 2.5 min in the AE separation is arsenosugar1 (OH-ribose). The protonated molecular ion with m/z 483, creating the fragments at m/z 465, 391, and 329 are characteristic of arsenosugar2 (PO₄-ribose) eluting at the retention time 5.2 min. The CID MS/MS spectra of the peak eluted at 9.1 min showing fragmentation of the protonated molecular ion at m/z 393 with the daughter ions at m/z 375, and 295 are the characteristic of arsenosugar3 (SO₃-ribose).

Table 5.9 Observations of the retention times, protonated molecular ions and fragmentation ions of AsB, and arsenosugar 1, 2 and 3 in the matrix-matched standard in algae samples.

Matrix Matched standard:

Compound	[M+H] ⁺ Observed	Retention time (min)	In source fragmentation (m/z)	Fragmentation Ions (m/z)
AsB	179.0	2.20	161.0	161.0, 137.1, 120.1, 105.0, 91.1
Arsenosugar 1 (OH-ribose)	329.1	2.47	237.0	311.1, 236.9, 219.0 194.9, 97.2
Arsenosugar 2 (PO ₄ -ribose)	483.1	5.16	391.0	465.0, 391.0, 329.1, 237.0, 194.9, 97.1
Arsenosugar 3 (SO ₃ -ribose)	393.1	9.12	237.0	375.1, 295.0, 236.9, 194.9, 97.1

Algae samples:

Sample	Retention Time (min)	[M+H] ⁺ Observed	Fragmentation (m/z)	Assigned Identity
Kombu	2.2	179.0	160.9, 105.2, 97.1	AsB
	2.5	329.1	311.1, 237.0, 218.9, 194.9, 97.1	Arsenosugar1 (OH-ribose)
	5.2	483.1	465.1, 391.1, 329.1, 237.0, 194.9, 97.1	Arsenosugar2 (PO ₄ -ribose)
	9.1	393.1	375.1, 295.1, 236.9, 194.9, 97.2	Arsenosugar3 (SO ₃ -ribose)
Wakame	2.2	178.8	160.9, 137.1	AsB
	2.5	329.1	311.1, 236.9, 218.9, 194.9, 97.2	Arsenosugar1 (OH-ribose)
	5.1	483.1	465.1, 391.0, 329.1, 237.0, 195.1, 97.2	Arsenosugar2 (PO ₄ -ribose)
	8.9	393.1	375.0, 295.1, 237.0, 194.9, 97.2	Arsenosugar3 (SO ₃ -ribose)
Nori	2.2	178.9	161.0, 137.1, 105.2,	AsB
	2.4	329.1	311.0, 236.9, 195.0, 97.2	Arsenosugar1 (OH-ribose)
	5.2	483.1	465.0, 391.0, 329.1, 237.0, 194.9	Arsenosugar2 (PO ₄ -ribose)
Sea lettuce	2.2	178.9	160.9, 137.1, 105.3	AsB
	2.4	329.0	311.1, 236.9, 219.0, 195.1, 97.3	Arsenosugar1 (OH-ribose)
	5.2	483.1	465.0, 391.0, 329.1, 237.0, 194.9, 97.1	Arsenosugar2 (PO ₄ -ribose)

In spite of weak signals acquired in the AE-LC-ICP-MS As profiles, the evidence of the presence of AsB in the algae extracts eluted at the retention time 2.2 min, can be observed from CID MS/MS spectra, which contains the ion fragments of m/z 161, 137, 105, and 97, as well as the protonated molecular ion at m/z 179. The typical fragmentation patterns of AsB and other organoarsenicals such as AsC, MMA, DMA, TeMA and TMAO are commonly known and reported elsewhere [79, 207-209]. However, attempts to characterize DMA using ESI-Ion Trap MS in the positive-ion mode were unsuccessful. This might be ascribed to the limited capability of trapping low molecular species to enable structural characterization. Additionally, it is possible that analyte signals were suppressed by the presence of severe matrix accidentally co-eluted. Despite the fact that the identification of arsenic is hampered by being mono-isotopic, ESI Ion Trap MS/MS providing fragmentation patterns allow unambiguous identification of the presence of arsenic species even in the limited availability of commercial standards.

5.6 SUMMARY

The consumption of edible marine algae either raw or in cooked products have gained increasing popularity, since they are in abundance with various nutrients. However, nutritionists raised a considerable concern in their consumption, because the high levels of arsenic concentrations, mainly arsenosugars, were found. *In vivo* studies reported the presence of a wide range of metabolites, primarily DMA in human urine following the intake of arsenosugars, implying an increasing toxicity. The metabolic transformation of arsenosugars in the course of gastrointestinal digestion and its bioavailability to human body remain unclear.

The development of methodologies for As speciation in edible marine algae was performed in four commercial marine algae: Kombu, Wakame, Nori, and Sea lettuce. Appropriate extracting solutions (water and 50%MeOH) and extraction time (1h, 2h, 4h, and 6h) were optimized. It was found that water gave better As extraction efficiency than 50% MeOH, while providing similar SEC-ICP-MS As profiles. Insignificant differences in sonication time were observed; 1 h was chosen for the As extraction time. The sample size to extractant volume was determined by the concentration levels of As in algae samples. The use of 0.05-0.20 g of algae in 5 mL of de-ionized water with 1-h sonication was chosen for this study. The arsenic extraction efficiencies in water extracts were dependent on the type of algae and

varied from 40-60%. Using SEC-ICP-MS, two arsenic peaks eluted at the retention times of 15 min, and 18 min were found in all algae extracts, suggesting all of extracted arsenic were smaller than 10 kDa. By varying the concentration of salt, and pH of the mobile phase, the AE-LC-ICP-MS method for As-species identification was developed using ammonium hydrogen carbonate solution as a mobile phase due to its compatibility to both ICP-MS and ESI-MS. The addition of 1% methanol in mobile phase can improve the ionization of arsenic in the plasma (2-3 times). The assignment of arsenic species in such a complex matrix was found to be difficult owing to the retention time shifts and loss the resolution of separation. To prevent misidentification of arsenic species, two-dimensional (SEC-AE)LC-ICP-MS was used to demonstrate as the means of matrix removal by fractionating algae extracts. This approach providing more purified fractions allowed the improved resolution of separation between AsB and arsenosugar1, thereby resulting in minimizing the wrong assignment by spiking experiments or retention time matching.

Preliminary As identification was performed by spiking experiment, indicating the presence of possible As species; AsB, DMA, arsenosugars 1-2 with negligible levels of inorganic arsenic in Sea lettuce and Nori. Arsenosugar 3 was found to be the additional As species in Kombu and Wakame. More than 90% of total arsenic was found to be arsenosugars in algae investigated, with the exception of Sea lettuce (~40%). Full identification of arsenic species in edible marine algae was performed by the use of AE-LC-ICP-MS in parallel with ESI-MS/MS. Using ESI Ion Trap MS, the CID MS/MS spectra providing typical fragmentation patterns confirmed the presence of arsenosugars 1-3 and AsB in algae. Moreover, both retention time matching and CID MS/MS spectra found in algae extracts and matrix matched standard were used to support the confirmation.

In order to gain understandings of their metabolic pathways, a simulated *in vitro* gastrointestinal digestion modified by Haro-Vicente *et al* (2006) [192] was employed in this study. In brief, minerals (<10 kDa) smaller than MW cut off are able to diffuse into a dialysis membrane, in which 0.15 M PIPES solution, pH 7.5 as dialyzing solution is contained. The total amount of arsenic dissolved in the dialyzing solution after dialysis represents the total amount of bioavailable arsenic. The results of the As bioaccessibility in the raw algae indicated that only 10-20% of arsenic were bioavailable. In dialyzates, it was found that no transformation of arsenic speciation in raw algae during the *in vitro* digestion; more than 90% of total dialyzable As were

arsenosugars in algae investigated, with the exception of Sea lettuce (~40%). However, the results obtained by the *in vitro* dialysis method need to be proved with those obtained by human *in vivo* studies to assess the accuracy of the method.

CHAPTER 6

DEVELOPMENT OF METHODOLOGIES FOR ARSENIC SPECIATION ANALYSIS IN TOBACCO LEAVES

Like other terrestrial plants, tobacco can accumulate metallic and non-metallic elements during growth from sources such as fertilisers and soils. Following harvest, the leaves are subjected to curing, a carefully controlled process involving the use of heat and light to achieve the texture, colour and overall quality of a specific tobacco type. Commercially, current cigarettes typically consist of three main types: Virginia, Burley, and Oriental, whose characteristics rely on tobacco variety, cultivation practices as well as environmental conditions. Details on leaf curing and leaf processing operations for tobacco products are described on the British American Tobacco company website (www.bat.com).

The fact that an estimated 3,000 constituents have been identified in tobacco leaves, approximately 4,000 found in tobacco smoke indicates that the urgent need to identify the important compounds in association with smoking related diseases [210]. In order to gain insights into smoke chemistry, the investigation of chemicals existing in tobacco leaves should be a priority. Tobacco has gained interest in containing arsenic since 1927 [211]. In USA before 1950's, lead arsenate was used as a pesticide was applied in tobacco cultivation, leading to high levels of As in tobacco products. Although a dramatic reduction for As concentrations in tobacco (from 40 ppm [212] to sub ppm [213] levels) has been reported, there are still cause concerns in human health because of its toxicity. The human health risk of tobacco consumption needs to be assessed in views of its quantity and speciation.

6.1 RESEARCH CIGARETTES 3R4F

The research cigarettes (3R4F), purchased from Kentucky Tobacco Research & Development Centre (KTRDC), were used in this study. After delivery, research cigarettes contained in cartons were kept in plastic bags and stored in a refrigerator at 4°C until analysis. A commercial electrical blender was utilized to blend tobacco leaves, which were separated from a carton of 200 cigarettes. Homogenized tobacco was then divided into 3 amber bottles, which was tightly sealed with parafilm and kept at a -80°C freezer. Fig 6.1 shows the appearance of reference cigarettes in a carton and longitudinal dissection of the cigarette.

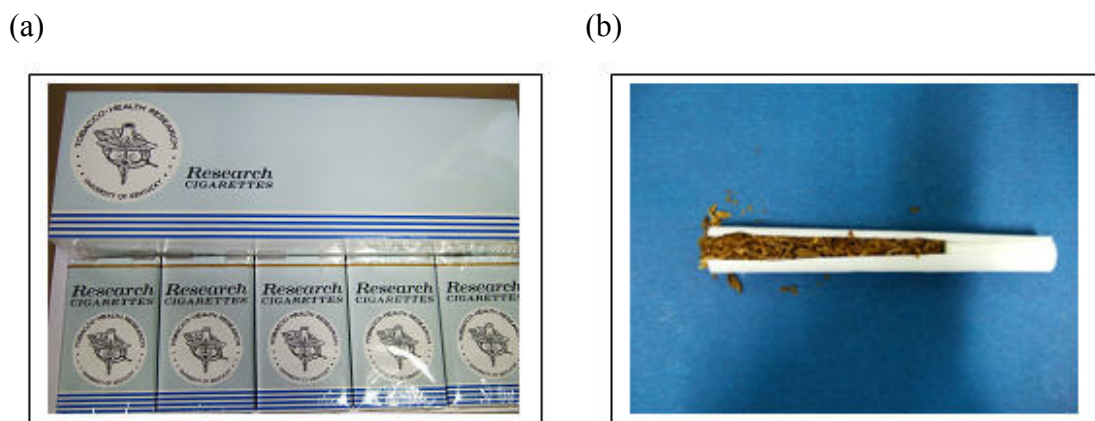


Figure 6.1 Research cigarettes (3R4F) used for method development showing (a) packaging contained in a carton and (b) tobacco in a rod and cellulose acetate filter wrapped with cigarette paper.

Prior to ICP-MS measurement, the moisture content of tobacco leaves was determined so that concentrations could be calculated on a dry weight basis. This was performed by heating 0.5 g of ground tobacco leaves at 100°C in an oven until constant weight was achieved [214, 215]. (Weight did not differ by more than 0.001 g.) The moisture content determined ($13.4 \pm 0.1\%$, $n=3$) was found in a good agreement with the preliminary analysis values reported by KTRDC (13%, see appendix 4). An early report indicated that possible errors for moisture determination might be due to the heterogeneity of the tobacco material, which was consisted of a blend of several different types (see Appendix 4). Since tobacco types vary in hygroscopicity, non-reproducible results are possible when the proportions of tobacco type in a cigarette are not the same as a whole. In order to obtain accurate quantification, moisture content needs to be maintained by avoiding sample exposure to air.

6.2 DETERMINATION OF TOTAL ARSENIC USING HE-MODE ICP-MS

The determination of total As in tobacco was proved to be challenging due partly to complexity of matrix. A number of studies have been published showing a wide variation of results. This might be caused by heterogeneity of samples itself, and /or lack of suitable methods providing high accuracy measurement.

6.2.1 Closed vessel microwave digestion

Blended tobacco leaves and terrestrial plants CRMs (NIST1575 Pine Needles and NIST1568a Rice flour), used for checking performance of methods, were digested using a microwave oven (Multiwave 2000, Paar Physica, UK). A 0.4 g subsample of blended material was accurately weighed into a PTFE tube and then 5 mL of a 50:45:5 (v/v/v) mixture of concentrated HNO₃, H₂O₂, and HF (ultrapurity; Romil) were added. A small amount of HF was added due to the presence of high levels of silica naturally found in tobacco products [214]. The microwave conditions used for achieving complete digestion are shown in Table 6.1. After mineralization, the digests were diluted to 15 g with de-ionized water (The HNO₃ concentration in this solution was 17% (v/v), 26,500 ppm TDS).

Table 6.1 Microwave parameter settings for digesting tobacco leaves and plant CRMs.

Step	Initial Power (W)	Time (min)	Final Power (W)
1	0	15	600
2	600	10	600
3	600	10	0
4	0	5	0
5	0	5	1000
6	1000	10	1000
7	0	15	0

6.2.2 Instrumental setup

Methods for determining low As concentrations in such a complicated matrix need to be evaluated. The ICP-MS equipped with collision cell and fitted with Pt/Ni sampler and skimmer cones due to the corrosive nature of the digests was used for total As analysis with He mode. Several calibration methods such as external calibration with internal standards: ⁷³Ge (60 ng g⁻¹) and ¹⁰³Rh (10 ng g⁻¹) commonly used and without internal standard (check standard with similar concentration to analyte in sample used) were investigated. Two sets of As standard calibration solutions were prepared,

to demonstrate the effect of matrix, at the concentration range: 0, 0.5, 1, 2, 5, 10 and 20 ng As g⁻¹ in 17% and 6.7% HNO₃ for high matrix (26,500 ppm TDS) and low matrix media (10,600 ppm TDS), respectively. The standard addition method at three spike levels was also used to confirm whether sample matrices have an impact on the determination using external calibration. After measuring each sample, the ICP-MS system was cleaned with blank solution (10% HNO₃) followed by analysis of a check As standard solution for monitoring instrumental drift. Agilent Technologies ICP-MS MassHunter software was utilized for calculation of the results of total As measurement.

6.2.3 Comparative studies on results obtained from different calibration methods

Results obtained from external calibration with internal standards (⁷³Ge and ¹⁰³Rh) and without (check As standard solution used) and those from standard addition in plant CRMs used as QC samples are compared in Fig 6.2.

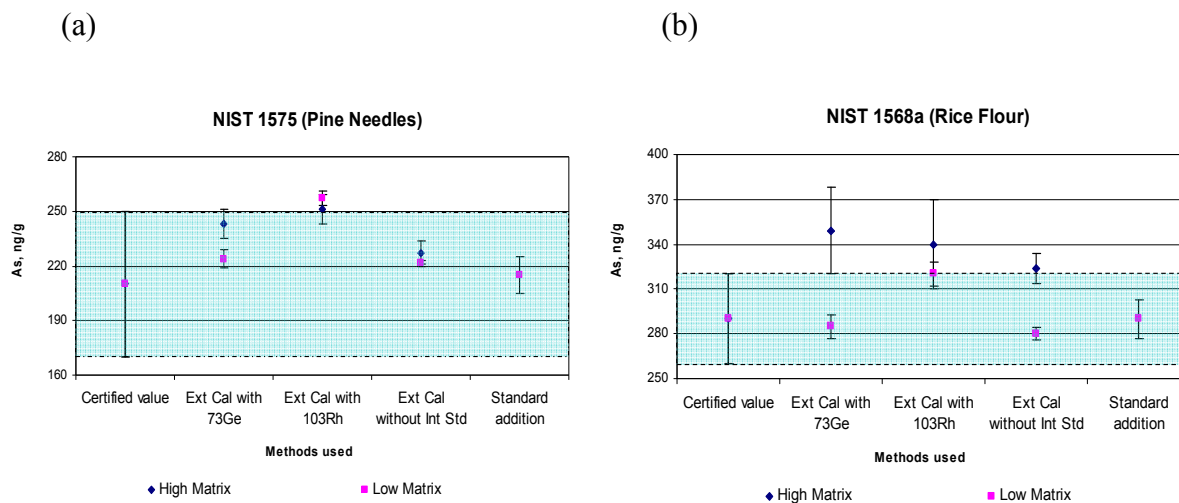


Figure 6.2 Effect of the calibration methods on the measured As concentration in plant CRMs (a) NIST1575 pine needles and (b) NIST1568a rice flour. *Error bars are SD (n=4).*

It is clearly seen that matrix in plant CRMs strongly affect precision and accuracy of As measurement particularly when performing in high matrix (26,500 ppm TDS). Closer results to certified values can be obtained if the matrix was diluted (in this case, 2.5 times more dilution equal to 10,600 ppm TDS). The use of ^{73}Ge as an internal standard seems to give better results than that of ^{103}Rh , whose results show top-ended values even performed in low matrix. In both plant CRMs used, there were no significant differences in the mean results obtained by the use of external calibration with Ge, external calibration without internal standard and standard addition in low matrix, at the 95% confidence level.

Results of total As determination in tobacco leaves (research cigarettes 3R4F) obtained from different calibration methods are presented in Fig 6.3. However, limited availability of the As certified value for the sample has been reported.

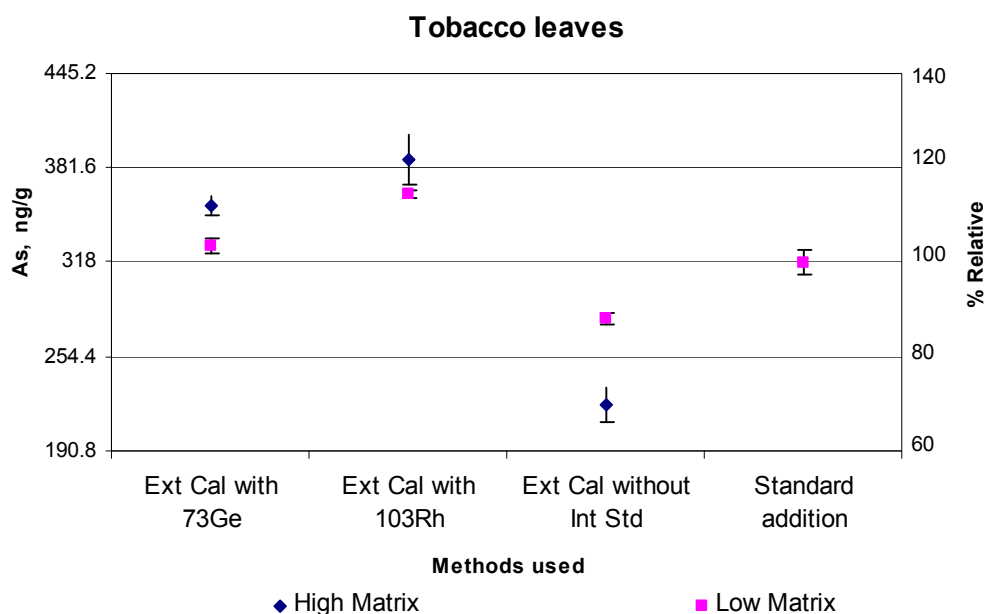


Figure 6.3 Effect of calibration methods on the measured As concentration in tobacco leaves. *Error bars are SD (n=8).*

Tobacco leaves were found to contain the most influential matrix on As determination. High variations of results ranging from 222 to 387 ng As g⁻¹ were observed, depending on calibration methods used and what extent of matrix exists in measured solutions. Results suggest that matrix effects are important, so standard addition calibration has to be used to obtain accurate results.

The use of the check As standard measured before and after sample measurement can be used to correct for the instrumental drift, but not for the matrix-induced effect. Although signal drifts arising from instrumental and matrix-induced effect can be traced by the use of internal standards, whether the correction of signal suppression from matrix is in the right way is dependent on the behaviors of analyte and internal standard used for a specific matrix. It is difficult to monitor the actual drift of As signals during sample measurement without a reliable internal standard (Unfortunately, As is mono-isotopic element). Differences between results obtained by external calibration without internal standard and standard addition method implied the 30% suppression of As signals arising from high matrix induced during measurement.

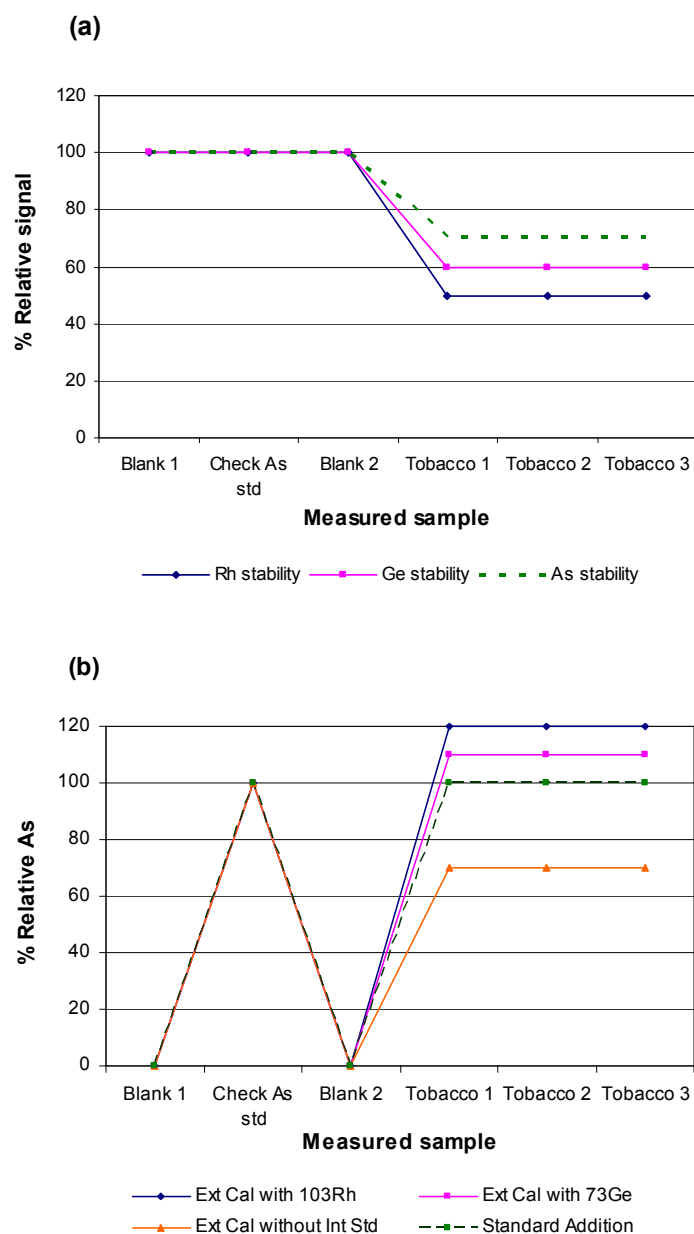


Figure 6.4 Demonstration on the matrix-induced effect on the signal stability of analyte (As) and internal standards (Rh and Ge) affecting the results of total As determination in tobacco digest (26,500 ppm TDS) using different calibration methods.

It can be seen in Fig 6.4 that Rh and Ge signals were suppressed by 50% and 40% in the presence of a high matrix, so a similar behaviour is therefore expected for arsenic. However, the degree of suppression in this case is 30%. It is therefore not surprising that the relative increases of measured As concentration obtained using

^{103}Rh and ^{73}Ge as internal standards were 20% and 10%, respectively, when compared with results obtained by standard addition.

The results in high matrix (26,500 ppm TDS) obtained from external calibration without internal standard (check standards used to adjust sensitivity) were found to 30% lower than those obtained from standard addition methods, suggesting serious effect arising from the matrix. However, this effect can be alleviated to 10% when performing in low matrix (10,600 ppm TDS). Suppression of analyte (As) and internal standard signals might be due to the presence of high levels of sodium in tobacco leaves. The results indicated that As is slightly less affected by the matrix than Ge and Rh, despite the fact that As have relatively a higher ionization energy. The reason behind this might be that tobacco digest was abundant in carbon. This can help the ionization of As due to a charge transfer between C^+ ions and arsenic atoms in the plasma [202, 203], as presented in the following reaction.



Although performing measurement in low matrix media provided better qualities of results, the selection of internal standard is also important to correct for signal drift from instrument and matrix for acquiring acceptable results. For example, external calibration with ^{103}Rh was not appropriate to use as an internal standard in this case. The use of Ge as an internal standard for tobacco digest in low matrix seems to be fairly acceptable, despite the fact that the ionization energy of Ge (762 kJ mol^{-1}) is much lower than that of As (947 kJ mol^{-1}). Although selenium might be used as an internal standard due to its proximity to As in terms of both mass and ionization energy seems to be ideal, certain amounts of selenium found in tobacco might led to spurious results. To check whether there is a difference between the mean results obtained by external calibration with Ge (10,600 ppm TDS) and standard addition methods, t test based on the pooled standard deviation was used. It was found that there is a significant difference between the results, at the 95% confidence level.

As part of method validation, a spiking experiment of As was conducted prior to sample digestion (by adding 100 μL of 650 ng As g^{-1}) to check whether there are any As losses during digestion. Complete recovery of total combined As in spiked tobacco leaves determined by standard addition indicated reliable methods for

digestion and measurement. The results of total As determination and recovery in tobacco and spiked tobacco and two CRMs are summarized in Table 6.2.

Table 6.2 Total As determination using standard addition method (precisions are standard deviations).

Samples	Certified value (ng g ⁻¹)	Experimental value (ng g ⁻¹)	% Recovery	n
Tobacco leaves (3R4F)	N/A	318 ± 9	N/A	8
Spiked tobacco leaves (3R4F) (185 ng As g ⁻¹ spiked)	N/A	506 ± 32	101 ± 5	4
NIST1575 (Pine Needles)	210 ± 40	215 ± 10	102 ± 5	4
NIST1568a (Rice Flour)	290 ± 30	290 ± 13	100 ± 4	4

6.3 PARTITIONING ARSENIC EXTRACTION

In biological tissues, arsenic species can be mainly divided into two types: polar and less-polar species, depending on its polarity. Water and methanol are usually used for polar As extraction, while hexane, chloroform, dichloromethane, and acetone appear to be used for extracting less polar species. In order to see distribution of As species according to polarity, partitioning As extraction, based on gravitational separation of two extractants (water/chloroform) possessing different density was performed.

This procedure was carried out by adding 2 mL of de-ionized water, followed by 2 mL of chloroform (99.9+%, Aldrich, Milwaukee) into 0.2 g of blended tobacco contained in a cleansed glass tube for mini CEM digestion. The mixture was shaken and then sonicated for 2 h in a water bath. Water and chloroform layers were separated, placed into an individual glass tube and evaporated by TurboVap at 40°C until dryness. The amount of As dissolved in the water and chloroform layers was quantified using the standard addition method, following acid digestion (0.5 mL of HNO₃) with mini CEM digestion unit. It was found that most of As species can be extracted in the polar fraction (130 ng g⁻¹ dry), whereas a tiny amount of As (6 ng g⁻¹ dry) can be recovered in less polar fraction. A single As extraction with water was also compared in terms of extraction efficiency with partitioning extraction. The preliminary study indicated that the use of water alone can extract As in tobacco leaves with 135 ng g⁻¹ dry, which was comparable to amount of As in the combined

fractions. It is probable that a small portion of arsenic dissolved in water can be partitioned and extracted into the less polar phase, so negligible amount of actual less polar species was believed to be present in the leaves. Consequently, effort for developing arsenic speciation methodologies was dedicated to polar As species.

6.4 DEVELOPMENT OF METHODOLOGIES FOR WATER-SOLUBLE ARSENIC EXTRACTION

There have been no previous reports on As speciation analysis using HPLC-ICP-MS in tobacco leaves. Studies of arsenic speciation detailing the actual species in tobacco leaves remain a challenge. Nevertheless, qualitative results of the oxidation state of As by X-ray absorption near-edge spectroscopy (XANES) revealed that most of As in cut tobacco leaves, mainstream cigarette smoke and cigarette ash were presented in oxidation state +5 [215]. Although X-ray absorption spectroscopy can provide details regarding bond distance and coordination number, its poor detection limit make it difficult to identify the extracted molecular arsenic forms.

6.4.1 Optimization of extraction method for water soluble arsenic species

Optimizing sample preparation condition is very important since there is a trade-off between species preservation and extraction efficiency. SEC-ICP-MS was used for fast-species screening for method development. Chromatographic condition for SEC-ICP-MS used was performed as described in section 4.4.1.

6.4.1.1 Effect of different extractants

For polar As-species extraction in plant materials, a number of studies have reported the use of a range of extractants such as water, buffer, 1% formic acid, and varying concentrations of MeOH. These extractants for water-soluble fraction in tobacco leaves were investigated. In this study, 0.2 g of blended tobacco was extracted in 10 mL of extractant for 1-h SON in a water bath. Following sonication, extracts containing methanol needed to be evaporated (at 40°C) until dryness, and then the crude extracts were diluted with water to obtain the same dilution factor as original. Without evaporation, sensitivity of As is affected due to charge transfer effects. All extracts were filtered through a 0.45 µm cellulose membrane before HPLC introduction. The results displayed, in Fig 6.5(a), show no differences in total peak area of SEC-ICP-MS for all extracts, except the extract with higher 50% MeOH ratio,

at which lower As extraction was obtained. The reason for this may be a limited capacity of high ratio of the MeOH for extracting polar As species.

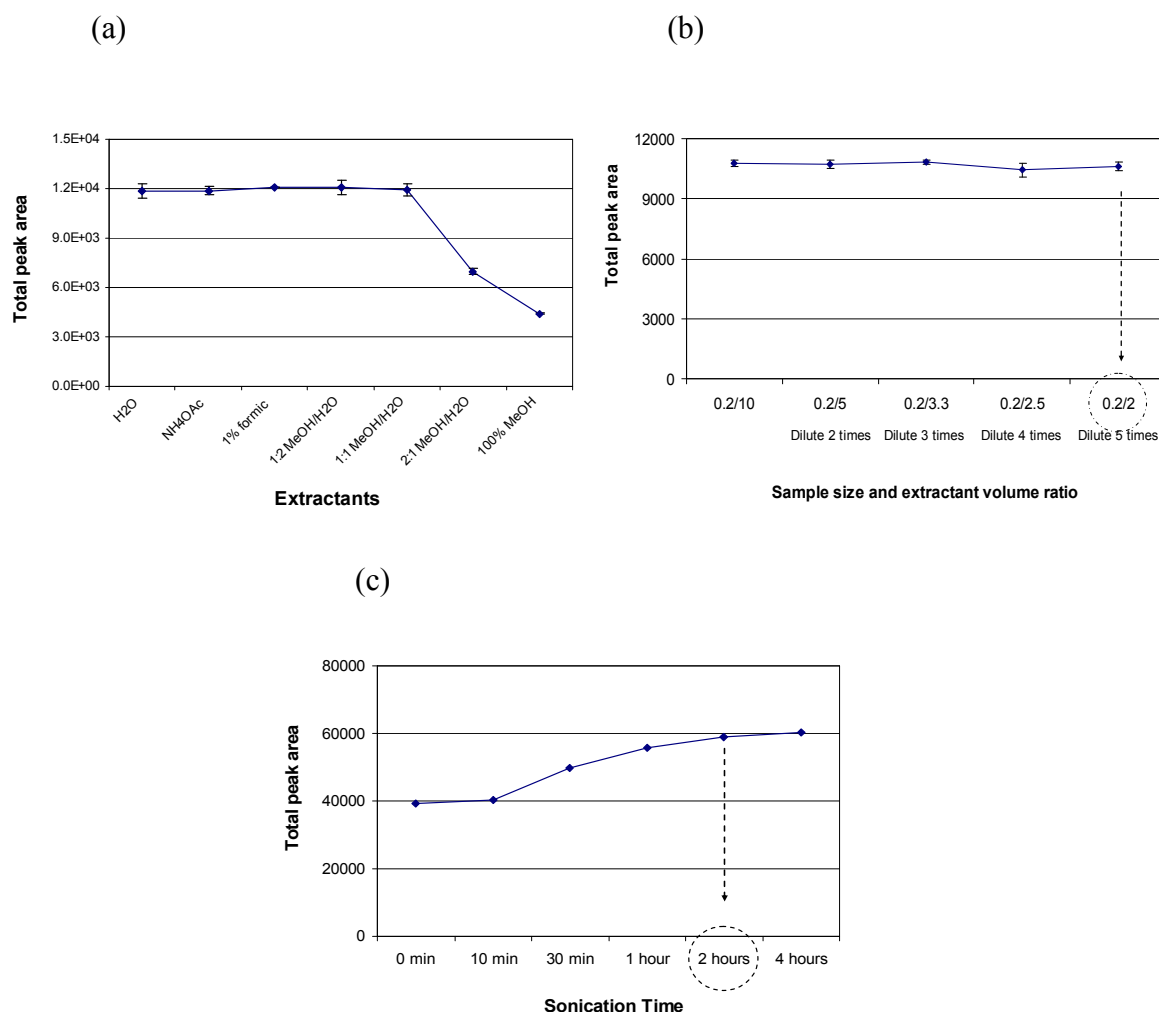


Figure 6.5 Optimization of (a) extractants, (b) sample size and extractant volume ratio, and (c) sonication time on As extraction (from total PA obtained by SEC-ICP-MS profiles). *Error bars are SD.*

Consequently, the most important criteria to justify the optimal extractant appears to be species preservation. As presented in Fig 6.6(a-c), optimization of different extractants was investigated by means of SEC-ICP-MS As profiles. Although 1% formic acid gave the efficiency for the extraction equal to water, it can cause almost complete degradation of As-containing biomolecules (HMW at t_r 11.5 min) to As(V) t_r ~14 min, while these molecules were preserved with water. Without such essential information obtained by SEC-ICP-MS profiles, losses of information of

HMW As species, and overestimation of inorganic As might be possible. Likewise, the use of MeOH in the extractant also led to degradation of the HMW compound. This might be due to MeOH evaporation by Turbovap prior to LC introduction. It was also observed that the degree of extraction of HMW As species was lower, when higher ratio of MeOH in extractants was used. Nevertheless, no significant differences were observed for the profiles obtained by extraction with water and NH_4OAc . For simplicity, de-ionized water was therefore chosen to be an extractant for further study.

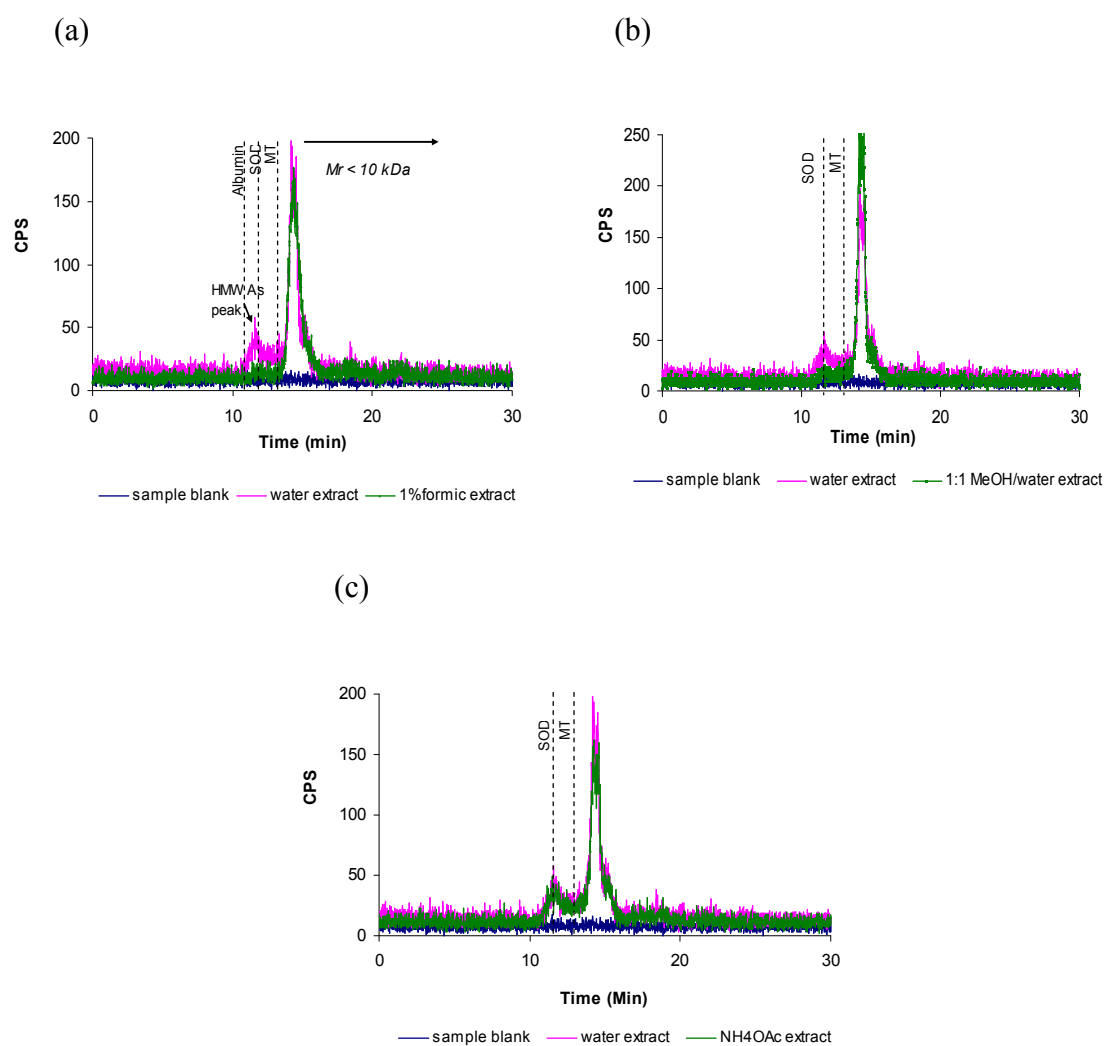


Figure 6.6 Comparison of SEC-ICP-MS As profiles of water with (a) 1% formic acid, (b) 1:1 MeOH/water and (c) NH_4OAc extracts.

6.4.1.2 Effect of sample size and extractant volume ratio

Since the measured As concentration in the solid samples was found at low ppb levels, the method development aimed at reducing the extractant volume and using the minimum sample amount without sacrificing extraction efficiency in order to improve precision of measurement. Effect of sample size to extractant volume ratio on As extraction efficiency, as presented in Fig 6.5(b), was investigated by varying the volume of water (10, 5, 3.3, 2.5 and 2 g) and keeping the solid weight constant (0.2 g). After 1-h sonication, dilution of extracting solutions was performed to obtain similar dilution factor for comparing SEC-ICP-MS peak area. This study shows that the use of sample size to extractant volume ratio at 0.2 g in 2 g of water provided efficiency of extraction equally to the original ratio. Then, 0.2 g of blended tobacco leaves in 2 g of de-ionized water was found to be optimal and then used for further study.

6.4.1.3 Effect of sonication time

In order to achieve better extraction efficiency for As whilst still preserving the species, the effect of sonication time was investigated at 0, 10 min, 30 min, 1 h, 2 h and 4 h. Similar SEC-ICP-MS As profiles were observed in Fig 6.7 and gave the total PA varying with sonication time. The total PA of water-soluble As extraction in tobacco leaves reached a plateau at 2 h, (Fig 6.5(c)). The As extraction efficiency was determined by ICP-MS after mini-CEM digestion (150W, 150°C, 10 min) of the dried extracts (with 0.5 mL of HNO₃) by standard addition method found to be 43±3% (n=3). Although over 2 h sonication slightly more As species can be extracted, the degradation of As-bound biomolecules (at t_r 11.5 min) seemed to be increasing. Using AE-LC-ICP-MS, the results indicated that the decomposition of HMW As compounds resulted in accumulation of As(III) at 4-h sonication. Two-hour sonication time was used for further experiments.

The optimum sonication condition for water-soluble As fraction in tobacco leaves is summarized as follows: 0.2 g blended tobacco leaves in 2 g of de-ionized water for 2-h sonication.

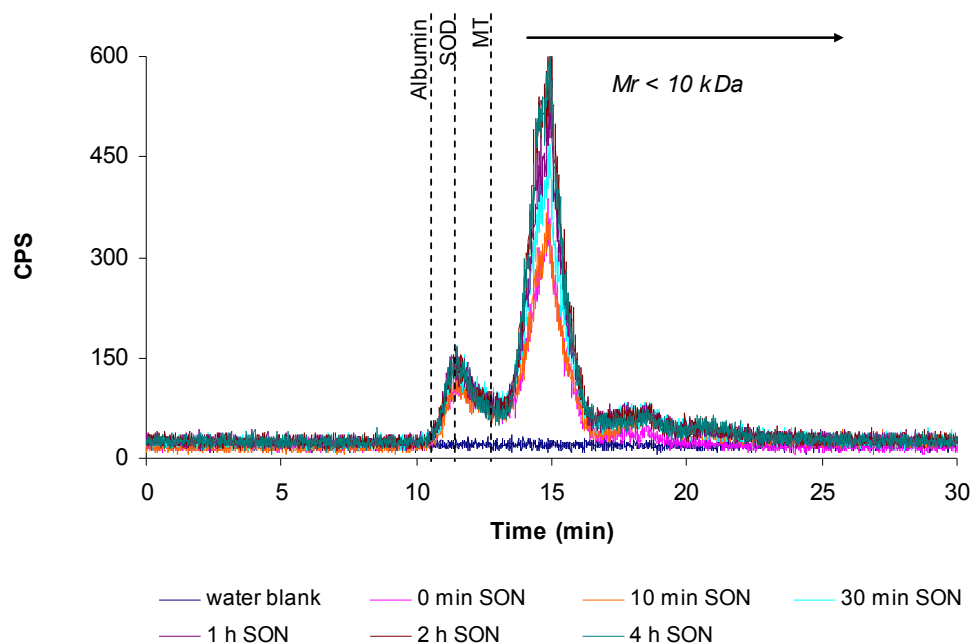
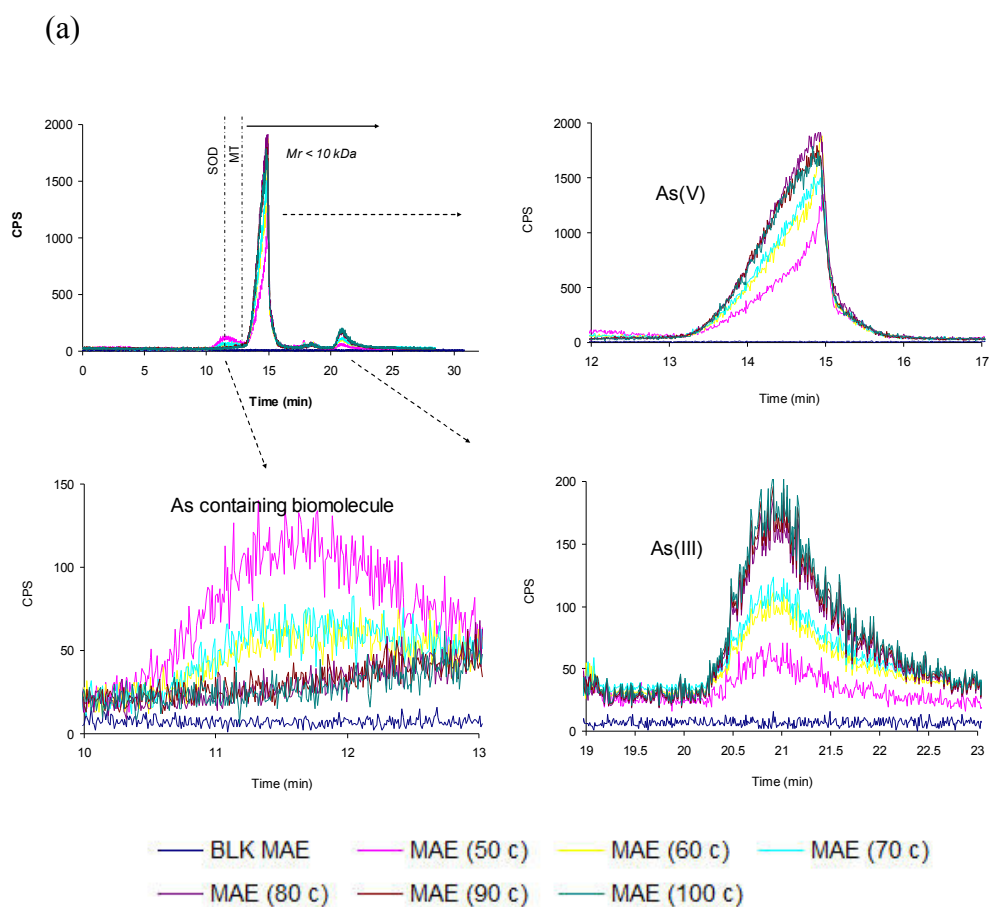


Figure 6.7 Effect of sonication time on As distribution on SEC-ICP-MS As profiles.

6.4.2 Optimization for water-soluble arsenic extraction using microwave-assisted extraction (MAE)

In order to see whether we can further increase extraction efficiency or minimize the extraction time, microwave-assisted extraction (MAE) was considered a priority. The first MW parameter investigated was MW temperature, believed to largely influence As extraction efficiency. This study was performed using 0.2 g of blended tobacco leaves in 2 mL of de-ionized water with mini CEM system by setting MW power at 150 W, and varying extracting temperature from 50°C to 100°C for 10 min. Three major regions can be seen in SEC-ICP-MS As profiles (Fig 6.8): (1) $t_r \sim 11.5$ min, this peak supposed to be As-containing biomolecules, (2) $t_r \sim 14$ -15 min, which was mostly comprised As(V), with small amounts of MMA and DMA, and (3) $t_r \sim 21$ min, As(III). Results show that increasing MW temperature led to building up of inorganic As peaks (As(III), and As(V)), whereas As-containing biomolecule peak was inclined to be decreasing. This study demonstrated how the use of SEC for method development of elemental speciation studies is important. Some studies reported the use of high MW temperature in order to achieve high extraction efficiency, without the evidence of preserving original forms of species [104]. The use of MAE at high

temperature is considered to be controversial, if the survival of HMW species is not tested.



(b)

Sample extract	Peak area at the retention time	
	11.5 min	21 min
Water blank	ND	ND
MAE 50°C	81505	48650
MAE 60°C	33213	106667
MAE 70°C	28927	111564
MAE 80°C	ND	183111
MAE 90°C	ND	184561
MAE 100°C	ND	186952

Figure 6.8 The SEC-ICP-MS As profiles of water extracts using MAE with varying temperature (a), the comparative peak areas at the retention time 11.5 and 21 min (b).

Considering the peak areas at both retention times, this study suggested that, over 50°C, not only the HMW As species at t_r 11.5 min but also some of As possibly bound to cell wall constituents and insoluble proteins were degraded to As(III) at t_r 21 min. At 80°C, the As-containing biomolecules were entirely decomposed. Not only MW temperature, but also MW power should be investigated. It was found that MAE condition at 150 W/50°C resulted in a slight degradation of As containing biomolecules. As presented in Fig 6.9, MAE condition at 50 W/50°C and extraction time of 10 min was found to be optimal for As speciation analysis in tobacco leaves, showing similar profile to 2-h SON extraction. Using MAE makes it possible to minimize extraction time from 2 h to 10 min.

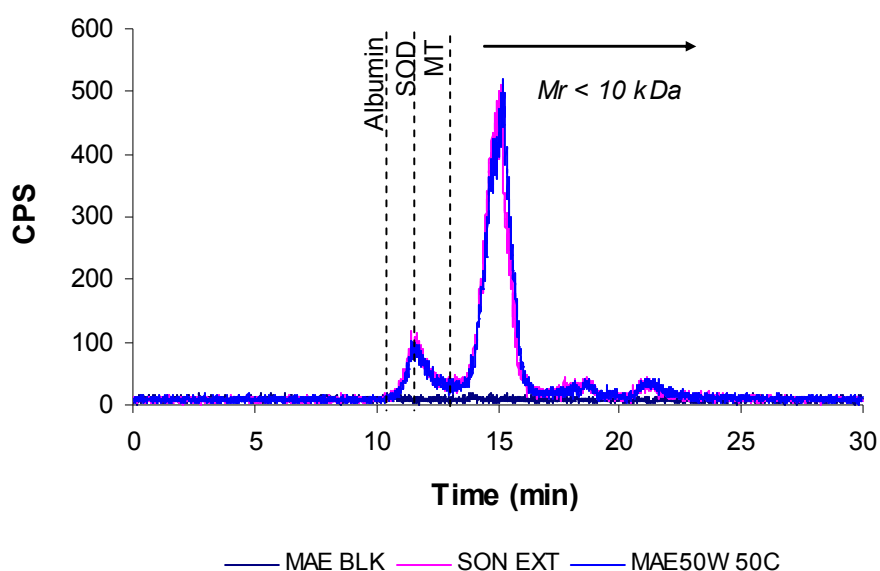


Figure 6.9 Comparison of SEC-ICP-MS As profiles obtained by MAE (50W, 50°C) with sonication.

6.4.3 Preliminary identification of arsenic species and its distribution in the water extracts

Due to limited separation of As-species on the SEC column, water-soluble As species in tobacco leaves were separated on an anion exchange column. The chromatographic condition used was described in the section 5.4.2, but to improve detection capability of As, the injection volume was increased from 50 μ L to 100 μ L.

The presence of extremely low concentration levels of water-soluble As species in tobacco leaves was found to be demanding to identify/characterize properly by organic mass spectrometry. Preliminary identification of As species was conducted by spiking experiment with As standards. Two sets of mixed As standards: the mixture of As standards (1): AsB, As(III), DMA, MMA, and As (V) standards; and the mixture of As standards (2): AsC, and 4 arsenosugars (A-D), were spiked into individual water-soluble As extracts, respectively.

The spiking AE-LC-ICP-MS As profiles of water extract with the mixed As standards 1, as shown in Fig 6.10, might suggest the potential existence of 5 As species: AsB, As(III), DMA, MMA, and As(V), whereas there were no As peaks matching with the retention time of mixed As standards (2). However, the assignment of peak in the vicinity of AsB should be considered carefully. Cation-exchange chromatography, which allows the species near the void volume in AE column to be more retained, and the use of organic MS are needed to confirm the species identity. Due to the lack of evidence in the presence of AsB and AsC in terrestrial plants, there is a possibility that the peak near the void volume is TMAO rather than AsB. Reported to elute very close to the void volume in anion-exchange column [184], TMAO has been identified in some plants [185] and detected as the dominant organoarsenical species in atmospheric particulate matters [184].

The distribution of As species in water extracts indicated that most of the As species (89%) are inorganic As ($\text{As}^{\text{III}} + \text{As}^{\text{V}}$), with small amounts of DMA and MMA (6%), the As species near the void volume (2%) and others (<3%). In common with other terrestrial plants like apple, rice, nuts and grass [100, 102, 104, 216], it was not surprising that the majority of As species found in tobacco leaves were inorganic arsenic, with small amounts of MMA, or DMA identified. This study is in agreement with XANES; most of As species were As(V) [215].

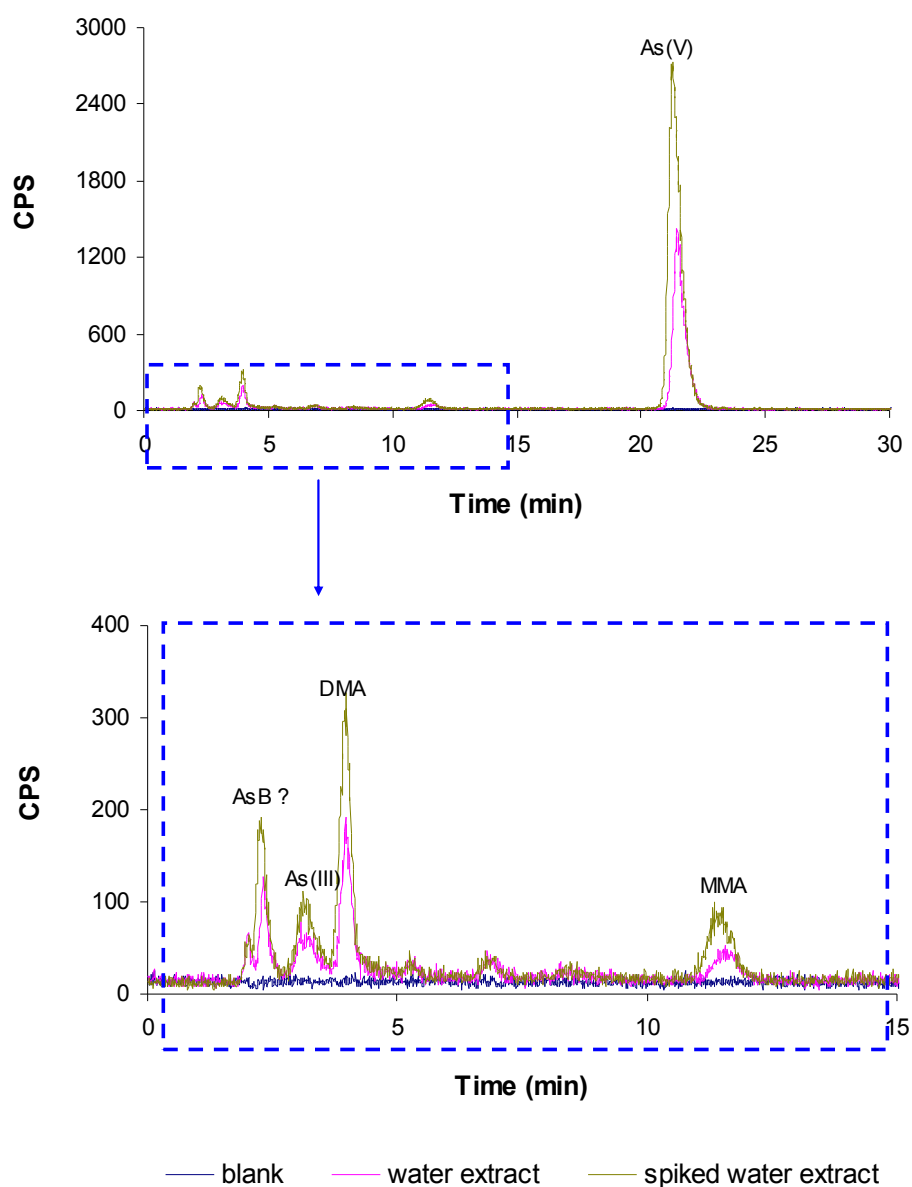


Figure 6.10 Distribution of arsenic species present in an aqueous extract of tobacco leaves, preliminarily identified by spiking experiment with a mixture of As standards (1) comprising AsB, MMA, DMA, As(III) and As(V).

6.5 ENZYMATIC AND SDS EXTRACTION

Over 50% of arsenic species in tobacco leaves were found to be difficult to extract with water. It was therefore considered where the remaining 50% are located. It is likely that As species which are not extracted might bind with cell wall components like cellulose, hemicellulose and pectin, or plant proteins, which are abundant in tobacco leaves and then form strong affinity [210]. The following extractants such as

driselase, SDS, protease and lipase were used to investigate the potential for improving the extraction of different classes of As compounds.

Brief descriptions of general information and chemical properties of the extractants mentioned are given as follows. Driselase, a multicomponent enzyme prepared from *Basidiomycetes* sp. containing cellulase, laminarinase, pectinase, xylanase and amylase, is known to selectively digest cell wall components. Sodium dodecyl sulphate (SDS), a surfactant, is able to leach water-insoluble protein complexes, whereas the protease type XIV, a group of proteolytic enzymes produced by *Streptomyces griseus* K1 containing at least 10 proteases in the mixture, is nonspecific and proved to be much more effective in digestion. Finally, lipase, a water-soluble enzyme comprising a subclass of the esterase, is able to catalyze the hydrolysis of ester chemical bonds in insoluble lipid substances.

To be effective for enzyme activities, these enzymes and SDS were dissolved in Tris buffer used as a buffer in biochemistry at pH 7.5. Although Tris-HCl has been extensively used in selenium speciation for garlic and yeast, it is not appropriate for the use in As speciation work due to the undesirable effect of chloride introduction. As the preliminary study found no differences between the use of water, Tris-HCl, and Tris base in terms of As extraction efficiency and species preservation (SEC-ICP-MS As profiles compared), Tris base was therefore chosen for a medium for enzymatic and SDS extraction.

The modified extraction procedure from literatures [108, 109], were carried out in duplicate and described as following.

1. Incubation at 37°C & sonication with 30 mM Tris base

Approximately 200 mg of blended tobacco leaves were placed in cleansed glass MW vessels followed by the addition of 2 mL of 30 mM Tris base (99.9+%, Sigma-Aldrich, St Louis, USA), pH 7.5. The mixtures were incubated in a hybridization oven Model HB-2 (Techne, Duxford, UK) at 37°C for 20 h, in comparison with sonication in a water bath for 2 h.

2. Incubation with 4% (w/v) driselase

Approximately 200 mg of blended tobacco leaves were placed in cleansed glass MW vessels followed by the addition of 2 mL of 4% (w/v) driselase solution in 30 mM Tris base pH 7.5 containing 1 mM phenylmethanesulfonyl fluoride (PMSF, >99% GC, Sigma-Aldrich) as the protease inhibitor. The mixtures were incubated in a hybridization oven at 37°C for 20 h.

3. Sonication with 4% (w/v) SDS

Approximately 200 mg of blended tobacco leaves were placed in cleansed glass MW vessels followed by the addition of 2 mL of 4% (w/v) SDS (>99% GC, Sigma-Aldrich) in 30 mM Tris base pH 7.5. The mixtures were sonicated in a water bath for 2 h.

4. Incubation with 0.4% (w/v) protease

Approximately 200 mg of blended tobacco leaves were placed in cleansed glass MW vessels followed by the addition of 2 mL of 0.4% (w/v) protease type XIV from *Streptomyces griseus* (Sigma Aldrich, St Louis, USA) in 30 mM Tris base pH 7.5. The mixture was incubated in a hybridization oven at 37°C for 20 h.

5. Incubation with 0.2% lipase

Approximately 200 mg of blended tobacco leaves were placed in cleansed glass MW vessels followed by the addition of 2 mL of 0.2% (w/v) lipase from *Candida rugosa* (Sigma Aldrich, St Louis, USA) in 30 mM Tris-base pH 7.5. The mixtures were incubated in a hybridization oven at 37°C for 20 h.

6. Incubation with a mixture of 0.4% protease and 0.2% lipase

Approximately 200 mg of blended tobacco leaves were placed in cleansed glass MW vessels followed by the addition of 2 mL of a mixture of 0.4% (w/v) protease and 0.2% (w/v) lipase in 30 mM Tris base, pH 7.5. The mixtures were incubated in a hybridization oven at 37°C for 20 h.

Following extraction, reaction mixtures were centrifuged at 3000 rpm for 30 min. Supernatants were decanted and residues were washed with 30 mM Tris base twice. Leaching buffer was collected in a supernatant vessel. Subsequently, supernatants were divided into 2 portions for speciation analysis and total measurement. The latter was evaporated to dryness at 40°C in order to facilitate digestion, using mini CEM system. Dried supernatants were digested with 0.5 mL of HNO₃, whereas residues were digested with 1.5 mL of a mixture of (2:1) HNO₃ and H₂O₂. Determination of total As in different extracts and residues were performed using standard addition method.

To investigate how efficiently these enzymes and SDS performed for As extraction, results of total As in the extracts and SEC-ICP-MS As profiles from individual enzymes and SDS were compared with those obtained by the use of 30 mM Tris base, pH 7.5 alone.

6.5.1 Selection of enzymatic extraction for formulating a sequential extraction scheme

Results obtained from the use of different enzymatic, and SDS extraction in comparison with that of Tris base alone are shown in Table 6.3. The SEC-ICP-MS As profiles of enzymatic and SDS extracts in comparison with their blanks are depicted in Fig 6.11(a-d).

With no enzymes, it was found that the use of either 2-h sonication or 20-h incubation at 37°C with 30 mM Tris base, pH 7.5 gave similar results in terms of As extraction efficiency and species preservation, which can be observed in SEC-ICP-MS As profiles. These methods gave 42% As extraction efficiency. Using lipase, there was no evidence of assisting the improvement of As extraction. These findings indicated that less amount of As species in tobacco leaves were bound to lipid, which was in good agreement with the early result (a negligible level found in the chloroform layer).

Nevertheless, the use of protease gave a slight increase in enhancing the As extraction from 42% to 46%. This might show the presence of As bound to plant proteins, known to be abundant in tobacco leaves. As expected, the result of the combined use of protease and lipase were found similar to that of protease alone. As a result, the increase in As extraction efficiency was believed to be ascribed to protease effect. However, it was found that the introduction of protease for assisting As extraction led to high procedural blank, since it was applied in the tobacco sample containing a low concentration level of As.

Insignificant differences in extraction efficiencies and the SEC profiles between incubation of sample with Tris base alone and driselase solution, were observed. Similar to several studies, it was indicated that driselase had a little assistance for As extraction in Reed Grass and Lady fern [217], and Se extraction for garlic [107].

By means of sonication, the improvement of As extraction can be made using SDS (from 42% to 47%). As shown in the SEC-ICP-MS profile, it was found an additional As peak appeared at the early retention time 10.5 min ($M_r \sim 66$ kDa), which was unnoticed in other extracts. This suggested that the surfactant can be used as a powerful reagent to leach HMW As species.

The use of enzymatic and SDS for As extraction in tobacco leaves, in general, can give at least equal extraction efficiency to that of water alone, and remain HMW

As species intact. Although the use of certain enzymes can give slightly better As extraction than the use of the buffer alone, single enzymatic extraction was found to be difficult to differentiate the source of extractable inorganic As species whether they were derived from readily free ions, or As bound to cell walls or insoluble protein. This is due to the fact that enzyme can cleave target biomolecules and release as As^V or As^{III}, which are mixed with readily extractable inorganic As.

Table 6.3 Results of As extraction efficiencies in tobacco leaves obtained using different enzymatic/SDS extractants.

Extractants	Amount of As in extract (n=2)		Amount of As in residue (n=2)		% Extraction Efficiency (n=2)
30 mM Tris base pH7.5 (incubation 37°C, 20 h)	130	138	177	180	42
4% Driselase (incubation 37°C, 20 h)	142	134	173	172	43
0.4% Protease (incubation 37°C, 20 h)	152	142	167	169	46
0.2% Lipase (incubation 37°C, 20 h)	130	124	185	191	40
0.4% Protease-0.2% lipase (incubation 37°C, 20 h)	157	147	167	168	48
30 mM Tris base pH 7.5 (sonication, 2 h)	141	129	183	189	42
4% SDS (sonication, 2 h)	153	147	158	164	47

(Total As in dried tobacco leaves $318 \pm 9 \text{ ng g}^{-1}$, n=8)

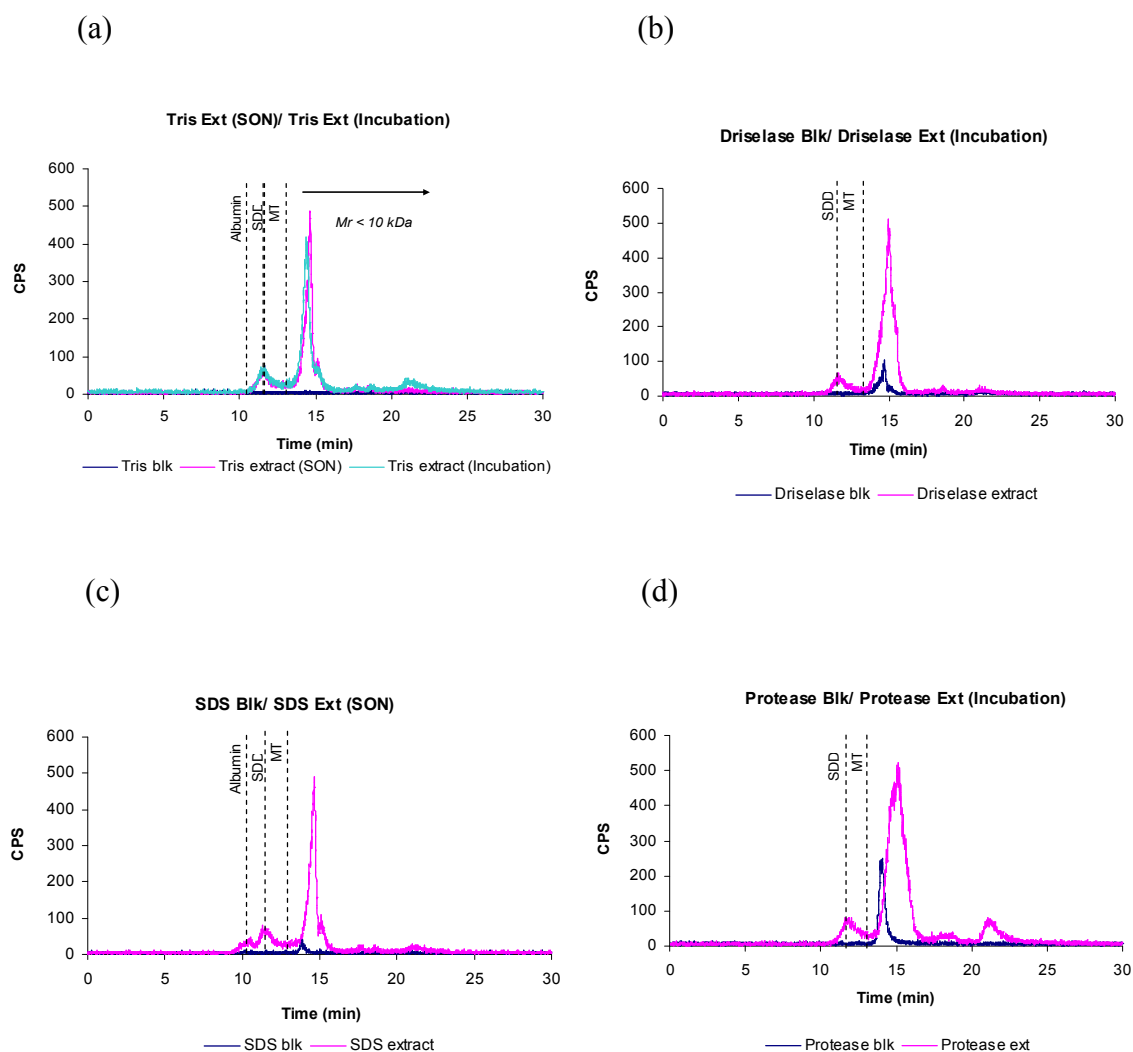


Figure 6.11 Comparative SEC-ICP-MS As profiles obtained from (a) Tris blank-Tris extract (b) Driselase blank-Driselase extract, (c) SDS blank-SDS extract and (d) Protease blank-Protease extract.

In order to characterize the bindings (cell wall bound, or insoluble-protein bound) of unextractable As and to know their ratios, a sequential As extraction involving the use of specific enzyme and SDS to consecutively leach the rest of As species strongly bound to cell walls and insoluble proteins was used. Carry-over can affect the accuracy of the sequential extraction method. To minimize this problem, rinsing residues several times and then combining supernatants in each wash was performed. The proposed sequential extraction scheme was formulated as following. A water-soluble extraction was the first step for As extraction in tobacco leaves due to

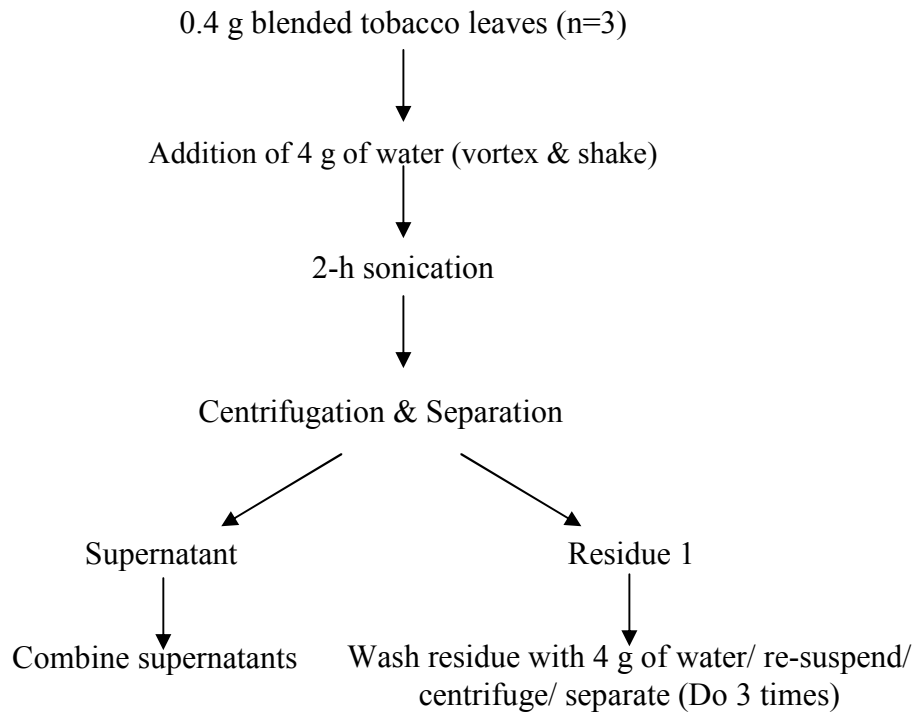
the large portion of water-soluble As species present. This fraction involves organoarsenicals such as As species near the void volume, MMA, DMA, and readily dissolved inorganic arsenic (As(III)+As(V)), which accounted for approximately 40%. Despite providing less improvement in As extraction by direct incubation with sample, lysis of cell walls was chosen for the second step because there was some of unextractable As believed to bind with cell wall components. Attempts to extract the residue using driselase after sonication with water were made. It was thought that driselase is more active for removing As bound to cell walls following textural changes during sonication. The SDS extraction step uses surfactant to leach insoluble proteins. As with previous results, SDS gave better As extraction efficiency than other extractants used. The sonication time with SDS was adjusted from 2 h to 1 h in common with literature reports [105]. The steps used were similar to the scheme used for the extraction of selenocompounds in selenized yeast [105]. Finally, proteolytic extraction employing protease was used to break down the rest of As bound protein remaining in sample residues.

6.5.2 Characterization of arsenic species in the leaves using sequential extraction scheme

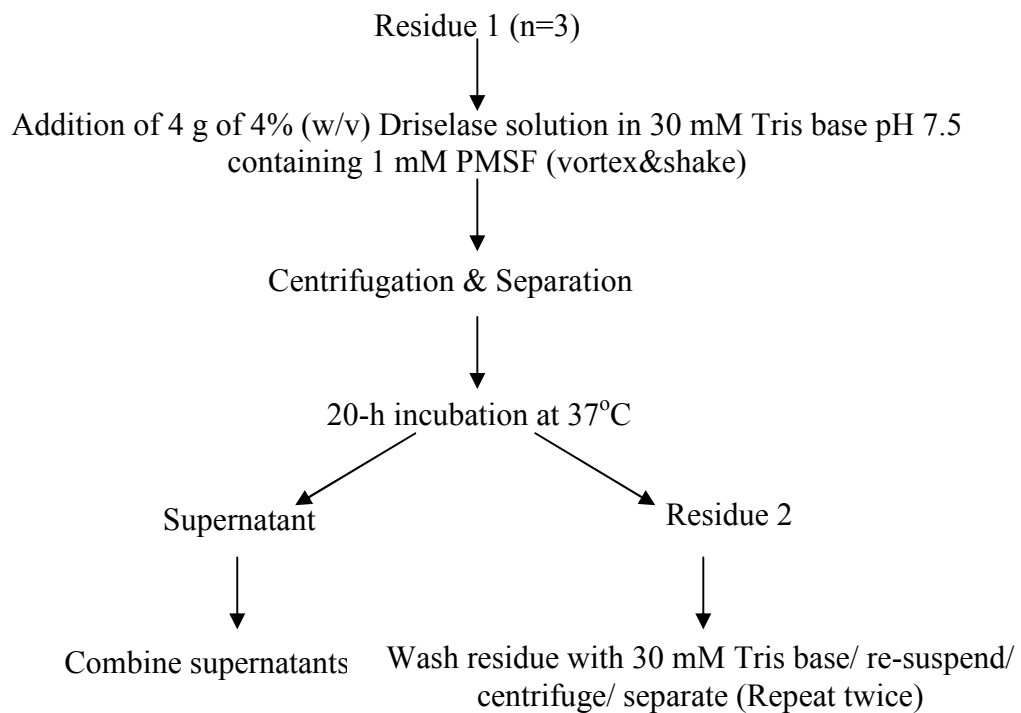
A sequential extraction scheme was not only designed for improving extraction efficiency, especially for plant materials but also used for fractionating As-species according to its affinity to organic ligands. The scheme consists of 4 steps: water soluble, lysis of cell walls, SDS extraction, and proteolytic extraction. To improve the precision and accuracy of total As measurement in the extracts, sample size and extractant volume ratio used in this study was increased 2-fold from the previous ratio and the extraction was carried out in triplicate.

This fractionation procedure consists of four following steps:

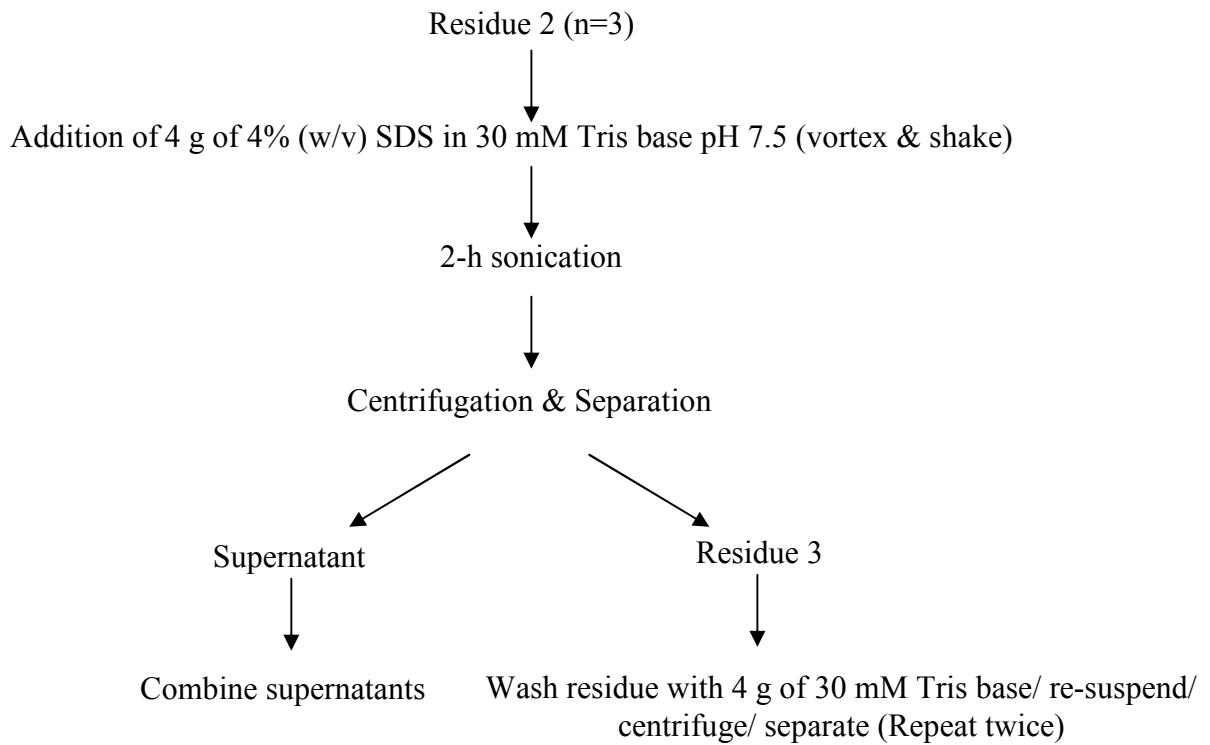
(1) Water soluble extraction



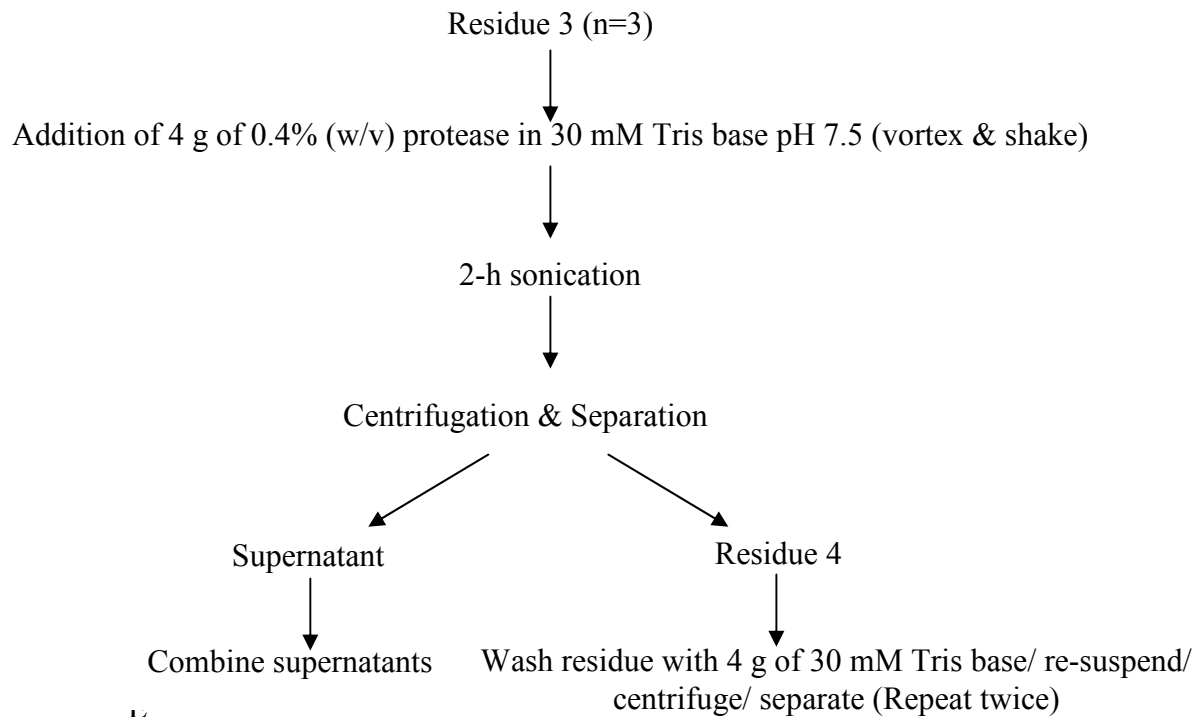
2. Lysis of cell walls



3. SDS extraction



4. Proteolytic extraction



Extracts in each stage were centrifuged at 3000 rpm for 30 min. Supernatants were decanted and residues were washed with 30 mM Tris base twice. Supernatants were then combined and separated into 2 portions for speciation analysis and total measurement. The latter was evaporated to dryness at 40°C to facilitate digestion using mini CEM system. Dried supernatants were digested with 0.5 mL of HNO₃, whereas residues were digested with 1.5 mL of a mixture of (2:1) HNO₃ and H₂O₂. Determination of total As in different extracts and residue were performed using the standard addition method. Using the sequential extraction scheme, results obtained from individual steps were reported as the extractable As amount and percent increased of As extraction efficiency as shown in Table 6.4, while the distribution of As categorized by extractants used can be illustrated as a diagram in Fig 6.12.

Table 6.4 As extraction efficiency results obtained by the proposed sequential extraction scheme.

No.	Steps	Amount of extractable As (ng g ⁻¹ , n=3)	% As extraction efficiency
1	Water soluble	135 ± 5	42
2	Driselase	40 ± 1	13
3	SDS	25 ± 2	8
4	Protease	4 ± 2	1
Total sequential steps		204 ± 10	64

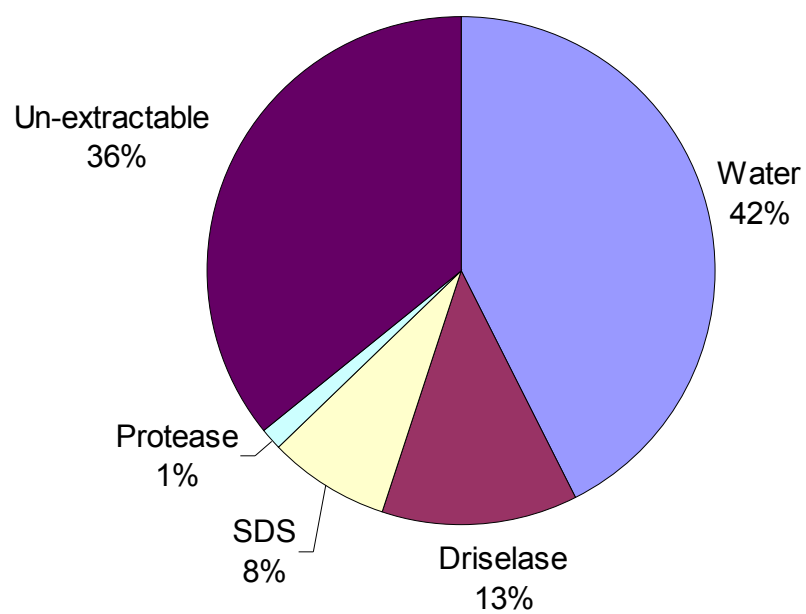


Figure 6.12 Distribution of As species categorized by extractants used.

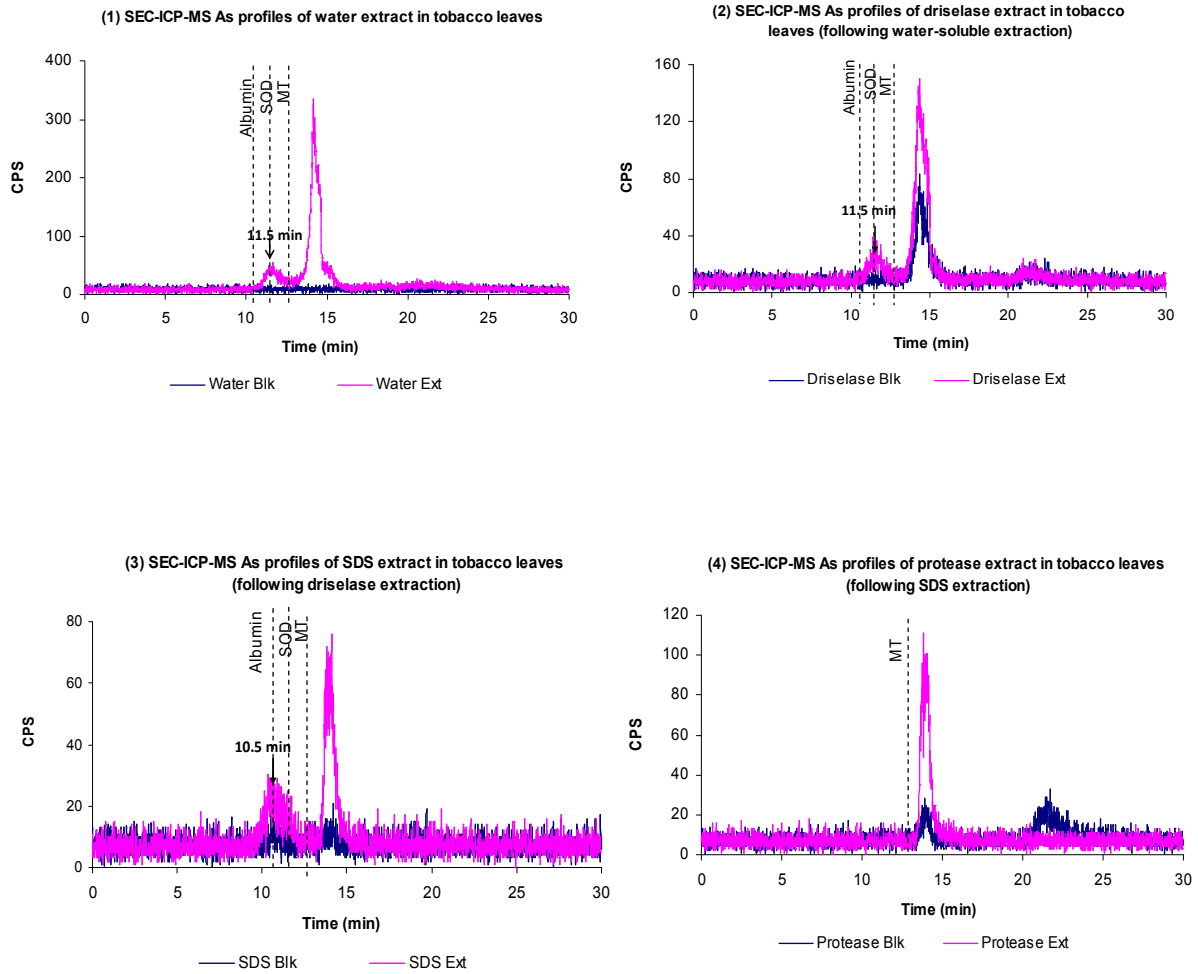


Figure 6.13 The SEC-ICP-MS As profiles of sequential extracts in tobacco leaves.

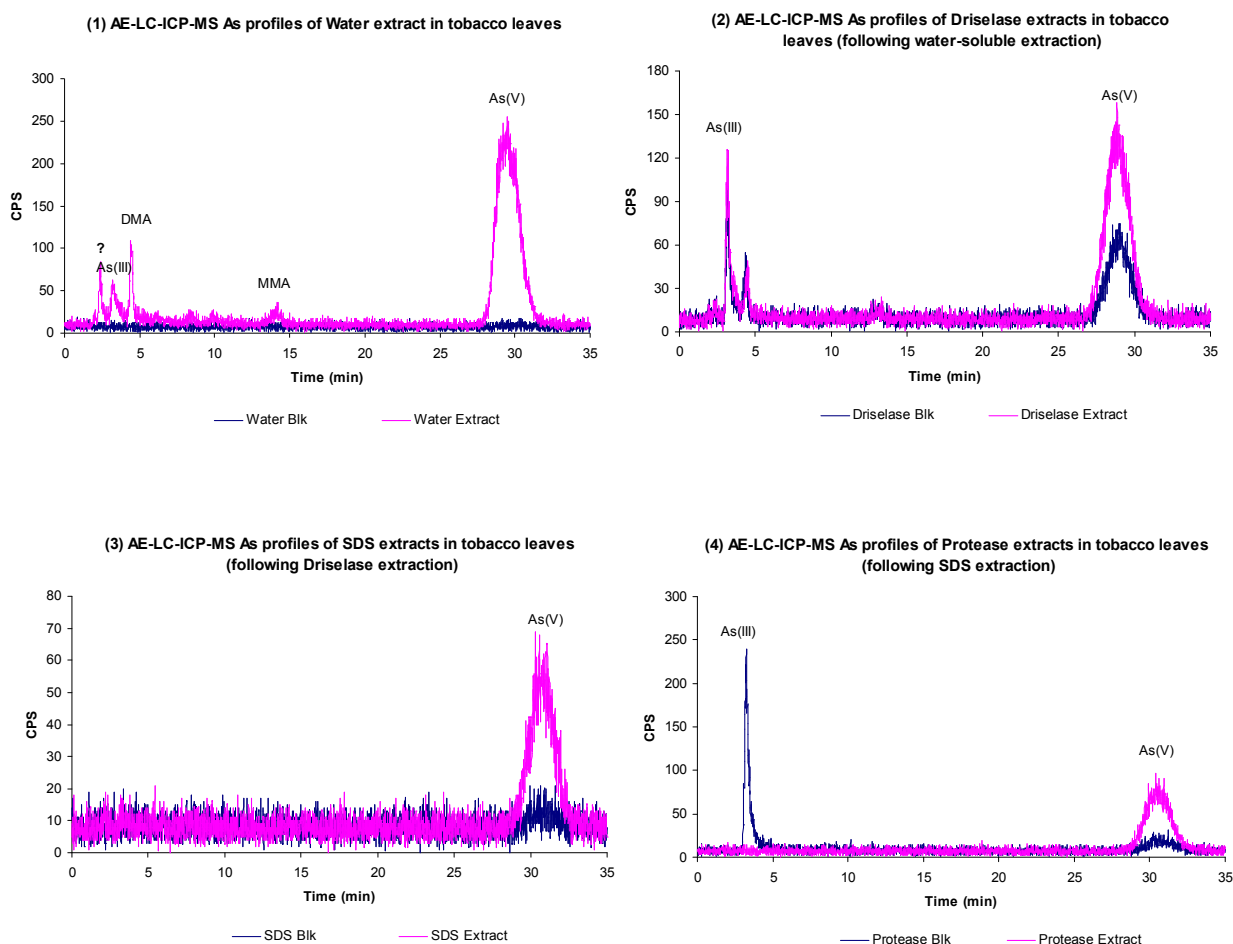


Figure 6.14 The AE-LC-ICP-MS As profiles of sequential extracts in tobacco leaves.

The proposed sequential extraction scheme overall provided 64% extraction efficiency, which was 50% improvement from the use of water alone. To see the distribution of As species, Fig. 6.13-6.14 show the SEC-ICP-MS, and AE-LC-ICP-MS As profiles of sequential extracts in tobacco leaves. The results show that incubation with 4% (w/v) driselase solution for 20 h to extract after the sample was previously sonicated proved to be more effective than direct incubation. The lysis of cell wall process can provide an additional 13% increase in the extraction efficiency. This may be due to the change in the surface texture caused by sonication, thereby readily releasing As species form from plant cell walls. The enzyme can cleave As bound polysaccharides, releasing HMW As peak at t_r 11.5 min ($M_r \sim 32$ kDa), which can be seen in SEC-ICP-MS profile, and inorganic As observed in AE-LC-ICP-MS

profile. Further 8% efficiency increased was given by SDS extraction. Using 4% (w/v) SDS solution, known as surfactant, with 1-h SON can leach even larger HMW As peak at t_r 10.5 min ($M_r \sim 66$ kDa), and inorganic As. Less extraction efficiency was improved (1%) using protease, implying that the use of SDS was sufficient to extract As containing insoluble proteins. Consequently, an improved sequential 3 step As extraction scheme for tobacco leaves is proposed comprising: water soluble, lysis of cell walls and SDS extraction.

6.6 SUMMARY

Methodologies for total As determination in tobacco leaves were investigated using different calibration methods (external calibration with/without internal standards: Ge and Rh, and standard addition methods), following a closed vessel MW digestion. Different behaviours of internal standards used and As were observed which resulted in a wide variation of total As results, which was thought to be ascribed to the plant matrix. Since matrix is essential, standard addition method has to be used to obtain accurate measurement.

Partitioning As extraction, for giving an idea of polarity of As species present, shows that the majority of As species were likely to dissolve in polar phase, while a negligible level of As was found in less polar layer. As a result, method development of As speciation in tobacco leaves was focused on water-soluble species. Optimization of sample preparation methods was made on the basis of extraction efficiency and species preservation. Due to detection capability of As species in low ppb levels required, several key parameters on the extraction efficiency such as type of extractants, sample size to extractant volume ratio and extraction time were investigated. SEC-ICP-MS As profiles were proposed to use as a powerful tool for fast species screening. Results suggested that the use of 1% formic acid as an extractant can cause degradation of HMW As species to inorganic As, while the species can be well preserved in water. Although over 2 h sonication, a slight improvement in extraction efficiency was observed, the HMW peak was inclined to be degraded. The use of 0.2 g in 2 g of de-ionized water with 2-h sonication was found to be optimal, giving approximately 43% As extraction efficiency. Preliminary study suggested the presence of inorganic arsenic (89%) as major species, with a minority of MMA, DMA and the peak in the vicinity of AsB.

Despite the superior capability of As extraction by microwave-assisted extraction compared with sonication, selecting the MW condition was principally considered from original species preservation point of view. Above 50°C, the HMW species became degraded to As(III), and these species were completely degraded at 80°C. With the optimum MW condition at 50W and 50°C, microwave-assisted extraction (MAE) was used as an alternative technique for accelerating the extraction from 2 h for sonication to only 10 min.

Efforts to improve the extraction efficiency were made using enzymatic and SDS extraction. Despite the fact that single extraction with protease and SDS can extract up to 50% of the As from tobacco samples, there is a difficulty in differentiating the source of leachable inorganic As whether they were derived from being readily extracted or from being cleaved by enzymes. A sequential extraction scheme was used to consecutively leach As species according to its affinity and to ensure the highest As extraction efficiency achieved (63% for tobacco leaves obtained). Since the use of LC-ESI-MS is unlikely to detect these compounds at low concentrations, the proposed procedure was utilized to characterize tobacco sample in terms of distribution of As among the different origins e.g., water soluble (42%), cell wall bound (13%) and insoluble protein bound (8%), allowing a comprehensive study of the speciation of As in the sample. Such information is essential for gaining insights into smoke chemistry of the transformation of As species from tobacco leaves during transference to cigarette smoke.

CHAPTER 7

APPLICATION OF WATER-SOLUBLE AS SPECIATION METHOD TO THE STUDY OF MAINSTREAM SMOKE CONDENSATE (3R4F)

Despite the fact that toxicity of cigarette smoke has been recognized for over a century, most efforts have been dedicated to the study on organic constituents. There is far less information on metal(loid)s available. Approximately 30 metal(loid)s have been detected in tobacco smoke [13], and details on percent transfer of these elements between tobacco and tobacco smoke are given in Appendix 5. However, due to measurement uncertainties over the chemical forms of the metal(loid)s and their oxidation states, it is currently difficult to establish the potential relevance of these materials to the toxicity of cigarette smoke.

Smoke chemistry analysis is instrumental in the research and development of the tobacco industry. Traditionally, the industry has mainly employed various spectroscopic techniques to quantify total levels of trace elements in cigarette smoke. However, these techniques provide no information on the chemistry and amount of the metal species in tobacco smoke. Extending the available knowledge in this area would be a valuable advance in understanding the drivers of cigarette smoke toxicity.

Arsenic toxicity is not only dependent on the chemical form and concentration of arsenic in tobacco leaf, but also its transformation products to tobacco smoke. One of the main challenges of arsenic speciation analysis in cigarette smoke is the presence of heavy organic matrix, in which more than 4,000 smoke constituents have been identified [210]. This may lead to potential alteration in speciation arising during sample preparation, ageing and storing environment. Moreover, the low levels of As concentration has to be determined among significant background contamination during smoke collection and sample pretreatment. The sample mass is also small presenting an additional analytical challenge. Methodologies that provide reliable information on the amount and identity of arsenic species in such samples are scarce and therefore, urgently needed to support upcoming regulation and for human risk assessment.

7.1 MAINSTREAM SMOKE CONDENSATE (3R4F)

Cigarettes used in this work were Kentucky Reference Cigarettes 3R4F (available from University of Kentucky, Kentucky Tobacco Research & Development Center). All the cigarettes were stored in a conditioned room (temperature 22 ± 2 °C and relative humidity 60 ± 5 %) for at least 48 h prior to smoking [218]. Principally, tobacco smoke can be divided into 2 main types according to the direction of smoke: smoke exhaled by smokers known as mainstream smoke, while smoke come out of the tip of burning cigarette is referred to as sidestream smoke. Preparation of mainstream smoke condensates was carried out in the British American Tobacco Company (BAT). Mainstream smoke condensates were prepared under the ISO standard machine-smoking conditions with puffing parameters (35 mL puff volume, 2 s puff duration, once every 60 s).

The most common method used to trap the particulate phase of smoke (i.e., TPM) is a circular glass fibre pad, commercially known as Cambridge filter pad (CFP) (Borgwaldt KC GmbH, Hamburg, Germany). However, this material is known to contain trace metals that may interfere with the measurement. Various other smoke TPM trapping techniques have been described in the literature, e.g. electrostatic precipitation or acid impinger [219]. However, these methods are not suitable for the purpose of this study. For example, electrostatic precipitation is known to cause electrical discharge and ozone production, which may cause undesired oxidation changes to the species of interests [220]. Acid impingers may alter the metal chemistry through reaction with the acid. In order to examine the oxidation state of the metals in smoke TPM, it is necessary to preserve the redox character of the trapped mainstream smoke. The particulate matter collected from cigarette mainstream smoke has been shown to be a complex mixture with reducing ability [221]. In order to minimize any metal redox reactions occurring in the trapped mainstream smoke during or after smoking, an impaction trapping device was used in study as shown in Fig 7.1.

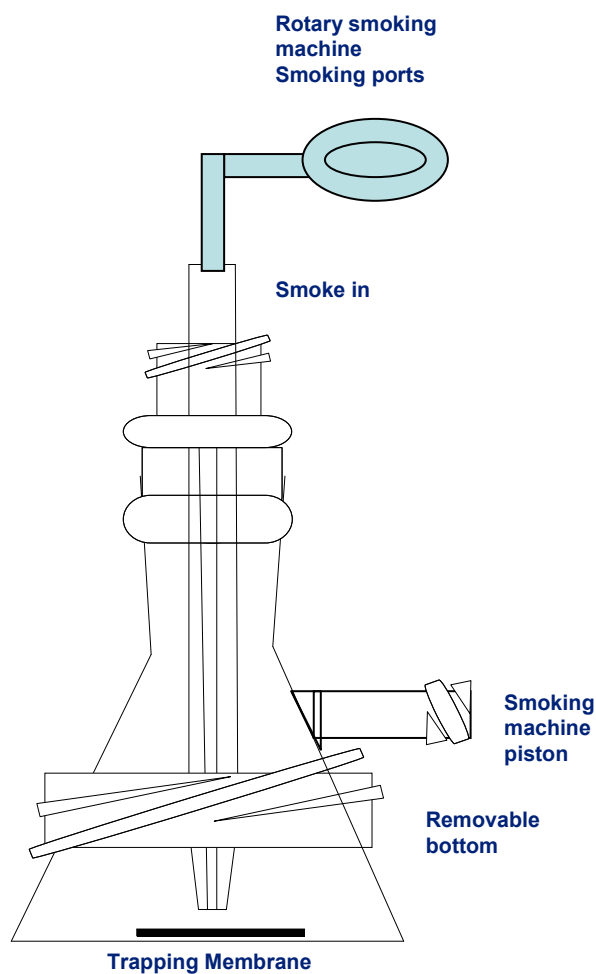


Figure 7.1 A schematic of the impaction trapping device, with a removable bottom to insert the polycarbonate membrane. Cigarettes were smoked by a 20 ports rotary smoking engine.

This device used a metal-free polycarbonate membrane (Millipore, Watford, UK) with pore size of 0.2 μm for smoke TPM collection to avoid any arsenic contamination. It was placed at the bottom of the flask (removable) and used as the sample substrate for subsequent analysis. Kapton tape (Fisher Scientific, Loughborough, UK) was used as the binding material to bind the sample and the sample holder throughout experiments. Smoking was carried out with a RM20CSR 20-port rotary smoking machine (Borgwaldt KC GmbH, Hamburg, Germany). During smoking, the flask was placed in a dry-ice box. The total volume of the tube connection from the rotary smoking machine to the impaction device was approximately 5 mL. Variability for trace elements in tobacco products is typically

high as it depends on their varieties, cultivation practices and environmental conditions. In order to minimize the effect of heterogeneity of tobacco blends in each stick and improve the detection capability of As, each mainstream smoke particulate sample collected on a plastic filter was produced from combusting 40 and 60 research cigarettes 3R4F according to the instruction above.

Six sticky dark brown samples of mainstream condensate on plastic membranes were kept in glass vials tightly sealed and kept in a dry-ice box for shipping until delivery to LGC. Five of which were subsequently transferred to a -80°C freezer prior to sample treatment, whereas the other was left at room temperature for 5 days.

7.2 SAMPLE PREPARATION

7.2.1 Water-soluble arsenic extraction

The procedure was performed by adding 5 g of de-ionized water into a centrifuge tube containing individual smoke condensate collected on a plastic filter and ensuring the condensate was covered. Following 2-h sonication, supernatants were separated from the condensate. The mixture was decanted and filtered through a 0.45 µm cellulose membrane. Supernatants were separated into two portions: one of which (3-4 g) was used for total water-soluble As determination, and the other (1 g) was kept for As speciation analysis by AE-LC-ICP-MS.

7.2.2 Closed-vessel microwave digestion

Water extracts of the mainstream smoke condensates were placed in mini-microwave glass vessels, which were soaked in 50% HNO₃ and then rinsed with de-ionized water to prevent possible contamination. In order to facilitate digestion, the extracts were evaporated at 60°C with N₂ purge using a TurboVap[®] until a crude extract was obtained. A 0.5 mL aliquot of HNO₃ was added to the microwave vessel and digestion conditions described in section 4.2.1 were used. Digests were finally diluted to 5 g with de-ionized water, giving a final matrix of 10% HNO₃. Since the smoke condensate is complex and to ensure reliable results were obtained, standard addition method with three spiked levels of arsenic standards (2, 5 and 8 ng As g⁻¹) was applied for the total water-soluble arsenic measurement.

7.3 TOTAL WATER-SOLUBLE ARSENIC IN MAINSTREAM SMOKE CONDENSATE

Results of total water-soluble As determination in the condensate are presented in Table 7.1. Blank extraction, performed together with the sample extraction, gave the As result below the limit of detection (0.03 ng g^{-1}). However, less correlation between the number of cigarettes and weight of smoke condensate were found, showing a large variation between 0.6 and $2.53 \text{ mg cigt}^{-1}$. This implies that the collection efficiency of the condensate seems to be inconsistent. Likewise, it was observed that total water-soluble As determined did not vary with the number of cigarettes. More than 50% RSD obtained indicated that the method for collection of smoke condensate used appears to be problematic when calculation was referred to cigarette number. The difficulty in preparation of smoke condensate may result in this variation of results. This may be due to a problem in collecting smoke particulate by the plastic membrane used. However, this membrane was selected to prevent As contamination from the determination. Consequently the study has focused on qualitative analysis of water-soluble As species in smoke condensate.

Table 7.1 Results of the total water-soluble As determination in mainstream smoke condensate (SC).

SC No	Cigt No	SC weight (g)	Wt of Mainstream smoke condensate SC ($\times 10^{-6} \text{ kg cigt}^{-1}$)	Total water-soluble As ($\times 10^{-12} \text{ kg cigt}^{-1}$, $n=2$)		Level of water-soluble As in SC (ppm)	SC storage
1	40	0.037	0.93	0.148	0.162	0.168	-80°C
2	60	0.095	1.58	0.214	0.226	0.139	-80°C
3	40	0.025	0.63	0.102	0.118	0.177	-80°C
4	60	0.07	1.17	0.124	0.136	0.111	25°C
5	60	0.0435	0.73	0.079	0.091	0.118	-80°C
6	60	0.1518	2.53	0.384	0.392	0.158	-80°C
Average $\pm$ SD			1.26 $\pm$ 0.71	0.181 $\pm$ 0.106		0.144 $\pm$ 0.026	
(%RSD)			(56%)	(58%)		(18%)	

Despite no relationship with cigarette number, a linear relationship between total water-soluble As determined and weight of smoke condensate was found, with good correlation $r^2 = 0.9715$, as presented in Fig 7.2. Therefore, it seems to reasonably report as the level of As in the mainstream smoke condensate. This study shows that the level of water-soluble As in smoke condensate was 144 ng As g^{-1} sample (sample LOD was 4 ng As g^{-1}). The conversion of detection limit in ng As g^{-1} sample was based on 100 mg sample mass and the dilution of the sample extract by a factor of 140. The percent RSD was, however, high (18%) because of a limited amount of sample size used. To minimize uncertainties of measurement, the suggested weight of smoke condensate should be at least 0.1 g .

Liu *et al* (2009) reported the level of total As in mainstream smoke condensate for research cigarette 3R4F being approximately 450 ppb (Appendix 6). However, due to poor variations of results obtained, it seems to be difficult to compare both results on the basis of extraction efficiency.

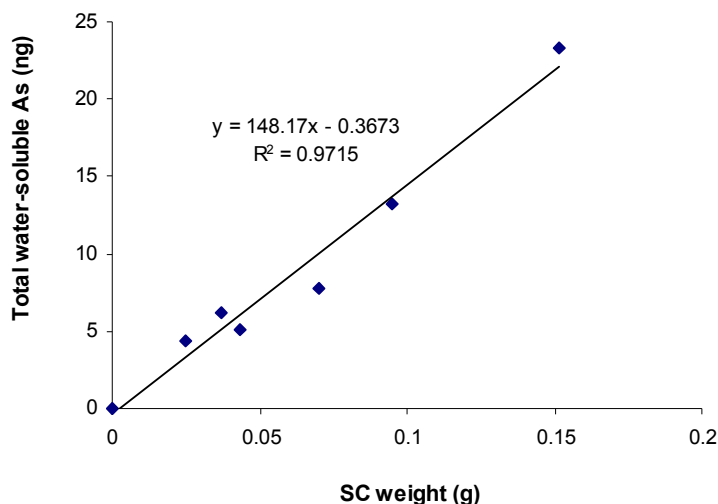


Figure 7.2 The relationship between total water-soluble As and mainstream smoke condensate weight.

7.4 ARSENIC SPECIATION IN MAINSTREAM SMOKE CONDENSATE

7.4.1 Arsenic-species distribution

As shown in Fig 7.3, the result from AE-LC-ICP-MS As profiles under spiking experiment indicated that all water-soluble As species existing in tobacco leaves extract can be found in smoke condensate extract, with the extra unknown As peak (40% of total peak area, at t_r 65 min). This study revealed that when a cigarette is smoked, arsenic in tobacco leaves can be transferred to smoke intact or create other As species.

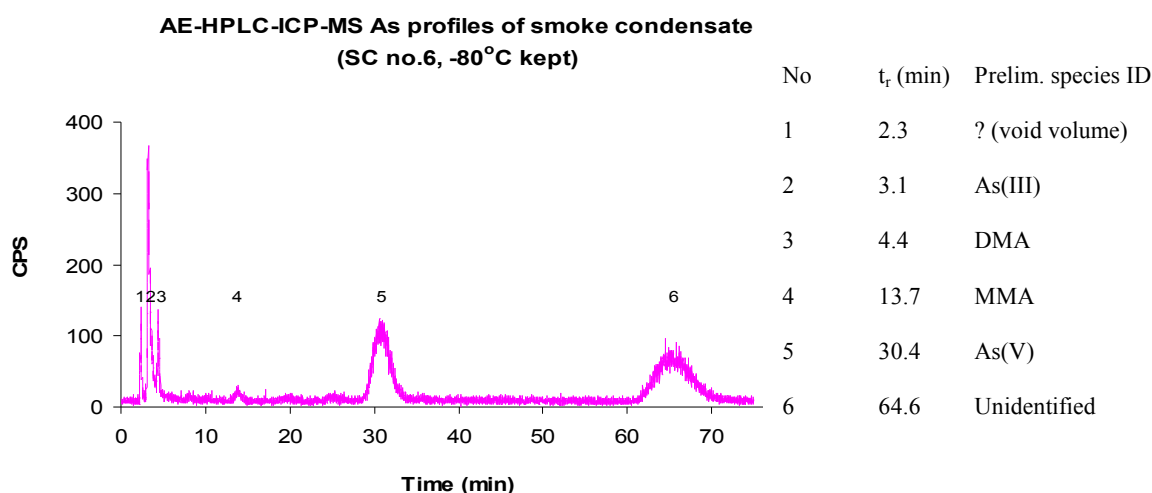


Figure 7.3 Preliminary As species identification in mainstream smoke particulate.

With an injection volume of 100 μL , and integration time for m/z 75 being 300 msec, instrumental limits of detection (LODs, 3σ criterion) of As(V) and As(III) were 0.05 and 0.01 ng g^{-1} , respectively. The approximate arsenic concentration in the undiluted extract (SC no.6) was 4.7 ng g^{-1} (Fig 7.3).

The unidentified As peak showed a very long retention time and broad peak on the separation using the anion-exchange column PRP-X100, which is packed with a backbone of polystyrene divinylbenzene copolymer. Considering the peak characteristics, it seems to be thio-arsenic species rather than oxo-arsenicals. However, due to its high background measured by He mode ICP-MS, ^{34}S peak cannot be detected at the retention time of the unidentified As peak. Li *et al* (2000) reported the amount of H_2S in cigarette smoke found to be $7.6 \times 10^{-2} \text{ mg cigt}^{-1}$ [222]. Although well below the lethal level, the H_2S level can allow As species ($5.0 \times 10^{-6} \text{ mg cigt}^{-1}$) to react and then form thio-arsenic species.

A reversed phase column [223] or organic solvent gradients with the anion exchange column [224] were developed to improve chromatographic separation of thio-arsenic compounds such as thio-DMA and thio-arsenosugars. Furthermore, there are several reports of determination of four soluble As-S species (thioarsenates) in environmental waters, using an IonPak AS-16/AG-16 column and gradient elution with NaOH, where the eluting As-S species with S/As ratios of 1-4 [225, 226]. In this study, our isocratic elution with the anion exchange (Hamilton PRP-X100) was thought to provide the adequate separation of both oxo-arsenicals and an unidentified As species (believed to be a thio-arsenic species) with low concentration. The use of gradient elution with organic solvents may lead to high background dominating such a low signal of unidentified As peak (<100 cps).

To try to understand the transformation of As species from cut tobacco leaves to mainstream smoke condensate as revealed by this work, examining the distribution of As species on the basis of total peak area of AE-LC-ICP-MS in the cut tobacco and that in the mainstream smoke condensate was carried out. As shown in Table 7.2, more than half of As(V) existing in cut tobacco were transformed into new As species and As(III), accounting for 41% and 15% of total peak area, respectively. The combustion process in a burning cigarette resulted in the unidentified As species becoming the predominant species in smoke particulate samples, while other minor species such as MMA, DMA and the species near the void volume (see discussion in 6.4.3) were insignificantly changed and found at similarly low levels. An obvious increase in the As(III)/As(V) ratio observed suggested a rise of reducing activity in the smoke condensate.

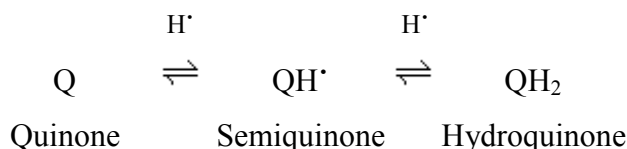
Table 7.2 Comparison of As-species distribution in smoke condensate (SC, -80°C, room temp) and cut tobacco leaves in the research cigarettes.

Sample Name	% Peak Area (AE-LC-ICP-MS)											As(III)/ As(V) ratio	n	
	Inorganic As			Unidentified As	DMA+MMA		Peak 1 (near void - volume)			Others				
	As(V)	As(III)					2	2	3	2				
Cut tobacco leaves	86	88	3	2	0	6	5	2	2	3	2	0.02	2	
SC (-80°C, 7 days)	35 ± 3		15 ± 3		41 ± 1	4 ± 3		3 ± 0			1 ± 2		0.43	10
SC (25°C, 5 days)	89	89	1	1	0	5	6	3	3	2	1	0.01	2	

Table 7.3 Comparison of As-species distribution in smoke condensate extracts (freshly prepared, kept at -80°C for 1 week).

Sample Name	% Peak Area (AE-LC-ICP-MS)							As(III)/ As(V) ratio	n
	Inorganic As		Unidentified As	DMA+MMA	Peak 1 (near void - volume)	Others			
	As(V)	As(III)							
Freshly prepared extract	35 ± 3	15 ± 3	41 ± 1	4 ± 3	3 ± 0	1 ± 2	0.43	10	
Extract kept at -80°C for 1 week	35 ± 4	11 ± 2	40 ± 2	5 ± 3	9 ± 1	1 ± 2	0.31	5	

Knowledge of the dynamic redox reaction in the smoke condensate is required to gain a better grasp of the phenomenon. Liu *et al* (2009) described the behaviour of As(III)/As(V) species in the smoke particulate matrix detected by XANES in accordance with redox properties [215]. It is realized that mainstream smoke is derived from the combusting zone, which is at the tip of the burning cigarette. This region lacks oxygen and is abundant in hydrogen [227], so fresh smoke condensate produced is supposed to be weakly reducing [228]. However, all of As(V) in tobacco leaves did not transform into As(III) completely. This might be due to being kinetically hindered by the reduction of As(V) to As(III). Schmeltz *et al* (1977) investigated the change of electrochemical potential on cigarette smoke particulate and found a rise in the reducing activity from the 2nd puff to the last puff (the 9th and 10th puff) and continue further for 500 s, as shown in Fig 7.4. The reason why the reducing activity was cumulated is believed to involve a radical-driven reaction of quinone/semiquinone species [228]. Hydrogen radicals generated during cigarette pyrolysis can reduce quinone compounds present in smoke constituents such as anthraquinone, naphthoquinone and benzoquinone to form semiquinone and hydroquinone, as the chemical equilibrium shown below. The resulting species, which serve as reducing agents, can convert As(V) present in tobacco leaves to As(III) and unidentified As species detected in the cigarette smoke.



Electrochemical potential for fresh smoke particulate studied using a standard calomel electrode reported ranges from +0.24 to +0.17 V, which was equivalent to 0.00 to -0.07 V using a standard hydrogen electrode [228]. The ‘pH’ values of the smoke particulate sample for a blend type of cigarette were estimated in the range of 5.5 to 6.5 [227]. Using this information, the potential phase of As (labeled region) can be predicted on the Eh-pH diagram for arsenic illustrated in Fig 7.5. For these reasons, it is likely that As(V) can be electrochemically reduced to be As(III) in the smoke condensate.

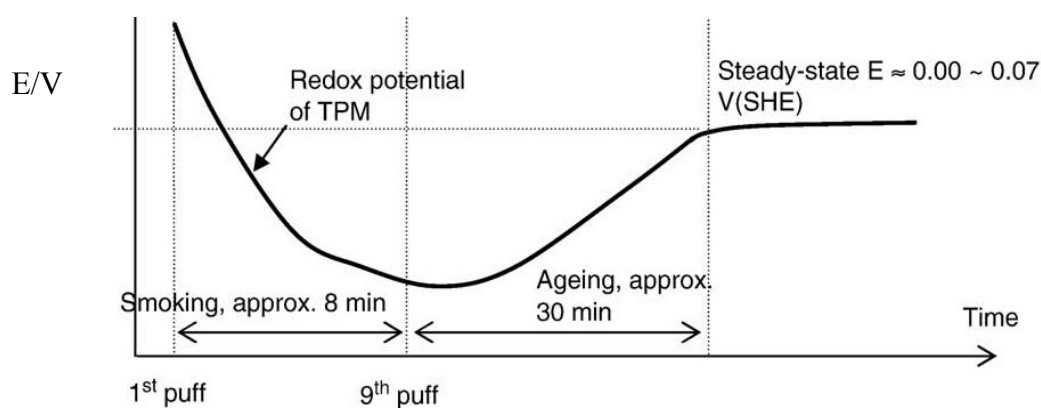


Figure 7.4 The schematic graphical plots showing the dynamic change of electrochemical potential on cigarette smoke particulate [228].

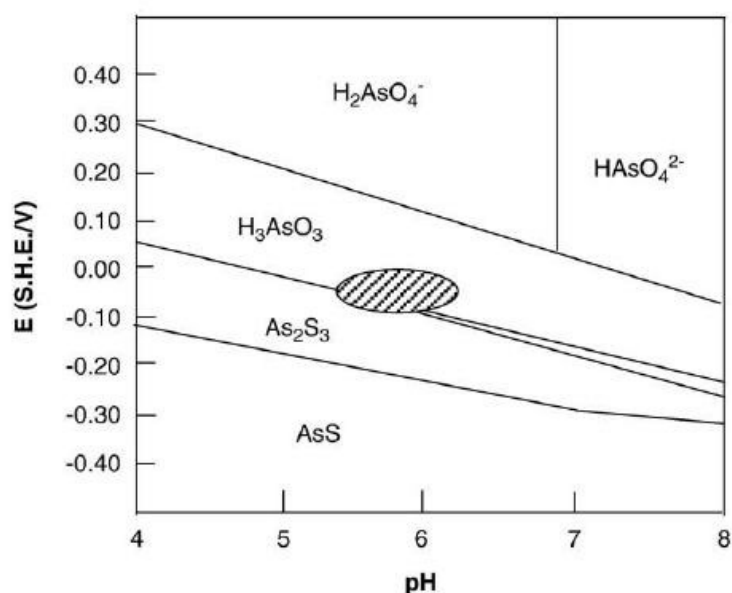


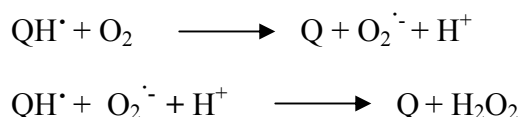
Figure 7.5 The Eh-pH diagram for arsenic showing the region set by the electrochemical potential reported in [228] and typical 'pH' values of smoke [227].

The shaded region shows the intercept between H_3AsO_3 (As^{III}) and As_2S_3 . It is probable that the unidentified arsenic species might be thio-arsenite species. However, more solid evidence is still necessary to confirm the species identity. Since redox activity in smoke condensate is thought to be sensitive for species transformation, it is worthwhile investigating the stability of arsenic species.

7.4.2 Arsenic-species stability

To investigate arsenic-species stability, the distribution of arsenic species on the basis of total peak area of AE-LC-ICP-MS As profiles in different storage conditions of mainstream particulate samples (-80°C freezer and 25°C for 5 days) were compared. As shown in Fig 7.6, the findings reveal that following the smoke condensate sample (no.4) kept at room temperature, As(III) species and unidentified As species found in the fresh smoke were mostly converted into As(V). Interestingly, the distribution of As in the smoke kept at room temperature was found to be similar to that in cut tobacco (Fig 7.4). It is suggested that the transference was derived from arsenic being released as aerosol particles [229, 230], which is not dependent on type of As species. A similar result was found when hydrogen peroxide (1% (v/v)) was added to a fresh smoke condensate extract, indicating the oxidation of the unidentified As species and As(III) to As(V).

The study of As-species distribution in smoke particulate samples after post smoking has been explained by Schmeltz *et al* (1977) [228]. When fresh smoke comes into contact with air, its redox potential tends to be increasing after 900 s post smoking. The phenomenon of self quenching involving the radical species in the smoke is thought to be responsible. This process can be accelerated by a small amount of oxygen present.



It was suggested that storing smoke particulate samples at cooled condition can retard this process [215, 228]. The reducing potential is quickly lost if the sample is exposed to air/or room temperature. Despite no actual measurement performed on electrochemical potential of the smoke condensate prepared, the results in this study were found in agreement with Schmeltz *et al* (1977) [228]. To conclude, the diagram in Fig 7.7 illustrates the transformation of water-soluble As species between cut tobacco and the mainstream smoke condensate.

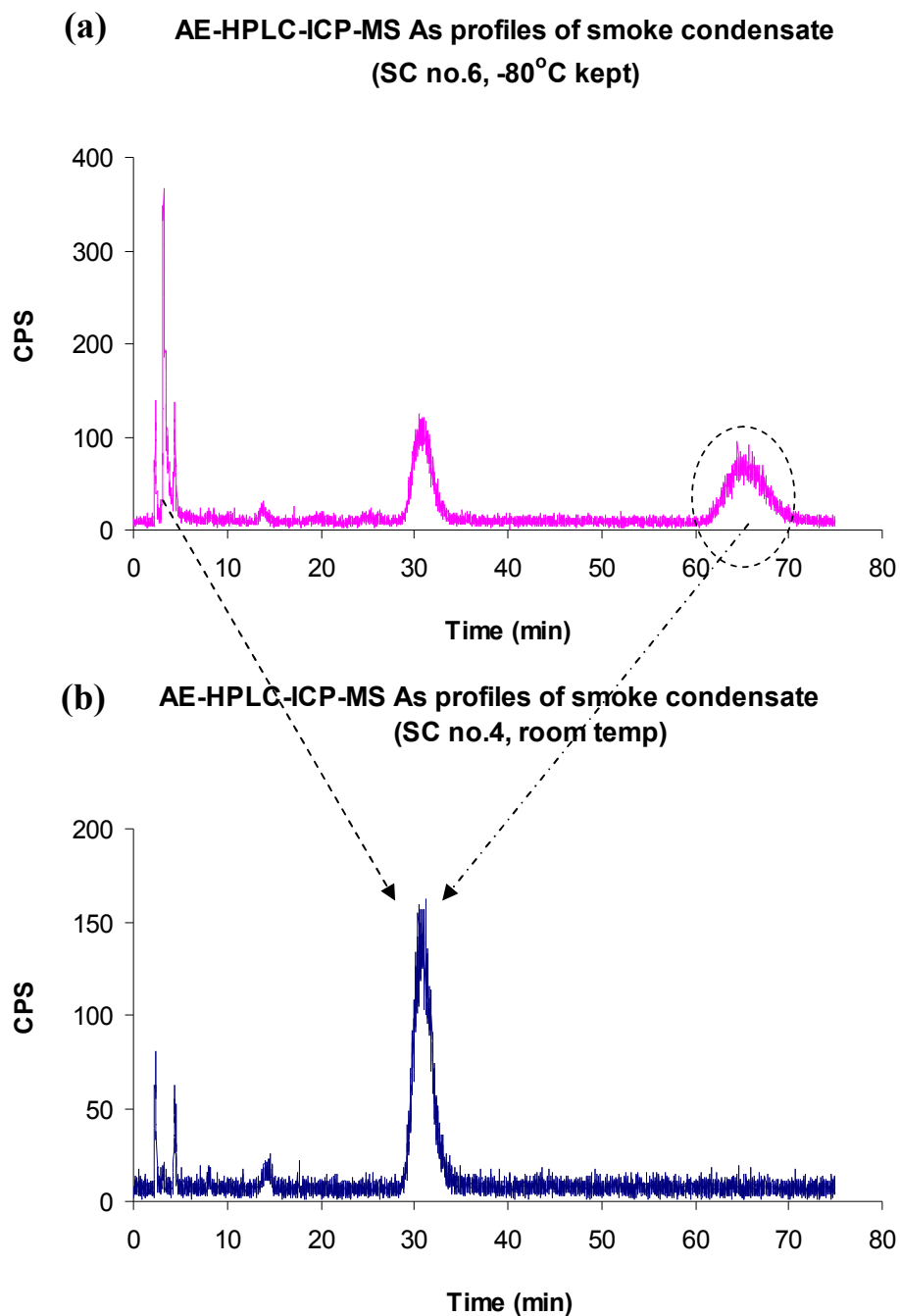


Figure 7.6 As-species stability testing using AE-LC-ICP-MS profiles (solid SC kept at (a) -80°C and (b) room temperature)

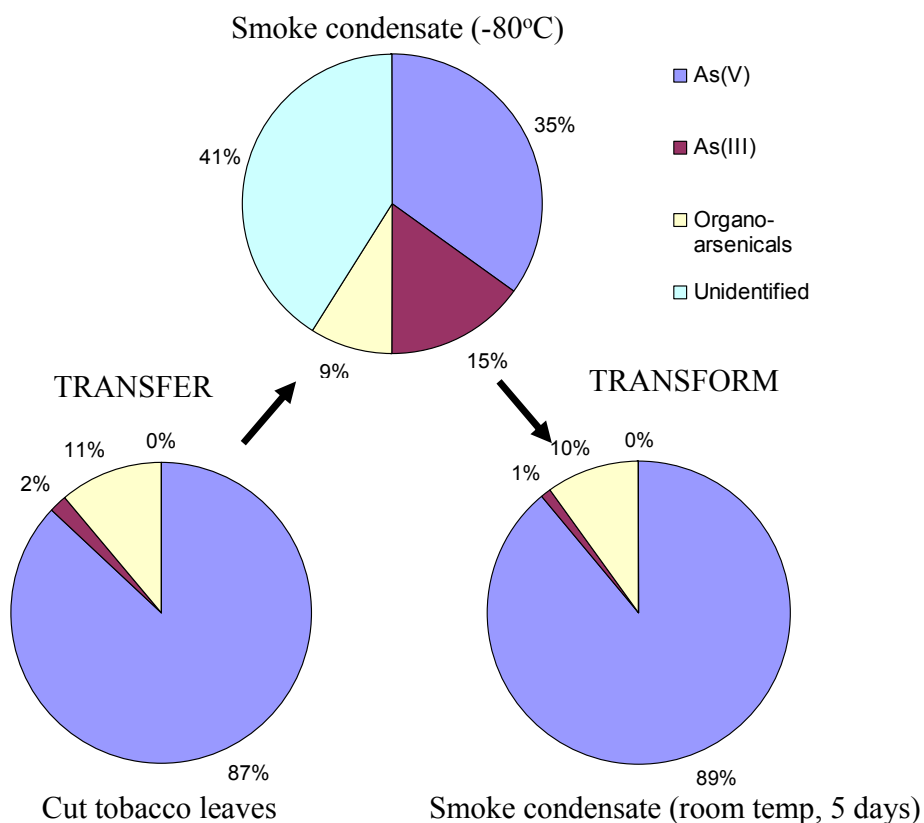
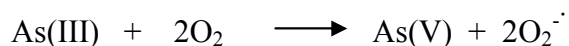
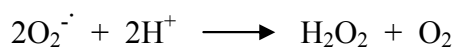


Figure 7.7 Diagram showing the distribution of water-soluble As species by AE-LC-ICP-MS, which are transformed during processing from cut tobacco leaves to smoke condensate. The complete degradation of the unidentified As species occurred when smoke condensate was stored at room temperature for 5 days.

Schmeltz *et al* (1977) indicated that cooling the fresh particulate not only slows down the reduction of arsenic by limiting activity of the radical species, but is also responsible for some gas-phase species being trapped in the frozen smoke. In general, the gas phase largely consists of ambient air with a minority of reactive oxygenated species. Although occasionally found in the range of 10^{-9} kg cigt⁻¹, their levels are significantly higher than the combined levels of all the transition metals, which are partly responsible for any change in redox states. The arsenic redox behavior, which is overwhelmed by the overall redox environment of the smoke condensate, seems to be more oxidizing. Reactive oxygenated species released in slightly acidic media can lead to the formation of hydrogen peroxide and oxygen. As

a consequence, arsenite and the unidentified As species (possibly thioarsenite species) were oxidized to arsenate.



The study of As-species stability was not only investigated in smoke particulate samples stored in different locations, but also in the fresh extracts and extracts after collecting at -80°C for 7 days. The percentage of arsenic species distributed using AE-LC-ICP-MS in the two different extracts were compared in Table 7.3. It was found that maintaining the smoke extracts at -80°C following 7-day storage can stabilize the unidentified As species, but approximately 25% conversion of As(III) to the As species near the void volume was observed (Fig 7.8). The mechanism of the transformation of the arsenic species remains unclear. Storing the smoke condensate extracts for 9 months resulted in As(III) and unidentified As species being completely converted to As(V). For arsenic speciation study, fresh extracts are recommended to avoid species interconversion.

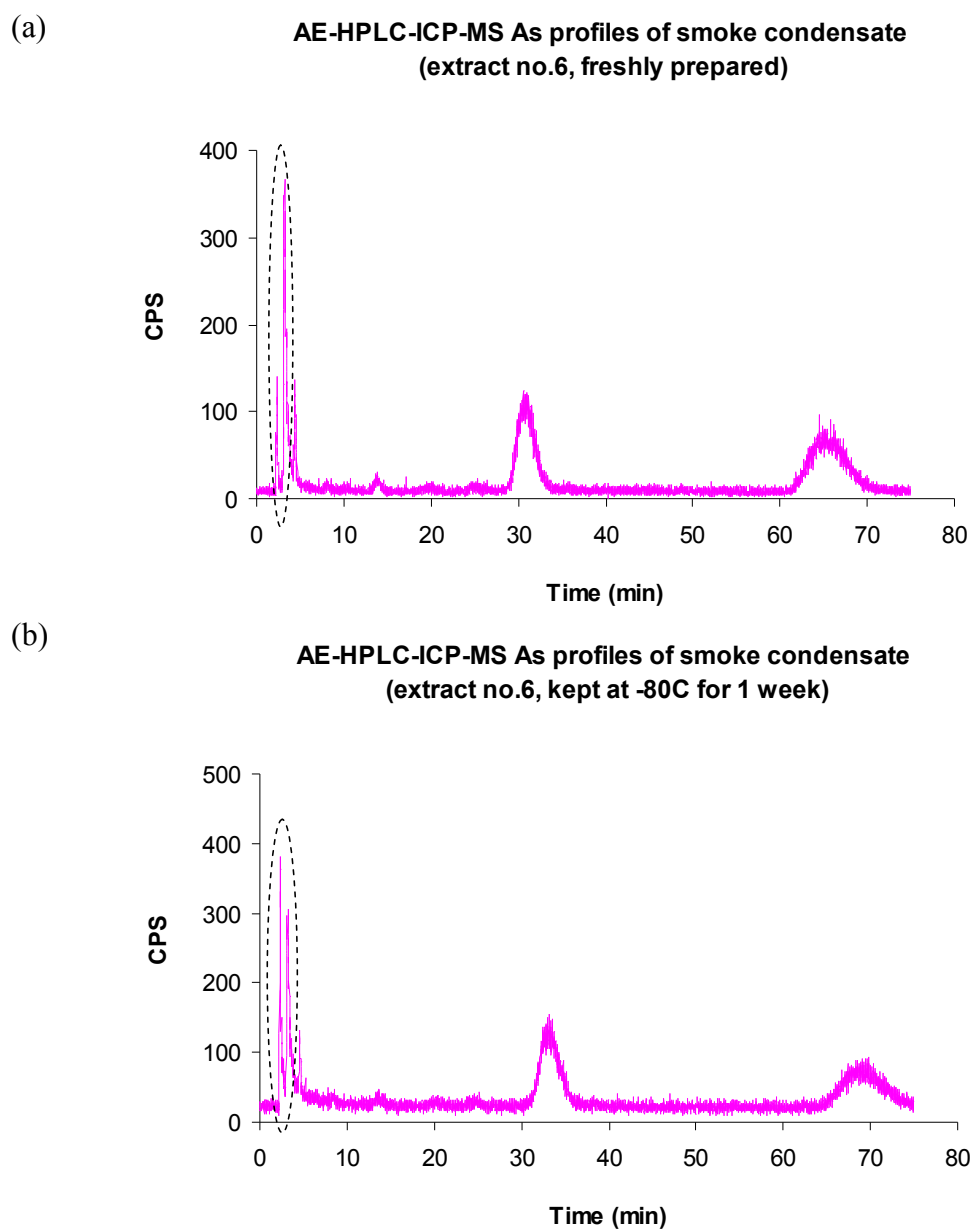


Figure 7.8 As-species stability testing using AE-LC-ICP-MS As profiles (a) freshly prepared extract; and (b) extract kept at -80°C for 1 week.

7.5 COMPLEMENTARY USE OF INFORMATION OBTAINED FROM SYNCHROTRON-BASED X-RAY ABSORPTION NEAR-EDGE STRUCTURE SPECTROSCOPY

Regarded as a direct speciation method, information obtained from XANES is a complementary method, which is used to support the findings. The spectra, as well as As K-edge positions, could allow us to predict the unidentified As species. Liu *et al* (2009) employed synchrotron X-ray absorption for providing XANES information to study the oxidation state of arsenic present in cut tobacco, mainstream cigarette smoke, and cigarette ash. They estimated the relative percentages of As(III) and As(V) in individual sample by modeling the XANES spectra as linear combinations of the two standard oxides at each edge. The percentage error (%R) for 10 cigarette TPM given by the modelling software (HASYLAB beam X1) were 28. XANES spectra obtained from the jet-impaction trapped smoke particulate sample is presented in Fig 7.9. Using the first derivative plots, two main peaks believed to be As(III) and As(V) are identified and in addition, several shoulder peaks in a vicinity of As(III) in the slightly lower energies can also be observed.

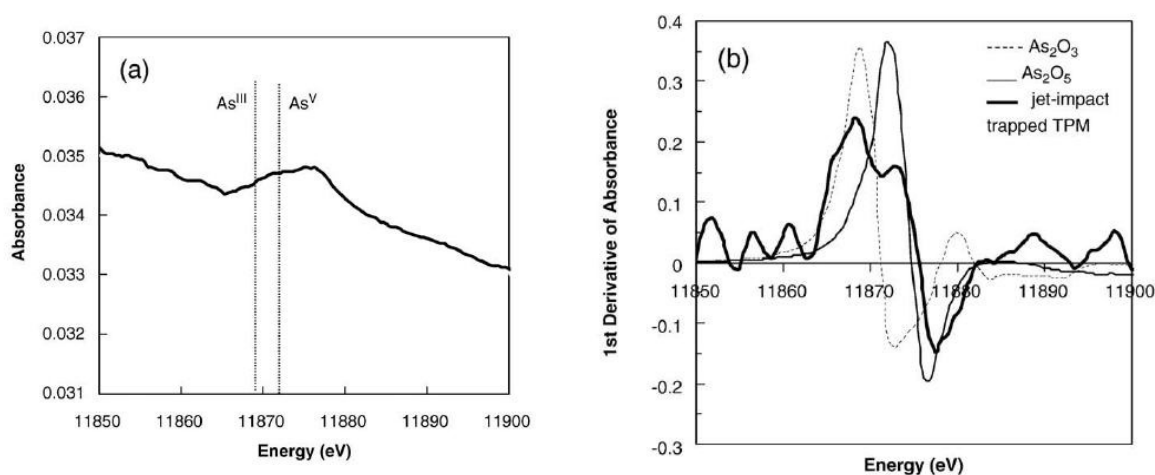


Figure 7.9 XANES spectra from the smoke particulate sample (a) XANES spectra; (b) First derivative of the XANES from the smoke particulate sample and two reference As standards, As(III), and As(V) edge positions based on the values obtained using As₂O₃ and As₂O₅ [215].

In high concentrations of arsenic, Suess *et al* (2009) demonstrated the use of X-ray absorption spectroscopy to discriminate thioarsenites and thioarsenates from arsenite and arsenate [76]. They found that the absorption near edge energy increases in the order of $\text{As}^{\text{III}}_2\text{S}_3$ (11866.8 eV) < As^{III} -sulfur species (11867-11867.5 eV) < Arsenite (11868.2 eV) < As^{V} -sulfur species (11869.3-11871.3 eV) < Arsenate (11872.3 eV). They also reported that thioarsenates was unstable upon acidification below pH 9.5, since they are converted rapidly to thioarsenite species. Consequently, it is likely that the unidentified As species might be a thioarsenite species such as $\text{As}_2\text{S}_{3(\text{aq})}$, HAs_2S_4^- and $\text{As}_2\text{S}_4^{2-}$ [231]. Using X-ray absorption spectroscopy (XAS), Liu *et al* (2009) found the As(III)/As(V) ratio of 0.59-0.67 for mainstream particulate kept in solid CO_2 for 7 days. One reason for the low ratio (0.43) (Table 7.2) found in this study might be that some of As(III) was converted into As(V) during sample preparation, which is commonly found in aqueous solution [86]. Another reason is that the measurement by XAS was carried out close to the detection limit. This might affect the accuracy of measurement, as the possible signals of other arsenic species near the As K edge of As(III) might be included in the As(III) signal by the software used.

Elucidation of the chemical structure of the unidentified As species is still required. The lack of thioarsenite as discrete reference compounds and levels of the As concentration in the smoke samples below detection limit of ESI-MS/MS make it demanding to characterize the unknown species. Furthermore, the use of EXAFS measurement for gaining structural information as to identity of ligand atom and bond distance is limited due to its very poor detection limits. To gain a better grasp of the transformation during transference, elucidation of the As species has to be done for health risk assessment of human consumption.

7.6 SUMMARY

A study of As speciation in cigarette smoke was first performed by trapping mainstream smoke particulate generated from research cigarettes 3R4F on a metal free membrane by jet impaction. Each smoke sample was derived from burning 40 or 60 cigarettes by a smoking machine in order to preconcentrate As species on the membrane and minimize the heterogeneity effect from different tobacco blends. With this type of membrane, collection efficiency was found to be inconsistent. As a result, this study was focused on qualitative analysis of arsenic speciation on the smoke condensate.

Water-soluble As extraction was carried out by sonicating the smoke sample on the membrane, covered with 5 mL of de-ionized water for 2 h. The level of water-soluble As in the total particulate matter of the smoke was found to be approximately 140 ng g^{-1} . The findings revealed that the changes of electrochemical potentials on the mainstream smoke particulate samples (studied by [228]) during transference and post smoking affected the transformation of As species distribution. More than half of As(V) species, which was a majority of As species in tobacco leaves were transformed into As(III) and an unidentified As species within the smoke condensate (kept at -80°C), whereas other minority of As species such as MMA, DMA and the peak near void volume remain unchanged. The increase in reducing activity in the smoke samples is believed to be ascribed to radicals, which are produced by the breakdown of many organic species [228]. In contrast, the unidentified As species and As(III) were almost entirely transformed into As(V) when the smoke particulate sample was maintained at room temperature for 5 days. It is suggested that self-quenching of radicals and reactions with oxygen are responsible for the oxidation of As species [228]. The cooling procedure of the smoke particulate sample can retard the rate of this process. The other study on As-species stability indicated that the unidentified As species can be well preserved even when the extracts were kept at -80°C for 1 week. However, approximately 25% of As(III) were found to be transformed into the As peak near void volume. Consequently, to minimize erroneous results, it is highly recommended to prepare fresh extracts to quantify correctly.

The unidentified As species is thought to be thioarsenite species such as $\text{As}_2\text{S}_{3(\text{aq})}$, HAS_2S_4^- and $\text{As}_2\text{S}_4^{2-}$ [231] with several assumptions. For example, first derivative of the XANES spectra from the smoke particulate sample similarly prepared to this study shows the presence of several shoulder peaks at As K edge

energy between 11866-11867 eV [215], which might correspond to As^{III} -S species [76]. Another assumption is that the likely As phase region for the smoke sample on the Eh-pH diagram for arsenic acquired from electrochemical potential and 'pH' of the smoke shows the interception of As(III) and As_2S_3 phases [227, 228].

At present, qualitative results obtained in this work can identify the actual water-soluble As species. These results revealed the complex reaction in which As species are involved during the cigarette smoking process. Results from the developed method were found to be consistent with the known redox properties of the smoke particulate matrix and those obtained from XANES spectroscopy [215]. Further work is also needed to improve collection efficiency of mainstream smoke particulate matter to be more effective and reproducible. Elucidation of the unknown As species needs to be done to assess human health risk for human consumption. Methods for sample preparation involving preconcentration without species conversion are therefore needed.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER WORK

Metallomics approaches based on the combined use of elemental and molecular mass spectrometry for arsenic speciation analysis in food-related and environmental samples such as edible marine algae, accumulating plants, cut tobacco leaves and cigarette smoke total particulate matters (TPM) have been developed.

These methods have been employed to gain a better grasp of the factor involved in the uptake and distribution of arsenic in a well-known accumulator, *Arabidopsis thaliana*. The plants, individually and simultaneously supplemented with As, Se, Hg, As-Se, and Se-Hg (1, 3, 5 $\mu\text{g g}^{-1}$), were grown hydroponically for 7 weeks in a controlled environment. The effect of the presence of Se on the As and Hg uptake by the plant leaves was investigated here for the first time; Se in the growing media was found not to affect As and Hg concentrations of the leaves in comparison with individual exposure to these elements. The use of 1% formic acid (1°C, 1 h), compared with the buffer extraction (2, 5, 12 h) by sonication, was found to be the choice as a compromise between extraction efficiency (~60%) and preservation of the compound entity. This is a new finding since most methods reported in the literature based on a 12-h extraction procedure and to the author knowledge, the influence of extraction time on the extraction efficiency of As from the plant leaves has not been reported before. The comparative SEC-ICP-MS As profiles indicated that the use of 1% formic acid in an ice bath appeared to be less harsh extraction and provided better preservation of the HWM As peak at t_r 12.6 min. The AE-LC-ICP-MS was used to investigate the distribution of water-soluble arsenic species, indicating that almost 90% of the total arsenic in formic extracts was inorganic arsenic. A deeper insight into the arsenic species distribution in the leaves was achieved using reversed phase HPLC in combination with ICP-MS and ESI Orbitrap MS/MS. It was found that arsenic-phytochelatin complexes representing a cluster of As peaks, which were accountable for small amounts of total arsenic in the 1% formic extract, were eluted using 20-35% MeOH content in the mobile phase. The need for accurate mass measurement in species identification has been demonstrated to minimize ambiguity in species identification, owing to the possibility of a diversity of biomolecules of similar composition and relative low stability present. The developed method is

proved to be successful for the detection of arsenic-phytochelatin complexes, even in the presence of low concentration levels.

The development of methodologies for arsenic speciation was performed in four edible marine algae. Although there was no significant difference in the distribution of arsenic species using SEC-ICP-MS found, when sonication with ammonium buffer and 1:1 (v/v) MeOH/H₂O were used, the higher As extraction efficiencies (~40-60%) were obtained using the ammonium buffer with 1-h sonication time. The application of the *in vitro* dialysis method, modified by Haro-Vicente *et al* (2006) [192] for predicting the As bioaccessibility in the marine algae, was investigated here for the first time. Despite the fact that the SEC-ICP-MS As profiles showed most of arsenic species extracted by the buffer solution were smaller than 10 kDa; therefore they were supposed to readily diffuse through the membrane (10 kDa, MW cut-off), the As bioavailability study showed the very low As dialyzability (only 10-20%). Compared to the results obtained by the solubility method previously reported [61], these results showed considerably lower values, which is thought to be ascribed to the permeable limitation and matrix effects that prevent the diffusion of arsenic species through the membrane. Nevertheless, results obtained by both methods found no transformation of arsenic species in common following the *in vitro* gastrointestinal digestion. The As distribution, based on the optimized AE-LC-ICP-MS, for algae extracts and dialyzates was similarly found, presenting the majority of arsenic species were arsenosugars. The optimized AE chromatography allowed the sufficient separation of nine As-species to prevent the misidentification; arsenosugar 1 can be separated from AsB and As(III). Two-dimensional (SEC-AE)LC-ICP-MS, involving the use of two different separation mechanisms in series, was used to demonstrate for matrix removal to achieve the chromatographic purity of algae samples. This approach is considered to be useful as a sample clean-up procedure prior to organic mass spectrometric detection. Although matrix effect is thought to be a significant barrier for the detection with organic MS, the characterization of arsenosugars in the original algae extracts using ESI Ion Trap MS/MS is found to be successful without any pretreatment step.

Several analytical challenges of developing the methodologies for arsenic speciation analysis in cut tobacco leaves (research cigarettes 3R4F) involve the high complexity of the sample matrix and the low ng g⁻¹ levels of As species. The results of total arsenic determination using different calibration methods indicated the

variability of the results, especially in high matrix media; standard addition method is recommended to use to obtain accurate measurement. Partitioning As extraction (by two different polarity extractants: $\text{CHCl}_3/\text{H}_2\text{O}$), for giving an idea of arsenic species according to their polarity, suggested that the majority of As were likely to dissolve in polar phase, while a negligible level of As was found in less polar layer. As a result, method development of As speciation in tobacco leaves was focused on water-soluble species. The optimization of water-soluble As extraction was performed for the first time, using different extractants, sample size to extractant volume ratio, extraction time, as well as extraction techniques. The use of SEC-ICP-MS was demonstrated for selecting the appropriate extractant as well as optimum extraction conditions. The use of 0.2 g in 2 g of de-ionized water with 2-h sonication was found to be optimal, giving approximately 43% arsenic extraction efficiency. Alternatively, microwave-assisted extraction (MAE), with the optimum MW condition at 50 W, 50°C, can be used to accelerate the extraction from 2 h sonication to only 10 min. The distribution of arsenic species in water extracts using AE-LC-ICP-MS indicated that almost 90% of the total water-soluble As was found to be present as As(V), which correlated well with XANES results [215]. Efforts to improve the extraction efficiency were made (from 43% to 63%) using sequential extraction (by leaching with water, followed by extraction with driselase and sodium dodecylsulfate). The proposed procedure was useful for characterizing tobacco samples as the distribution of arsenic among different origins e.g., water soluble (42%), cell wall bound (13%) and insoluble protein bound (8%). The results suggested that ca. 40% of arsenic remained strongly bound to the plant matrix. The quantitative extraction method offering better efficiency while maintaining species identity is also needed.

It is important to note that the use of the harsh condition like MAE at high power-temperature to achieve highest extraction efficiency is not convincing for this purpose. Although such a harsh condition can allow arsenic, which is possibly strongly bound to cell wall components, proteins and lipids, to be extracted, the released arsenic from sample matrix might be almost exclusively inorganic arsenic species. This leads to the difficulty to differentiate its origins whether inorganic As is readily dissolved in water or they are bound to cell walls or proteins. Unlike the sequential extraction method, MAE with extreme conditions does not provide important information such as the distribution of arsenic among different origins for assessing risks of human consumption. In general, risk assessment of human

consumption should take the arsenic species, which are potentially bioavailable in human gastrointestinal tract, into consideration. In fact, humans do not have enzymes to digest the cellulose. Therefore, taking the inorganic arsenic released from cell wall components into account may lead to the overestimation in the risk assessment associated with human consumption.

The study of arsenic speciation by HPLC-ICP-MS for the water-soluble fraction in the mainstream smoke was presented here for the first time. The cigarette smoke TPM sample (research cigarettes 3R4F) was prepared using an impaction-trapping device with a metal-free polymer material as a supporting membrane for the smoke collection. The extraction can be achieved using 5 mL of degassed water to cover the sample for 2-h sonication. The levels of water-soluble As in the cigarette smoke TPM was found to be approximately 140 ng g^{-1} . However, the report for As level per cigarette was found to be problematic due to the limited efficiency of the smoke collection. Both the hyphenated MS and XANES techniques were used to obtain information on the speciation of As in smoke condensates. The results showed that the tobacco smoke contained a mixture of As(III) and As(V); As(III) being found as arsenite and, possibly, thio-arsenite by HPLC-ICP-MS. The reduction of As(V) to As (III) during dynamic cigarette smoke formation can be explained by the overall smoke redox properties in accordance with the cigarette combustion process. The stability study suggested that thio-arsenite species was found to be rapidly converted into As(V) when the samples were exposed to ambient condition. Although thio-arsenite species remained stable when the TPM or fresh extracts were kept at -80°C for at least 1 week, approximately a quarter of As(III) were found to be transformed into other species (near void volume). Because the possible change in redox state influences the native forms and distribution, it is therefore highly recommended to prepare fresh cigarette smoke TPM and fresh extracts to quantify species correctly.

Recommendations for further work have been highlighted in the relevant chapters of this thesis. Most of arsenic speciation studies were carried out in the water-soluble fraction. The work on arsenolipids in marine algae samples using less-polar solvents is suggested to obtain full information of a range of arsenic species according to the polarity. Information on the presence of arsenolipids in marine algae is still scarce; there might be the implication of the consumption in toxicity due to their difficulty to excrete. The challenging in development of direct measurement for

intact arsenolipids by HPLC-ICP-MS is therefore required for providing information of the actual speciation.

The improvement in collection efficiency for cigarette smoke to be more effective and reproducible remains an additional challenge. In addition, the enhancement of the efficiency of As-species extraction from mainstream smoke condensate is also needed. In order to confirm the unknown As species by ESI-MS/MS (possibly thio-arsenite species), methods for preconcentration maintaining redox properties of As species are required. Furthermore, the reduction in the chromatographic time for the “thio-arsenite” detection without the sacrifice of signal to noise ratio needs to be considered.

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APPENDIX 1 Growing conditions.

Growing conditions for the arsenic accumulating plants reviewed.

Arabidopsis thaliana

D. L. LeDuc *et al.*, Overexpression of selenocysteine methyltransferase in *Arabidopsis* and Indian mustard increases selenium tolerance and accumulation, Plant Physiology, 2004, 135, 377-383

Seeds sterilized by rinsing in isopropanol for 2 min, in 20% hypochlorite/Tween 230 for 15 min and in sterile deionised water five times for 5 min, on a rocking platform. A total of 100 seeds were sown per 150-mm plate of Murashige and Skoog medium containing 10 g/L Suc, 0.5 g/L of 2 N-morpholinoethanesulfonic acid monohydrate, 8 g/L of bactoagar and 25 to 100 μ M selenate, 25 – 100 μ M selenite, 25 μ M SeCys or 25 μ M SeMet. Selenocysteine was synthesised by reducing one volume selenocysteinine with two volumes dithiothreitol in 1M hydrochloric acid under nitrogen for 10 min. After 8 d at 25°C under continuous light, seedlings were harvested and washed with deionised water.

For Se accumulation and volatilisation in mature plants (both *Brassica juncea* and *Arabidopsis Thaliana*), seedlings were instead transferred to 4-inch pots containing coarse sand and covered with a plastic dome. Pots were maintained in a greenhouse with controlled temperature (25°C) and long-day (16 h) photoperiod. Plants were watered twice a day, once with tap water and once with half-strength Hoagland solution. After 1 week the dome was removed. After an additional week, plants were transferred to half-strength Hoagland solution in aerated plastic boxes. After 1 week the nutrient solution was replaced with fresh solution containing 20 or 50 μ M concentrations of selenocompounds. After 8 days of Se treatment, plants were harvested and weighed for Se accumulation or transferred to individual gas-tight glass volatilisation chambers for volatile Se analysis. Harvested plants were washed in running deionised water to remove any Se externally bound to the roots, dried at 70°C, cut into small pieces, and acid digested. Volatile Se was collected from plants in 200 mL of Hoagland solution, through which a continuous air flow (1.5 L min⁻¹) was passed and trapped in alkaline peroxide. 10 mL aliquots of trap solution were collected after 24 h and heated at 95°C to remove the peroxide. Selenate was reduced to selenite by adding an equal volume of concentrated HCl and heating at 95°C for 30 min.

APPENDIX 2 Protocol for growing *Arabidopsis thaliana*.

Experiment Title	METAL SPECIATION IN PLANTS USING A HYDROPONIC SYSTEM
Objectives	The purpose of this project is to develop and validate methodology for the extraction, measurement and characterisation of metal-containing species in plant materials. This will facilitate the exploitation of metal-accumulating plants for phytoremediation (an emerging technique for land reclamation and effluent treatment) for the production of new fortified animal feeds. More specifically the aims are to: - Develop and validate novel extraction procedures (e.g. enzymatic probe sonication and accelerated solvent extraction) and mass spectrometry strategies. - Develop two-dimensional HPLC methods in combination with ICP isotope dilution MS for the accurate quantification of major key compounds in plant materials. - Develop GC/HPLC-ICP-MS and GC/HPLC-MS methodologies for the extraction, accurate measurement and identification of Se-containing species in plant materials.
Location	Poly-tunnels # 1



Plant species	Indian or brown mustard (<i>Brassica juncea</i> L.)		
			Thale cress (<i>Arabidopsis thaliana</i> L.)
Treatments	Plants will be grown hydroponically with Hoagland solution and spiked with sodium selenite ($\text{Na}_2\text{O}_3\text{Se}$), sodium arsenite (NaAsO_2), mercury nitrate ($\text{Hg}(\text{NO}_3)_2$), sodium selenite-sodium arsenite ($\text{Na}_2\text{O}_3\text{Se-NaAsO}_2$) and sodium selenite-mercury nitrate ($\text{Na}_2\text{O}_3\text{Se-Hg}(\text{NO}_3)_2$) with the following metal concentrations:		
	Hoagland solution 1 mg Se L ⁻¹ 3 mg Se L ⁻¹ 5 mg Se L ⁻¹ 1 mg As L ⁻¹ 3 mg As L ⁻¹ 5 mg As L ⁻¹ 1 mg Hg L ⁻¹ 3 mg Hg L ⁻¹ 5 mg Hg L ⁻¹ 1 mg Se L ⁻¹ + 1 mg As L ⁻¹ 3 mg Se L ⁻¹ + 3 mg As L ⁻¹ 5 mg Se L ⁻¹ + 5 mg As L ⁻¹ 1 mg Se L ⁻¹ + 1 mg Hg L ⁻¹ 3 mg Se L ⁻¹ + 3 mg Hg L ⁻¹ 5 mg Se L ⁻¹ + 5 mg Hg L ⁻¹		
Design	Each species (all treatments, i.e. 5 replicates of 16 treatments) is being treated as a randomised block design.		
Solution preparation	Material Sodium selenite anhydrous ($\text{Na}_2\text{O}_3\text{Se}$, 172.94 g mol ⁻¹) Mercury nitrate anhydrous ($\text{Hg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$, 342.70 g mol ⁻¹) Sodium arsenite 98% (NaAsO_2 , 129.91 g mol ⁻¹) 9 × 1 litre bottles, CJK (product code: 310-770097 HDPE BLACK) http://www.cjk.co.uk/Bottles-(Round)---for-Liqui-5.php 15 × 3 litres jerricans, CJK (product code: 720.066.00 HDPE) http://www.cjk.co.uk/Jerricans-1.php 5 × 10 litres jerricans, CJK (product code: 720.062.01 HDPE) http://www.cjk.co.uk/Jerricans-1.php Hoagland, Sigma (product code: H2395-10L) http://www.mpbio.com/product_info.php?cPath=491_8_56_146&products_id=26218&depth=nested&keywords=hoagland		

Method

Label (Hoagland solution + date) five 10-litre jerricans and prepare the Hoagland solution by dissolving 10 bags of Hoagland powder into 10 litres of de-ionised water (ensure that the de-ionised water has an electrical conductivity less than 0.1 mS).

Label (metal name + concentration + date) nine 1-litre bottles and prepare metal concentrated solutions using the following table:

Metal Name	Target concentration (g L⁻¹)	Mass required (g)	Volume required (L)
Na ₂ O ₃ Se	0.1	0.2190	1
Na ₂ O ₃ Se	0.3	0.6571	1
Na ₂ O ₃ Se	0.5	1.0950	1
NaAsO ₂	0.1	0.1734	1
NaAsO ₂	0.3	0.5202	1
NaAsO ₂	0.5	0.8670	1
Hg(NO ₃) ₂	0.1	0.1708	1
Hg(NO ₃) ₂	0.3	0.5124	1
Hg(NO ₃) ₂	0.5	0.8540	1

Weigh the desired mass into a 1-litre flask and make up to 1 litre with de-ionised water (ensure that the de-ionised water has an electrical conductivity less than 0.1 mS). Place a cap and shake end-over-end by hand for few minutes. Empty the contents of the flask into the corresponding labelled 1-litre bottle. Store in a cool and shaded location.

Label (metal name + concentration + date) fifteen 3-litre jerricans and prepare final metal solutions using the following table:

Metal Name	Target concentration (g L⁻¹)	Volume of solution (L)	Volume required (L)
Na ₂ O ₃ Se	0.001	30 ml	3
Na ₂ O ₃ Se	0.003	30 ml	3
Na ₂ O ₃ Se	0.005	30 ml	3
NaAsO ₂	0.001	30 ml	3
NaAsO ₂	0.003	30 ml	3
NaAsO ₂	0.005	30 ml	3
Hg(NO ₃) ₂	0.001	30 ml	3
Hg(NO ₃) ₂	0.003	30 ml	3
Hg(NO ₃) ₂	0.005	30 ml	3
Na ₂ O ₃ Se + NaAsO ₂	0.001 + 0.001	30 ml + 30 ml	3
Na ₂ O ₃ Se + NaAsO ₂	0.003 + 0.003	30 ml + 30 ml	3
Na ₂ O ₃ Se + NaAsO ₂	0.005 + 0.005	30 ml + 30 ml	3

$\text{Na}_2\text{O}_3\text{Se} + \text{Hg}(\text{NO}_3)_2$	0.001 + 0.001	30 ml + 30 ml	3
$\text{Na}_2\text{O}_3\text{Se} + \text{Hg}(\text{NO}_3)_2$	0.003 + 0.003	30 ml + 30 ml	3
$\text{Na}_2\text{O}_3\text{Se} + \text{Hg}(\text{NO}_3)_2$	0.005 + 0.005	30 ml + 30 ml	3
Hoagland solution			10

Add the appropriate volume of metal concentrated solution into a 3-litre flask and make up to 3 litre with Hoagland solution. Place a cap and shake end-over-end by hand for few minutes. Empty the contents of the flask into the corresponding 3-litre jerricans. Check the target concentration (by LGC).

Ensure the metal concentrated bottles are left in a cool, shaded location after use.

Seeds
germination

Material

Tap water. Check that the pH of the water is acceptable.

Poly-tunnel # 1

Four 60 x 60 x 5 cm deep tray

20 litres of bedding compost

1 bag of 25g of *Arabidopsis thaliana* seeds (about 450 seeds g⁻¹),
Herbiseed

(Product code: 9160). <http://www.herbiseed.com>

1 bag of 25g of *Brassica juncea* seeds (about 150 seeds g⁻¹), Herbiseed
(Product code: 9202). <http://www.herbiseed.com>

Method

Sprinkle seeds of *Brassica* or *Arabidopsis* onto two trays (two for each species) with bedding compost (subject to modification by Steve). Lightly water and place trays in the poly-tunnel 8. (Light levels will be kept at about 400 $\mu\text{mol PAR m}^{-2} \text{ s}^{-1}$ on a 16h/8h photoperiod with an air temperature and a relative humidity set to 20/15°C and 80%, respectively) existing condition of poly-tunnel 8. Check trays every day and water as necessary.



**Plant
material
Preparation**

Material

160 1-litre plastic pots, CJK (product code: P01-WE-RD)

<http://www.cjk.co.uk/Pails-and-Buckets-1.php>

Poly-tunnel # 1

160 litres of perlite

20 litres of pea shingle

Method

Label 160 1-litre plastic pots with the number shown in Appendix A. Add 0.9 litres of perlite in each pot. For each plant species, prick out five seedlings per pot (subject to modification by Steve) from the stock supply, into 1 batch of 80 pots. Cover the surface of the perlite with clean pea shingle (will see). Place all pots in the poly-tunnel 1 as shown in Appendix A (i.e. divide all pots into 2 batches of 80 pots). The light level will be kept above $400 \mu\text{mol PAR m}^{-2} \text{s}^{-1}$ on a 16/8 h photoperiod cycle (for an air temperature set to 20/15°C) (will see).



Treatments

Material

Fifteen 3-litres of the following final metal solutions:

$\text{Na}_2\text{O}_3\text{Se}$ 1
 $\text{Na}_2\text{O}_3\text{Se}$ 3
 $\text{Na}_2\text{O}_3\text{Se}$ 5
 NaAsO_2 1
 NaAsO_2 3
 NaAsO_2 5
 $\text{Hg}(\text{NO}_3)_2$ 1
 $\text{Hg}(\text{NO}_3)_2$ 3
 $\text{Hg}(\text{NO}_3)_2$ 5
 $\text{Na}_2\text{O}_3\text{Se} + \text{NaAsO}_2$ 1+1
 $\text{Na}_2\text{O}_3\text{Se} + \text{NaAsO}_2$ 3+3
 $\text{Na}_2\text{O}_3\text{Se} + \text{NaAsO}_2$ 5+5
 $\text{Na}_2\text{O}_3\text{Se} + \text{Hg}(\text{NO}_3)_2$ 1+1
 $\text{Na}_2\text{O}_3\text{Se} + \text{Hg}(\text{NO}_3)_2$ 3+3
 $\text{Na}_2\text{O}_3\text{Se} + \text{Hg}(\text{NO}_3)_2$ 5+5
 10 litres of Hoagland solution

Method

According to the label on each pot, add about 200 mL of the corresponding final metal solutions or Hoagland solution in each pot. The light level will be kept above $400 \mu\text{mol PAR m}^{-2} \text{ s}^{-1}$ on a 16/8 h photoperiod cycle (for an air temperature set to 20/15°C) (will see). Check the temperature and light level (light sensor and hand-held meter provided by E. Casella) every weekday. Check all pots every day and fill them with the appropriate final metal solutions.



Harvest**Material**

40 litres of de-ionised water
Permanent markers
One 0.25 litre plastic wash bottle
Aluminium paper
Ice blocks
one cool box
200 Plastic bags
5 litres cryogen tank
40 litres of liquid nitrogen

Method

Place the ice blocks in the -80°C freezer for all night. Transfer the ice blocks onto the cool box and add about 1 litre of liquid nitrogen. Close the cool box.

For each plant, remove the entire plant from the perlite, wash carefully the root compartment with de-ionised water and separate the roots from the plant and the leaves from the shoot. Label (plant fraction) three 20 x 20 cm foils to wrap separately the three plant fractions. Plunge samples in the 5 litres cryogen tank filled with liquid nitrogen. After few minutes, remove the samples from the cryogen tank and place them quickly in a labelled (pot label + date) plastic bag. Store quickly the plastic bag in the cool box (add liquid nitrogen if necessary). Store the plastic bags inside a box located in the -80°C freezer.

Health and Safety Disposable rubber gloves should be worn at all times when handling contaminated plants.
All laboratory work should be carried out by a trained member of Forest Research staff who has read, understood and signed the appropriate risk assessments provided by Mark Oram.

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List of
Appendices

Appendix A: randomised block design
Appendix B: timetable

Appendix A: Position of the 160 pots in the poly-tunnel

B 9 /4	A 13 /2	B 5 /2	B 4 /3	A 2 /4	A 8 /2	B 11 /5	A 8 /4	B 11 /3	A 12 /4
B 14 /1	A 2 /1	B 1 /3	A 14 /2	A 7 /4	A 16 /5	A 15 /1	B 10 /4	A 4 /2	B 8 /4
A 12 /2	B 6 /1	B 11 /1	A 7 /5	A 15 /4	A 6 /2	B 3 /5	B 10 /3	A 16 /4	A 5 /5
A 3 /4	A 16 /3	B 13 /5	A 4 /3	B 1 /4	B 7 /4	B 7 /2	B 2 /5	B 7 /5	B 12 /1
B 4 /4	B 6 /3	B 9 /1	B 6 /4	A 5 /4	B 9 /5	A 11 /5	B 8 /1	B 7 /3	B 1 /5
A 1 /3	A 2 /5	A 12 /5	A 14 /5	B 6 /2	B 10 /5	B 16 /1	A 7 /2	A 9 /4	A 10 /1
B 1 /1	B 15 /1	B 10 /1	A 4 /1	A 14 /1	A 11 /2	A 10 /5	A 8 /3	A 3 /2	A 8 /1
A 6 /1	A 11 /4	B 14 /2	A 16 /1	A 10 /3	B 12 /3	A 6 /5	B 11 /4	A 2 /2	B 16 /2
B 14 /5	A 7 /1	B 8 /3	A 12 /1	A 9 /2	B 15 /4	B 8 /5	A 5 /3	B 5 /5	B 13 /2
A 13 /1	B 5 /1	A 13 /3	A 9 /5	B 6 /5	B 14 /3	B 5 /4	B 2 /3	B 9 /3	B 2 /1
B 16 /4	A 13 /4	A 8 /5	B 3 /4	A 16 /2	B 10 /2	B 16 /5	B 9 /2	B 13 /1	B 4 /1
B 13 /4	A 5 /1	A 3 /5	A 1 /5	A 11 /1	B 12 /4	A 15 /5	B 11 /2	B 2 /4	A 10 /4
B 15 /5	A 10 /2	A 6 /4	B 3 /1	A 15 /3	B 1 /2	B 5 /3	B 4 /5	B 12 /5	A 9 /1
A 2 /3	A 6 /3	A 11 /3	A 3 /1	A 14 /4	A 5 /2	B 4 /2	A 1 /2	A 1 /1	B 13 /3
A 4 /4	A 14 /3	B 12 /2	B 3 /3	B 16 /3	B 2 /2	A 4 /5	A 3 /3	B 8 /2	A 13 /5
B 3 /2	A 15 /2	B 7 /1	A 9 /3	B 15 /3	B 14 /4	A 7 /3	B 15 /2	A 1 /4	A 12 /3

A *Arabidopsis thaliana* L.
B *Brassica juncea* L.

- 1 Na₂O₃Se 1 mg L⁻¹
- 2 Na₂O₃Se 3 mg L⁻¹
- 3 Na₂O₃Se 5 mg L⁻¹
- 4 NaAsO₂ 1 mg L⁻¹
- 5 NaAsO₂ 3 mg L⁻¹
- 6 NaAsO₂ 5 mg L⁻¹
- 7 Hg(NO₃)₂ 1 mg L⁻¹
- 8 Hg(NO₃)₂ 3 mg L⁻¹
- 9 Hg(NO₃)₂ 5 mg L⁻¹
- 10 Na₂O₃Se + NaAsO₂ 1+1 mg L⁻¹

- 11 Na₂O₃Se + NaAsO₂ 3+3 mg L⁻¹
- 12 Na₂O₃Se + NaAsO₂ 5+5 mg L⁻¹
- 13 Na₂O₃Se + Hg(NO₃)₂ 1+1 mg L⁻¹
- 14 Na₂O₃Se + Hg(NO₃)₂ 3+3 mg L⁻¹
- 15 Na₂O₃Se + Hg(NO₃)₂ 5+5 mg L⁻¹
- 16 Control in Hoagland solution

- /1 replicate 1
- /2 replicate 2
- /3 replicate 3
- /4 replicate 4
- /5 replicate 5

Appendix B: Timetable

APPENDIX 3 pH measurement in the dialyzate and non-dialyzate fraction using PIPES to fill the dialysis bag (Dominguez-Gonzalez *et al* (2010) [70]).

Dialysis bag content	Initial pH	Final pH ^a	
		pH of dialyzate	pH of residual fraction
0.15 N PIPES	6.4	6.3 ± 0.0089	6.1 ± 0.15
0.15 N PIPES	6.9	6.8 ± 0.0060	6.6 ± 0.11
0.15 N PIPES	7.5	7.3 ± 0.017	6.9 ± 0.72
0.15 N PIPES	7.85	7.6 ± 0.045	6.9 ± 0.10
0.15 N PIPES	8.5	7.8 ± 0.041	7.1 ± 0.025

^a n = 2.

APPENDIX 4 Basic information and preliminary study of research cigarettes 3R4F.**Cigarette Design Criteria**

Cigarette Length	84 mm
Tobacco Rod Circumference	24.8 mm
Tobacco Rod Length	57 mm
Filter Length	27 mm
Total Resistance to Draw (RTD)	128 mm H ₂ O
“Tar”	9.5 mg cigt ⁻¹

Cigarette Rod		Cigarette Filter	
Filter	3R4F Blend	Tow	Cellulose Acetate 2.9/41,000
Cuts per Inch	30	Plasticizer	9% Triacetin
Paper Permeability	24 CORESTA units	4-up Resistance to Draw	115 mm H ₂ O
Paper Citrate	0.60%	Circumference	24.45 mm
Tobacco Weight (13% OV)	0.783 mg	Length	27 mm
Length	57 mm		

Target Total Cigarette

Tipping Paper Length	32 mm, white
Dilution	30 ± 5%
Circumference	24.8 mm
Length	84 mm
Resistance to Draw	128 mm H ₂ O
Weight	1.06 g
Pack Moisture	13% Oven Volatiles

Final FTC Smoking Results Average (n=12)

Puff Count	9.0 ± 0.15
Total Particulate Matter, TPM	11.0 ± 0.33 mg cigt ⁻¹
“Tar”	9.4 ± 0.30 mg cigt ⁻¹
Nicotine	0.73 ± 0.013 mg cigt ⁻¹
Carbon Monoxide	12.0 ± 0.48 mg cigt ⁻¹

Final Filter Analysis (n=23)

Total Alkaloids (as-is)	2.05 ± 0.04% at 11.6% Oven Volatiles
Reducing Sugars	8.4 ± 0.4% at 11.6% Oven Volatiles
Glycerin	2.7 ± 0.1% at 11.6% Oven Volatiles

Blend Summary	3R4F
Flue Cured	35.41%
Burley	21.62%
Maryland	1.35%
Oriental	12.07%
Reconstituted (Schweitzer Process)	29.55%
Glycerin (dry-weight basis @ 11.6% OV)	2.67%
Isosweet (Sugar)	6.41%

APPENDIX 5 Percent transfer of selected metallic and nonmetallic elements between tobacco and tobacco smoke (The chemical components of tobacco and tobacco smoke, p 913[232]).

Elements	Transference to smoke (%)	Elements	Transference to smoke (%)
Aluminium	0.009-0.0014	Iron	0.014-1.3
Antimony	0.003-19	Lanthanum	ND-11
Arsenic	[0.016]-7.0	Lead	0.16-6.3
Beryllium	0-[4.0]	Magnesium	0.0025
Bromine	0.02-2.41	Manganese	0.004-0.006
Cadmium	7-22	Nickel	<0.1-2.4
Calcium	ND-0.001	Potassium	0.2-0.51
Cerium	ND	Rubidium	0.18-0.78
Cesium	1.27	Samarium	ND
Chlorine	1.2-2.2	Scandium	0.018-2.6
Chromium	0.43-1.74	Selenium	2.5-5.2
Cobalt	0.5-4.2	Silver	0.60-1.08
Copper	0.71-1.7	Sodium	0.25-1.06
Gold	0.002	Zinc	0.4-2.7

[] = Limit of detection

ND = Not detected

APPENDIX 6 Levels of trace elements and other selected analytes in mainstream smoke (3R4F) under ISO standard machine-smoking conditions (Liu *et al* (2009) [215]).

	3R4F	3R4F-CV ₋₁
As, x 10 ⁻¹² kg cigt ⁻¹	5.0	56
Cd, x 10 ⁻¹² kg cigt ⁻¹	25.5	28
Cr, x 10 ⁻¹² kg cigt ⁻¹	1.7	66
Pb, x 10 ⁻¹² kg cigt ⁻¹	6.3	30
Ni, x 10 ⁻¹² kg cigt ⁻¹	0.9	88
Se, x 10 ⁻¹² kg cigt ⁻¹	2.2	64
Total particulate matter, x 10 ⁻⁶ kg cigt ⁻¹	11.0	-
CO, x 10 ⁻⁶ kg cigt ⁻¹	12.0	5
Nicotine, x 10 ⁻⁶ kg cigt ⁻¹	0.73	5
Tar, x 10 ⁻⁶ kg cigt ⁻¹	9.4	6

* Trace elements were measured by ICP-MS. 20 replicates per cigarette for the trace element determination, whereas 5 replicates per cigarette used for total particulate matter, CO, nicotine and tar measurement.