Speciation Analysis of Arsenic and Selenium by HPLC and Mass Spectrometry

By

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Abstract

New methodologies have been developed for the determination of arsenic and selenium species in a variety of environmentally important matrices. A simple liquid chromatographic separation technique based upon mini-column technology was developed to obtain a simultaneous, fast, efficient and reliable separation of relatively toxic from relatively non-toxic arsenic and selenium species. The relatively toxic arsenic and selenium species studied were inorganic As^{V}, As^{III}, Se^{VI} and Se^{IV}. The relatively non-toxic species of arsenic and selenium studied were AsBet, DMA and SeMet. Optimum conditions were found to be the use of a Hamilton PRP X100 12-20 μm anion-exchange resin with column dimensions of 100 x 3 mm. The mobile phase utilized a 10 mM K_2SO_4 solution at pH 10.2 with a flow rate of 1 ml min^{-1} and a sample injection loop of 100 μl. Total analysis time was under 7 minutes with limits of detection in the range of 2.0 – 10 μg kg^{-1} for arsenic and selenium species, respectively.

Work was undertaken, using HPLC-ICP-MS instrumentation, as part of a feasibility study, into the production and certification of six new reference materials; these being analyzed for the species of arsenic, in chicken, fish, rice and soil samples, and selenium, in wheat and yeast samples. Enzyme extraction techniques were used throughout, except for soil where a microwave H_3PO_4 extraction was used. Efficiencies were in the range 90-100%. The results obtained provided speciation information as well as total elemental concentrations with no operationally defined limits.

Speciation analysis requires that the endogenous species are extracted without modification of their chemical form or disturbance to the equilibrium existing between the various species present. Work was undertaken to identify and quantify the selenium species present in two samples of novel, previously unstudied, bio-natured nutrients, these nutrients being: i) a selenized yeast from a new process and: ii) a probiotic bacteria-based dried milk sample (Biogurt®). Specific interest was directed towards enzyme, MeOH and KOH and TMAH extraction efficiencies together with retention of species information. Selenium speciation was performed using ion-exchange HPLC-ICP-MS. It was found that the selenium content, in the form of SeMet, was adequately extracted from the yeast (Pharma Nord) that was used for method validation using protease, which yielding 90% of the total selenium. However, the determination of selenium and selenium species in the bionatured nutrients proved to be quite problematic. Methods that avoided species conversion with the highest extraction efficiencies were found to be: i) the use of protease for the yeast sample (19%) and; ii) the use of 0.01 M HCl for the Biogurt® (71%). Information obtained from speciation of these samples by anion and cation-exchange HPLC-ICP-MS was limited due to the low extraction efficiencies of any procedure undertaken for the samples, by the retention of the analyte on-column and by the lack of standards available for matching of retention times.

HPLC-ICP-MS has proved an efficient tool for the identification and determination of arsenic and selenium species providing detection limits at μg kg^{-1} levels. However, a major concern with this instrumentation is the unambiguous assignment of peaks which relies on the chromatographic purity of the signal and the availability of standards. Anion-exchange chromatography employing Hamilton PRP X100 resin with NH_4HCO_3 (10 mM, pH 10.2 for arsenic and 10-50 mM, pH 5 for selenium species) with methanol (10 %, v/v) as the mobile phase allowed separation of the arsenic and selenium species investigated under conditions that were compatible for both HPLC-ICP-MS and HPLC-ESMS. Molecular ions and structural fragmentation patterns of these by tandem MS have facilitated the identification of chromatographic peaks obtained using HPLC-ICP-MS. In the analysis of marine algae, arsenosugars were the major species found, and in yeast the dominant species was found to be selenomethionine.

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Acknowledgements

I would like to take this opportunity to thank the many people that have been involved in this thesis. To my supervisors Prof. Les Ebdon, Dr. Mike Foulkes and Dr. Les Pitts – thank you for your support, knowledge, wisdom and wit without which I would be reclining in a sanatorium as I write. A heartfelt thanks goes to Dr. Andy Fisher for giving freely of his time, vast wealth of knowledge and practical experience in teaching me all that I know in the field of ICP-MS. I also thank him for mostly anticipating what mistakes I would make before I made them, thereby saving me a lot of time and further embarrassment - and for his many free trips to the pub! A big thanks goes to Mr. Rob Harvey for keeping the instruments working regardless of what manner of insult they had undergone with me using them. I would like to thank Philip Oppong for his assistance in the laboratory whilst he was on a summer work placement and to James Brown for his work in the field of ESMS.

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Finally, I turn my attention towards my family without whom this journey would have been infinitely more difficult to travel. A special thanks goes to Becky and Simon for their insistence in pointing out the humour in many a desperate situation and for their overwhelming generosity. I must thank my eldest sister, Helen, for nearly always mentioning Ockham's razor regardless of the topic of conversation. Only joking, Hel. You were a formidable source of encouragement and inspiration. My thanks also to my parents, both of whom I love dearly, for making me realize from an early age that anything worth having in life would never be easily attained. And last, but by no means least, to my daughter who feels that she has been irreparably damaged due to the loss of income incurred whilst I have been at university. So to her, I must say that I'm sorry! Hopefully, it will all have been worth it in the end.

Author`s Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Chapter one

1 Introduction

1.1 Trace element speciation

Trace elements are present in nature in a number of different forms or species. The adequate functioning of life can be critically dependent on these elements in numerous ways. Some are considered to be highly toxic [1] whereas others are considered to be essential [2]. The significance of the toxicological, nutritional and biochemical impact of any element on a biological system depends on the chemical forms present [3]. One of the main reasons is that the toxicity or bioavailability of an element can be several orders of magnitude different, depending on the chemical form. Thus the field of trace metal or elemental speciation has become an area of increasing interest in analytical chemistry. In response to this, a European Union network has been established that aims to bring together scientists with an interest in speciation of a diverse range of elements with potential users from areas that include industrial process control, the food industry, biomedical and pharmaceutical disciplines as well as the biological and environmental sciences [4].

Two elements of particular interest are arsenic and selenium. Arsenic has long been regarded as a toxin and long-term consequences of exposure, in particular to inorganic forms, are of importance as it is now recognized as a carcinogen [5]. Arsenic-contaminated drinking water has been responsible for countless cases of chronic arsenic poisoning in countries such as Bangladesh, China and Taiwan [6]. Selenium, on the other hand, is recognized as an essential ultra-trace element in

the human diet [2]. It is an integral component of several enzymes, including glutathione peroxidase, which are responsible for disease prevention due to their anti-oxidant properties [7]. However, it has a narrow therapeutic window.

Research in identification and determination of the various chemical forms of arsenic and selenium will assist in understanding the relationships that link speciation with the biochemical and environmental cycling of these important elements.

1.2 Arsenic compounds

1.2.1 Occurrence of arsenic in the environment

Arsenic is a ubiquitous element in the environment having been introduced via natural and anthropogenic routes. Arsenic can be found in rock, soil, dust, water and air. Phenomena, such as weathering of minerals, biological activity and volcanic activity, are largely responsible for the emission of arsenic into the biosphere from natural sources. Anthropogenic sources arise predominantly from the mining and smelting of copper, lead, cobalt and gold ores. Other anthropogenic sources are given in Table 1-1. Recent estimates have placed the ratio of emission from natural compared with anthropogenic sources at approximately 60:40 [8]. Despite the now known toxicity of arsenic, particularly in the inorganic form, production of arsenic has remained static over the last 60 years [3].

Table 1-1. Common uses of arsenic compounds [3].

Area	Uses
Agriculture	Pesticides, insecticides, defoliants, wood preservatives, soil sterilant
Livestock	Feed additives, disease prevention (swine dysentery), cattle and sheep dips, algaecides.
Electronics	Solar cells, optoelectronic devices, semi-conductor applications, light-emitting diodes.
Industry	Glassware, electrophotography, catalysts, pyrotechnics, anti-fouling paints, dyes, ceramics.
Metallurgy	Alloys, battery plates.

Levels of arsenic in different environmental compartments are often quoted in the literature but can differ significantly with regard to location and nearby industry. This is clearly demonstrated by the values given in Table 1-2.

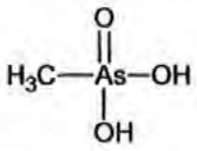
Table 1-2 Arsenic levels determined in environmental compartments [9]

	Background levels	**Contaminated sites**
Soil	≈ 7 mg kg^{-1}	1000 mg kg^{-1}
Air	1 – 10 ng m^{-3}	20 – 1000 ng m^{-3}
Freshwater	0.15 – 0.45 μg l^{-1}	up to 3000 μg l^{-1}
Seawater	0.09 – 24 μg l^{-1}	

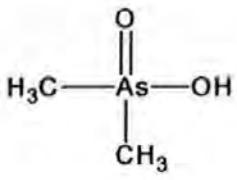
Considering the physicochemical similarities between arsenate and phosphate it is not unusual for organisms to inadvertently take up arsenate. However, there is a significant difference in the concentrations of arsenic found in terrestrial organisms to that found in marine organisms. Terrestrial organisms rarely contain more than 1 mg kg^{-1} (dry weight) [10] whereas marine organisms are often reported as containing much higher levels; with concentrations in marine animals generally lying in the range 10-500mg kg^{-1} but have been known to exceed 1000mg kg^{-1} for organisms living in contaminated areas [11]. The highest risk of exposure for humans comes from the consumption of seafood [12]. It would, therefore, be applicable to focus on the biochemical cycling of arsenic in the marine environment.

1.2.2 Distribution of arsenic in the marine environment

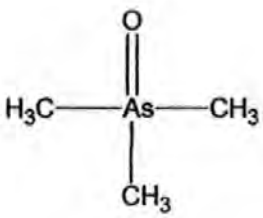
The presence of arsenic in marine samples was first comprehensively presented by Jones [13] in 1922. He remarked on the fact that arsenic was present in an organic form. Since then a vast amount of research has been undertaken and a variety of arsenic compounds have been identified. The most common organic arsenic compounds found in the marine environment are monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine oxide (TMAO), tetramethylarsonium ion (TeMA), arsenobetaine (AsBet), arsenoylribofuranosides (trivial name of arsenosugars) and less frequently, although still significant, arsenocholine (AsC). Structures of these organoarsenic species are shown in Figure 1-1. The distribution of arsenic compounds in seawater, marine fauna and flora are given in Table 1-3.



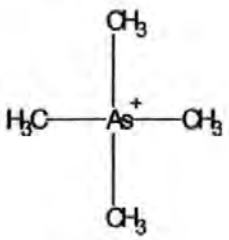
MMA



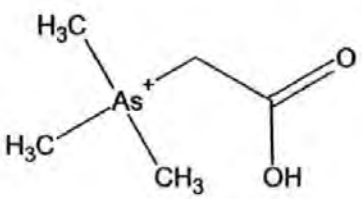
DMA



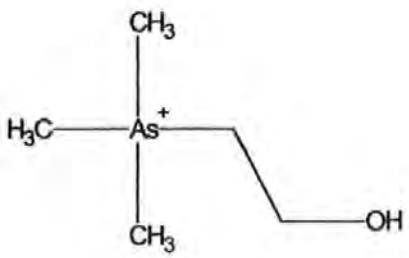
TMAO



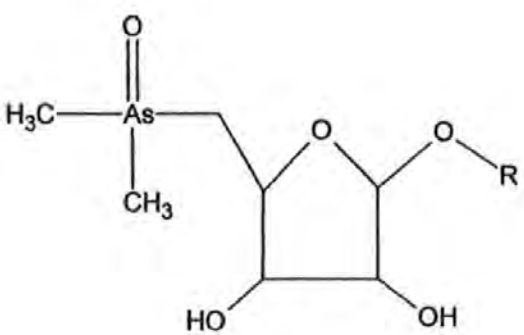
TeMA



AsBet



AsC



Arsenosugars

R = $CH_2CHOHCH_2OH$

= $CH_2CHOHCH_2OPO_3HCH_2CHOHCH_2OH$

= $CH_2CHOHCH_2SO_3H$

Figure 1-1 Common organoarsenic compounds [14]

Table 1-3 Arsenicals in different marine compartments [15]

	Major	Minor	Trace	Not detected
Sediments and porewater	As^{V}, As^{III}	MMA, DMA	TMAO	AsBet, TeMA, AsChol, arsenosugars
Seawater	As^{V}, As^{III}	MMA, DMA		AsBet, TeMA, AsChol, arsenosugars
Marine flora	Arsenosugars	As^{V}	MMA, DMA	AsBet, TeMA, AsChol
Marine fauna	AsBet	TeMA, arsenosugars,	As^{V}, As^{III}, TMAO, AsChol, DMA	

Bioaccumulation of arsenic is high in zooplankton, benthic organisms, seaweed and algae. However, there does not appear to be any biomagnification along food chains as levels of arsenic in predators are usually found to be no higher than those found in organisms lower down the food chain [16]. This suggests that the biochemical cycling of arsenic compounds by marine biota provide a route of detoxification with the added ability of the organism to excrete, or degrade to excrete, these compounds.

1.2.3 Biotransformations of arsenic compounds.

Arsenic readily undergoes conversions mediated by microorganisms, plants and animals where biotransformations give rise to the variety of arsenical species seen. Research has been abundant in this field and many arsenic compounds have been identified and mechanistic pathways for their formation proposed [15, 17].

The high levels of arsenic found in macroalgae, particularly brown algae, and its chemical form was first established in 1981 by Edmonds and Francesconi [18]. The two compounds identified by NMR spectroscopy were dimethylarsinoylribosides. Since then many other compounds of similar structure have been elucidated from marine algae and come under the broad heading of arsenosugars [19, 20, 21].

Classic studies by Challenger [17] on microbial methylation of arsenic still provide the basis for current understanding of the transformation of inorganic arsenic to the organic forms. Challenger's mycological studies indicated that arsenate could be transformed to trimethylarsine by sequential reduction and oxidative methylation, with MMA, DMA and TMAO forming the intermediate compounds. S-adenosylmethionine (AdoMet) was thought to be the active methyl donor. Methylation by methanogenic and non-methanogenic bacteria has also been shown to occur with mechanisms likely to be similar to that in fungi [10].

The biogenesis of arsenosugars from arsenate in marine algae follow a similar pathway to that described by Challenger [17]. A sequential reduction and oxidative methylation of arsenate with S-adenosylmethionine (AdoMet) donating its methyl group and adenosyl group to arsenic is given in Figure 1-2. This pathway is supported by the detection of both MMA and DMA in algae.

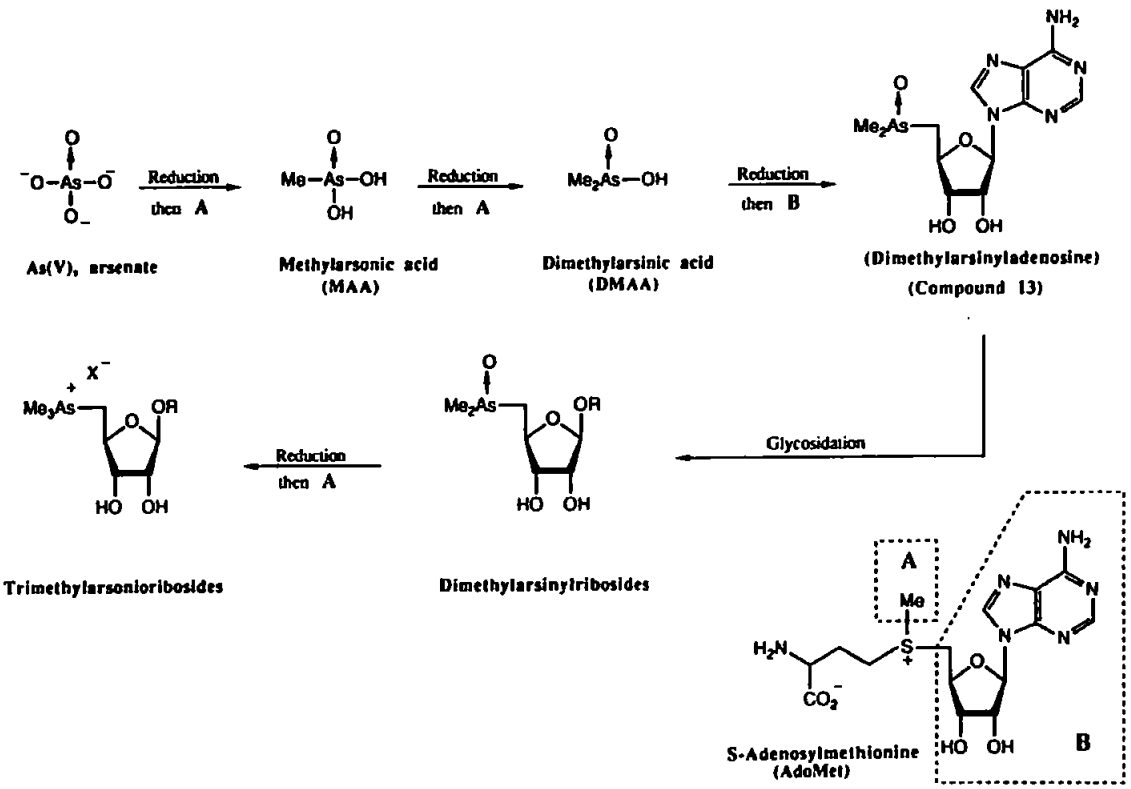


Figure 1-2 Proposed pathway for the biogenesis by algae of arsenic-containing ribosides from arsenate. The key intermediate (compound 13) undergoes glycosidation with available algae metabolites to give the range of dimethylarsinylribosides found in marine algae [14]

Arsenobetaine (AsBet) was first identified in marine animals by Edmonds *et al* [22], in 1977 . Since its discovery it has been found to be ubiquitous among marine animals. It is present at all trophic levels and is the predominant form of arsenic found. Other organoarsenicals may also be present as described in Table 1-3. Although many hypotheses have been proposed the pathway for its biogenesis remains unsubstantiated. Studies on fish have suggested that arsenobetaine is formed by microbial activity either in the seawater, although it is not detected in this compartment, or in the host itself and is then accumulated by the host [15]. Possible pathways for the formation of arsenobetaine by micro-organisms result from the cleavage of carbon-carbon bonds in the furan ring of arsenosugars followed by the oxidation of the resultant alcohol, arsenocholine. MMA and DMA have also been shown to be precursors of arsenobetaine [23]. Arsenobetaine is thought to be the final metabolite in this part of the arsenic cycle. It is itself degraded back to inorganic arsenic by the action of micro-organisms [24, 25] through a series of intermediates as shown in Figure 1-3, and by ultra-violet (UV) radiation [26].

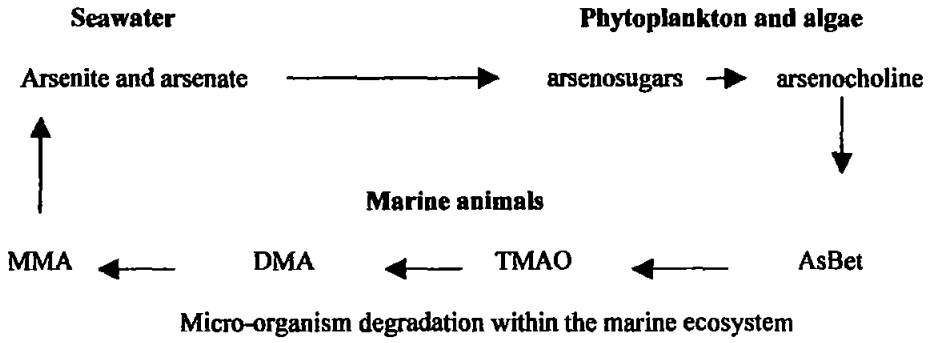


Figure 1-3 Proposed arsenic cycle in the marine environment [24].

1.2.4 Toxicology of arsenic compounds

It is known that the toxicity of arsenic can vary by several orders of magnitude depending on its chemical form, and that exposure to the more toxic species can give rise to mutagenic, teratogenic and carcinogenic effects [12]. Inorganic forms of arsenic, As^{III} and As^{V}, are considered to be the most toxic [27]. Organic species display decreasing toxicity with increasing derivatization. The LD_{50} values in rats, mg kg^{-1} body weight, in decreasing levels of toxicity has been given as: arsenite, 1.5; arsenate, 5.0; MMA, 50; DMA, 600 [28]. Arsenobetaine and arsenocholine have been shown to be essentially non-toxic with $LD_{50} > 10g\ kg^{-1}$ [11]. It is also thought that arsenosugars found in seaweed and algae are relatively non-toxic [29].

The degree of gastrointestinal absorption of arsenic depends on the species present. Greater than 95% of inorganic arsenic is absorbed, approximately 75% of MMA and DMA is absorbed whilst arsenobetaine is not readily absorbed by the body and is excreted unchanged [30].

Inorganic arsenic absorbed by humans is detoxified via a similar pathway to that described by Challenger with methylation to MMA and DMA occurring by hepatic enzymatic transfer of methyl groups from S-adenosylmethionine [31].

Toxicological effects in marine organisms vary but in general show a moderate tolerance to the presence of arsenic [32]. There does not appear to be biomagnification along the food chain and bioaccumulation of species will largely depend on the presence of phytoplankton and micro-organisms which are capable of transforming toxic to less toxic forms of arsenic [32].

1.3 Selenium compounds

1.3.1 Occurrence of selenium in the environment

Although selenium appears to be ubiquitous in the environment its uneven distribution results in regions with very low or very high natural levels. This is reflected in the levels found in endogenous food sources, which have been found to vary widely depending on the availability of selenium in the immediate environment. Soils derived from sedimentary rocks tend to have higher levels of

selenium than do igneous and metamorphic rocks. These soils tend to be alkaline in reaction and favour the presence of selenate. There is variable analytical data available for levels of selenium found in differing environmental compartments. However, Table 1-4 gives some indication of values expected [33].

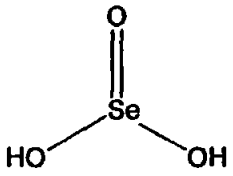
Table 1-4 Levels of selenium found in different environmental compartments [33].

Source	Levels
Air	< 10 ng m^{-3}
Water	$\approx 1 - 5$ µg l^{-1}
Foodstuffs:	
Meat and seafood	0.4 – 1.5 mg kg^{-1}
Cereals	$< 0.1 - > 0.8$ mg kg^{-1}
Dairy products	$< 0.1 - 0.3$ mg kg^{-1}
Fruit and vegetables	< 0.1 mg kg^{-1}

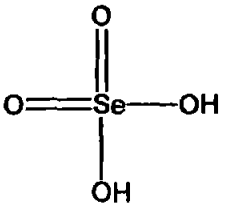
Anthropogenic sources of selenium arise from its use in agriculture and from products and/or byproducts of industry. The addition of selenium to animal feedstuffs and fertilizers to rectify naturally occurring low selenium levels is not thought to contribute significantly towards an environmental pollution risk. However, an incident at the Kesterton reservoir in California, where accumulation of selenium-laden water from agricultural run-off, led to toxic effects being demonstrated in birds and fish [34, 35].

The main industrial sources of selenium come from oil refineries and the burning of fossil fuels, mining and smelting of metal ores and in the production and purification of selenium itself. Ground water can be directly polluted from industrial effluent and atmospheric emissions may also be important in the contamination of open-water reservoirs.

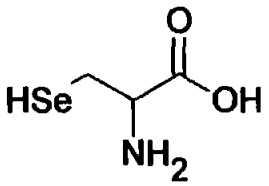
Selenium exists in the environment in several oxidation states, predominantly (-II), (IV) and (VI). Inorganic forms of selenium are more commonly present as selenite, Se^{IV}, and selenate, Se^{VI}. Organic selenium compounds are ordinarily found in the –II oxidation state as selenides and as analogues of sulphur-containing compounds. Selenium is present mainly in the aminoacids selenomethionine and selenocysteine. Selenomethionine and selenocysteine are bound covalently to proteins where they carry out a number of important biological functions. The major product of selenium metabolism is the trimethylselonium ion which is excreted via the urine. Selenium also exists in volatile forms, predominantly as dimethylselenide. Structures of some environmentally important selenium compounds are shown in Figure1-4.



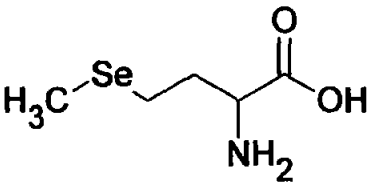
Selenous acid



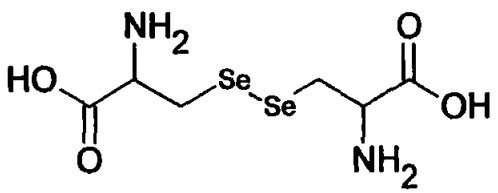
Selenic acid



Selenocysteine



Selenomethionine



Selenocystine

Figure 1-4 Structures of some environmentally important selenium compounds [36].

1.3.2 Biotransformations of selenium in plants and humans

Although biogeochemical cycles for selenium are not fully characterized, a review of the available research gives an insight into some of the mechanistic pathways that allow inclusion of selenium into the food chain. It is known that bacteria in soils can convert between elemental selenium, selenite and selenate [33]. Prevailing redox conditions in soil also allow for the presence of selenium as selenite and selenate. Selenium in these forms is bioavailable and uptake from soil by plants can occur. Plants vary considerably in their physiological response to selenium. Some plant species are selenium accumulators as they have a degree of tolerance to selenium whereas other plant species can be selenium-sensitive and, therefore, avoid selenium accumulation. In this respect, plants can play a pivotal role in the food chain as there is a narrow margin between the beneficial and harmful levels of selenium required in maintaining optimum human health. Plants may also take up organic forms of selenium such as selenomethionine [37]. Selenium accumulators can be used in areas where naturally occurring selenium levels are low providing a useful way of supplementing the diet.

Plant roots accumulate selenate by active transport. Selenite and organic forms of selenium can also be accumulated actively. Selenate readily competes with sulphate uptake [38] and a sulphate transporter therefore, mediates its accumulation. In selenium accumulators selenate is taken up preferentially over sulphates. Selenate is easily transported within the plant to the leaves whereas selenite and organic selenium compounds tend to remain in the root system. It is thought that

selenite is rapidly converted to organic selenium and thereby accumulates in the roots [39]. Plants can also release and absorb volatile selenium compounds to and from the atmosphere. The production of selenocysteine most probably occurs within the chloroplasts whereas selenomethionine is more likely to be synthesized in the cytosol; both are incorporated in proteins. Many other selenium compounds are also found in plants that include intermediates in metabolic pathways (selenocystathionine, selenohomocysteine) and end-products in themselves (dimethylselenide).

The major selenoamino acid found in plants is selenomethionine [40] and is assimilated by animals that feed on the vegetation. However, selenocysteine is the major selenoamino acid found in animals [40]. This implies that nutritionally derived selenium is used in metabolic processes within animals to achieve the required selenoprotein status. Selenium function within animals is primarily as selenoproteins.

In humans, the major biological functions of selenium are achieved through its redox activity when present as selenocysteine at the active sites of selenoproteins [7]. These proteins are selenium-dependent as replacement with the sulphur analogue renders them enzymatically inactive [2]. At least thirteen selenoproteins have been identified in humans, including glutathione peroxidases, selenoprotein P and iodothyronine deiodinases, all of which contain selenocysteine. Selenocysteine can be obtained from exogenous sources, however only endogenously derived selenocysteine is incorporated into the selenoproteins [41].

Although metabolic pathways are not fully characterized it is known that humans are capable of synthesizing selenocysteine, which has become known as the 21st amino acid, using a genetic code via the UGA codon [42]. Ultimately, selenocysteine is derived from the amino acid serine and selenide, which suggests that selenium compounds from dietary intake, must enter a metabolic pathway that pass through a common selenide intermediate [2].

Selenomethionine cannot be formed *in vivo* and is therefore exclusively obtained from the diet. It is incorporated non-specifically into body proteins in place of methionine, as met-tRNA. Excess selenomethionine is able to enter the metabolic pathway where its selenium can be incorporated into selenoproteins [2].

Selenides can be methylated to the trimethylselonium ion, which is the major route for excretion via the urinary tract [43]. If this process becomes overloaded then the methylation is to that of dimethylselenide which is excreted via the respiratory tract and hence the 'selenium/garlic breath' associated with high intake levels. Other metabolites such as monomethylselenol and dimethyl diselenide have been identified and, therefore, it can be inferred that methylation is a detoxification process. A selenium metabolic pathway, as described by Ip [44], is shown in Figure 1-5.

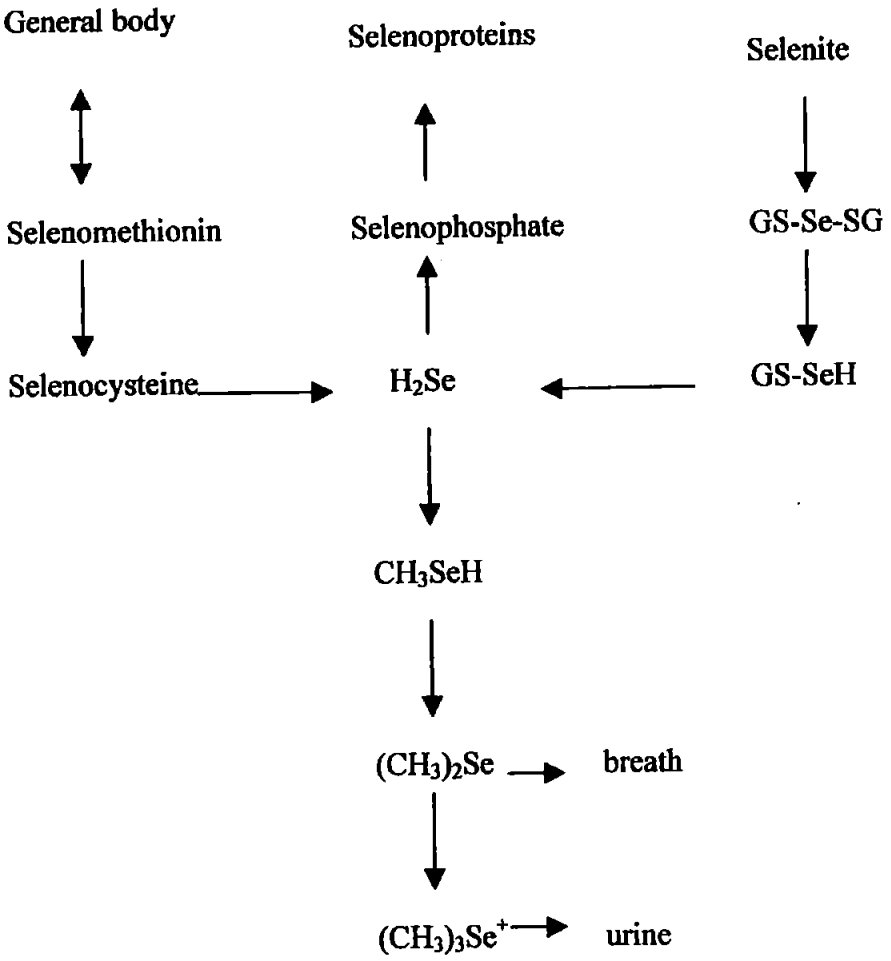


Figure 1-5 Selenium metabolic pathway. GS-Se-SG = selenodiglutathione, GS-SeH = glutathione selenopersulphide [44]

1.3.3 Selenium as an essential element in the diet

Selenium is widely recognized as both a toxic and an essential element depending on its chemical form and concentration. Although organic and inorganic forms of selenium have a common metabolic pathway it is generally thought that organic

forms of selenium are less toxic [45]. The gap between toxic and essential levels of selenium in humans is narrow. Diseases related to selenium deficiency were first identified in 1979 when Chinese researchers established an association between low selenium status in humans and the presence of Keshan disease, a form of cardiomyopathy, and Keshan-Beck disease, a deforming osteoarthritis [46]. A major clinical development of the 1990`s was the findings of Clark *et al.* [47] that human dietary supplementation with selenium-enriched yeast decreased cancer incidence and mortality by up to 50%. The cancer chemopreventative effect of selenium has been tentatively attributed to the biological anti-oxidant functions of selenoamino acids [48]. However, the form of dietary selenium most appropriate to confer anti-oxidant properties remains unclear.

Plasma GSH-Px activity is often used as an indicator for selenium levels as there is a close correlation between the two. GSH-Px activity plateaus out at similar intake levels for both organic and inorganic dietary forms. However, selenomethionine has a stronger and more stable effect on raising and maintaining GSH-Px [44]. Whilst this may be true, it has also been noted that it may not confer any greater degree of anti-carcinogenic properties than do other forms [49]. A more comprehensive explanation may be arrived at when considering that there is differential regulation of the selenoproteins; this ensures that those, which perform the most important functions, are preferentially preserved. Selenium supply to the brain, endocrine and reproductive organs is as far as possible maintained at optimum levels with the synthesis of selenoproteins such as iodothyronine deiodinases taking precedence [50]. GSH-Pxs are the most

dispensable and, therefore, when these levels are at an optimum it can be conferred that all other selenoproteins are also at an optimum. Any further selenomethionine taken up in the diet is incorporated non-specifically into selenium-containing proteins. Excess levels of other forms of dietary selenium are excreted in the urine [43].

Humans are exposed to selenium mainly through their diet. Studies have shown that the body absorbs approximately 95% of ingested selenomethionine whereas only 60% of selenite is absorbed with selenate absorption being around 90% [51]. Although selenomethionine is absorbed from the diet more rapidly and to a greater extent than any other form of selenium it is also retained in the tissues following non-specific incorporation more strongly than other forms. The dietary selenium species other than selenomethionine are therefore more readily available for biosynthesis of the active selenoproteins that contain selenocysteine.

The UK Reference Nutrient Intake (NRI) of 75 μg per day for men and 60 μg for women [52] has been determined as the intake believed to be necessary to maximize the activity of the anti-oxidant selenoprotein GSH-Px which occurs at a plasma concentration of around 95 μg l^{-1}. Toxic symptoms occur at approximately 10,000 μg l^{-1} [53].

Seafood and offal provide a large source of dietary selenium together with yeasts and mushrooms. However, there is limited information available on the proportions of organic to inorganic forms of selenium in these foods and of their

bioavailability. Dietary supplementation of selenium has become an increasingly popular choice as a route to redress the balance of naturally low selenium levels in food. Supplements can be found with varying amounts and forms of selenium. Most contain single chemical entities. Conversely, selenized yeast preparations may contain a mixture of compounds, some of which may be unspecified. Research has shown a diversity in yeasts ranging from 20 – 80% selenomethionine with selenocystine, *Se*-methylselenocysteine and selenoethionine (amongst others) comprising the remainder [54, 44]. Selenized yeast was used in the Clarke *et al.* [47] trials but it is not known exactly which form of selenium is responsible for reducing the incidence of cancers.

As the cancer chemopreventative effect of selenium has been tentatively attributed to the biological functions of selenoamino acids further understanding of the efficacy of dietary supplementation relies on the development of analytical methodology for the separation, identification and determination of the various species present in selenium-enriched food-stuffs. There is no doubt that the absorption, transport, metabolism, retention and elimination of selenium compounds is dependent on its chemical form but further work in these areas is needed to develop a more informed view regarding selenium supplementation.

1.4 Methods for the speciation of arsenic and selenium

It is recognized that the need for trace element analysis requires very sensitive analytical techniques for quantification and qualitative identification. Speciation of elemental compounds is essential for the assessment of their toxicological impact. Methods to separate and identify various species are now becoming a necessity for any elemental analysis. There are numerous review papers published annually dealing with analytical methods used for the identification and determination of arsenic and selenium compounds [55, 56]. Analytical approaches generally involve the use of separation techniques coupled with a sensitive element specific or molecular detectors for quantification purposes. Recent successful applications of electrospray mass spectrometry (ESMS) for species characterization and identification have confirmed the potential opportunities offered by this technique [36, 57]. Chromatographic techniques, for separation of analytes, comprise liquid and gas chromatography. Both are high performance methods but the most important distinction between the two is that GC relies on compounds that are volatile or can be evaporated intact at elevated temperatures, and can separate approximately 20% of known organic compounds without prior derivitization, whereas for LC most organic and inorganic compounds can be dissolved in a solvent and are therefore amenable to separation using this technique. HPLC together with ICP-MS and ESMS provide an exciting route for analytical chemistry, particularly in the area of speciation.

1.4.1 High performance liquid chromatography

Chromatographic separations have been used successfully since the 1930's [58]. Since its inception, chromatography has developed so that a vast array of complex matrices can be determined. This is due to the versatility of the technique. Liquid chromatography is a generic term used to describe any chromatographic procedure in which the mobile phase is a liquid. Examples of these techniques include ion-exchange, ion-pairing, reversed phase and size-exclusion chromatography. Ion-exchange chromatography will be used throughout the experimental chapters of this thesis and, therefore, a brief description of this will follow.

1.4.1.1 Ion-exchange chromatography.

Ion exchange comes under the umbrella of liquid chromatographic techniques. It is a process wherein a solution of an electrolyte (mobile phase) is brought into contact with an ion exchange resin (stationary phase) and active ions on the resin are replaced by ionic species of a similar charge from the electrolyte solution. Competition between ions of the mobile phase and that of the analyte allow chromatographic separation to occur, separations being based on the difference in migration rates among the analyte components. To achieve a successful separation of analyte ions by ion chromatography the effects of pH, the counter ion and the ionic strength of the eluent can be manipulated until optimum conditions are obtained.

Column packing materials used for ion exchange are characterized by the presence of charged groups covalently bound to the stationary phase. Anion-exchange columns carry a positive charge, usually a quaternary ammonium group (strong anion-exchange) or an amine group (weak anion-exchange). Cation-exchange columns carry a negative charge, usually a sulphonate group (strong cation-exchange) or a carboxylate group (weak cation exchange). Strong ion-exchange groups are more commonly employed as they retain their properties over a wide range of pH. The driving force for the separation is the charge on the analytes, which depending on the pH may or may not be fully ionized. For weak acids or bases retention behavior is pH dependent. However, if the analyte molecules are strong acids or bases their retention behavior is not affected by changes in pH of the mobile phase as they remain fully charged over a wide pH range. The pK_a values for compounds provide an indication of how they will behave and this knowledge can be used to optimize separation conditions based on the pH of the mobile phase.

Ion-exchange will also be influenced by the choice of the counter ion. As a rule, multiply charged ions are bound more strongly than singly charged ones. The retention time in anion-exchange increases if a counter ion is exchanged with another, for example, in the following sequence: citrate – sulphate – oxalate – tartrate – iodide – borate – nitrate – phosphate – bromide – cyanide – nitrite – chloride – acetate. For example citrate solutions elute analyte anions more rapidly than phosphate solutions. Ion exchangers have a preference for ions with a higher charge, smaller diameter and greater polarizability.

Ion-exchange equilibria can also be displaced by changes in the ionic strength of the buffer solution. The ionic strength affects the capacity factor, k', and causes it to drop with increasing ionic strength; the k' values of samples are inversely proportional to the ionic strength [59]. As the counter ion concentration increases the analyte ion will spend less time in the stationary phase due to increased competition and hence k' will decrease.

Other effects on ion-exchange chromatography include column temperatures. The higher the temperature the faster the rate of diffusion which gives rise to better peak shapes and shorter elution times [60]. This is not directly attributable to ion-exchange equilibria but a kinetic effect on the rate at which equilibria are attained. Column efficiency is also improved when eluent viscosity is decreased [60].

1.4.2 Inductively coupled plasma-mass spectrometry (ICP-MS)

The argon ICP is the most widely used atomic spectroscopic source in analytical chemistry [61]. A schematic of the instrumentation is shown in Figure 1-6. It is increasingly used as a high-temperature ion source for mass spectrometry. Practical considerations for the generation of ICPs were originally addressed by Reed [62] and refined for spectrochemical analysis in the early 1960`s. Greenfield successfully used ICP as an ion source for atomic emission spectrometry [63] and the technique was further development by Date and Gray [64] for use as an ion source for mass spectrometry. Since then many elements of the Periodic Table have been analyzed with success, including As and Se [65, 66].

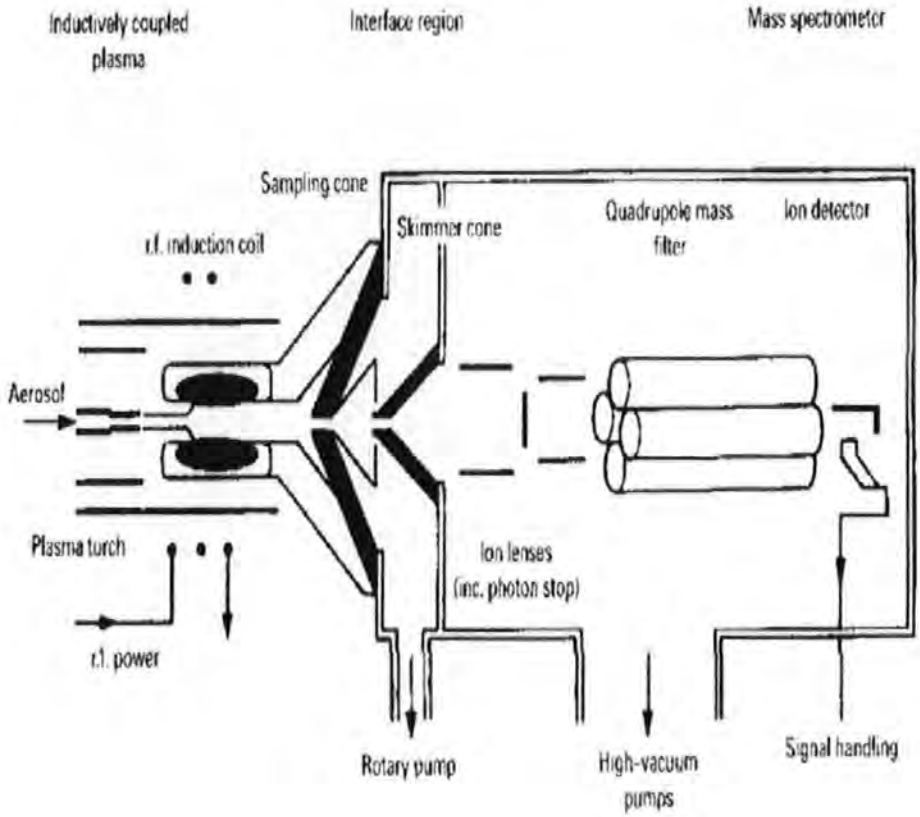


Figure 1-6 Schematic of an ICP-MS instrument [67].

An inductively coupled plasma is a relatively well characterized, high-temperature source, suitable for the atomization and ionization of elemental species. Generation of the plasma occurs within a quartz torch, which consists of a series of concentric tubes, through which argon gas flows. The coolant and auxiliary gases enter tangentially creating a vortex that produces the distinctive annular plasma characteristic of an ICP. The torch is encircled at the top by an induction or load coil, which is connected to a radiofrequency (rf) generator. The magnetic field generated by the rf current induces a current in the gas stream. The

seeding of the gas with electrons forms the plasma by initial excitation from a Tesla coil attached at the coolant gas inlet. The electromagnetic vectors giving them sufficient energy to cause ionization of the gas accelerate these initial electrons. Further collisions that ensue enable the plasma to become self-sustaining as long as the electromagnetic field is sufficiently high and the gas flows in a symmetrical pattern. The ionization conditions within the plasma result in highly efficient ionization of most elements in the periodic table (dependant upon their ionization energies) and, importantly, these ions are almost always exclusively singly charged [68].

Liquid sample introduction is the most common way for presenting samples to the plasma. Typical sample introduction systems consist of a nebulizer (e.g. crossflow, V groove) and spray chamber (e.g. double or single pass, cyclonic). The nebulization process creates a fine aerosol of the liquid sample and the spray chamber then separates out large droplets. The spray chamber may also be cooled facilitating the removal of solvent. The small droplet aerosol is then transported to the plasma by the nebulizer gas flow where it undergoes desolvation, vaporization, atomization and finally ionization. The analyte ions are subsequently introduced into the MS by a series of chambers held at consecutively lower pressures where they are focused by ion lenses then separated according to their mass:charge ratio either by using a quadrupole (low resolution MS) or magnetic sector (high resolution MS) analyzer and finally detected by an electron multiplier or Faraday cup detector (depending on analyte concentrations).

ICP-MS is generally regarded as a sensitive and selective multi-element detector with a wide linear range capable of providing low limits of detection [61]. However, spectroscopic and non-spectroscopic interferences can limit the utilization of this technique; an important case in point is the interference of $^{40}Ar^{16}O^{+}$ on $^{56}Fe^{+}$. By careful selection of operating conditions or sample modification most interferences can be minimized or reduced in magnitude.

Spectroscopic interferences include isobaric overlap of element isotopes and polyatomic ions which can be formed from water, plasma gases and from compounds present in the sample matrix. Such interferences can cause an erroneously large signal at the m/z of interest. Some polyatomic interferences for selenium and arsenic are shown in table 1-5.

Table 1-5. Spectroscopic interferences of arsenic and selenium in ICP-MS [69].

Molecular ion interference	*Analyte ion interfered with*	*Nominal m/z*
Cl_2^+, CaO_2^+	Se^+	74
Ar_2^+, CaO_2^+	Se^+	76
$ArCl^+$, CaO_2^+, Ar_2H^+	Se^+	77
Ar_2^+, CaO_2^+	Se^+	78
Ar_2^+, CaO_2^+, $ArCa^+$	Se^+	80
$Ar_2H_2^+$	Se^+	82
$ArCl^+$,CaO_2^+, Ar_2H^+	As^+ (monoisotopic)	75

Spectroscopic interferences can be overcome by employing a mixed gas plasma. Evans and Ebdon [70] have proposed that the reduction of $^{40}Ar^{35}Cl^{+}$ in the presence of N_2 can be explained by the competitive formation of ArN^{+}. Increasing the nebulizer flow rate can also reduce polyatomic interferences as there is less time spent in the plasma and therefore less time for the formation of these interferents. Other techniques to reduce spectroscopic interferences include matrix removal prior to analysis or the use of a high resolution MS.

Non-spectroscopic interferences refer to matrix-induced changes in signal intensity that are unrelated to the presence of a spectral component. They manifest themselves by signal suppression or enhancement although most commonly by suppression. These interferences are mainly attributable to space charge effects. Space charge effects arise from the mutual repulsion of ions in the ion beam, which influence ion trajectories.

The matrix effect is quite general in that almost any concomitant in high concentration will cause an effect. To reduce matrix effects the injector gas flow rate can be reduced in the ICP torch and ion lens modification can be utilized to enhance the throughput of certain mass ranges thereby avoiding interfering matrix elements. As with spectroscopic interferences matrix removal where possible is always beneficial. The use of internal standards, although they do not reduce or eliminate matrix effects, can be used to compensate for the changes that may occur.

1.4.3 Electrospray ionization mass spectrometry (ES-MS)

Electrospray ionization mass spectrometry has developed to become a widely used analytical technique since its introduction in the 1980's by both Yamashita and Fenn [71] and Aleksandrov *et al* [72]. Electrospray ionization is a technique that allows the transfer of ions from solution to the gas phase whereby they can be analyzed by mass spectrometry. The fundamental features of ES are that: i) it can produce extensively multiply charged ions enabling the analysis of large molecular weight compounds; ii) they are introduced in solution making ES compatible with many types of separation techniques: and iii) it is a 'soft' ionization technique retaining molecular structure. ESMS as a source for elemental speciation relies on its ability to preserve the formal oxidation state of metal ions and the molecular form of the species.

The ion formation process is the starting point for mass spectrometric detection. There are three steps involved in the production by electrospray of gas-phase ions from electrolyte ions in solution: i) production of charged droplets at the ES capillary tip; ii) shrinkage of the charged droplets by solvent evaporation; and iii) the production of gas-phase ions from the now small, highly charged droplets. The production of charged droplets at the capillary tip is achieved by the application of a high electric field to the flow of liquid. This is achieved by the application of a potential difference of approximately 3-6 kV between the capillary tip and the counter electrode located at approximately 0.3-2 cm away. When the capillary tip is in the positive mode positive electrolyte ions will drift

towards the liquid meniscus and negative ions will drift away from the surface, the charge separation being electrophoretic in nature. The mutual repulsion between the positive ions at the surface overcomes the surface tension of the liquid and the surface begins to expand. The liquid is drawn out into a cone known as the 'Taylor' cone. If the applied electric field is high enough a fine jet of charged droplets will emerge from the cone tip. The charged droplets produced then drift downfield towards the counter electrode. At this point the droplets undergo solvent evaporation without change in charge. The decrease of droplet radius at constant charge leads to an increase in the electrostatic repulsion of the charges at the surface until the droplets reach the Rayleigh stability limit, the condition at which the electrostatic repulsion becomes equal to the force due to surface tension which holds the droplet intact. Droplets undergo fission when they are close to the Rayleigh limit. This fragmentation is caused by Coulombic repulsion of the charges on the droplet. The energy required for solvent evaporation is provided by the thermal energy from the ambient air. These small, highly charged droplets ultimately provide gas phase ions. A schematic of this process is shown in Figure 1-7. However, currently there are two schools of thought as to how this process occurs; the ion evaporation model (IEM) proposed by Iribarne and Thompson [73] and the charge residue model (CRM) developed by Dole *et al* [74]. In brief, the CRM depends on the formation of extremely small droplets containing only one ion. Solvent evaporation from this droplet will give rise to a single gas phase ion. The IEM predicts that after the radii of the droplets decrease to a given size, direct emission from the droplets becomes possible and this process, ion evaporation, becomes dominant over coulomb fission for

droplets with radii > 10 nm. Much work has and is being carried out in this area of research but it is still not possible to say with any certainty which theory most closely matches the available evidence [77, 75, 76]. Although the emission of ions to the gas phase is highly endothermic and endoergic this process of evaporation is gentle enough not to fragment the parent molecule.

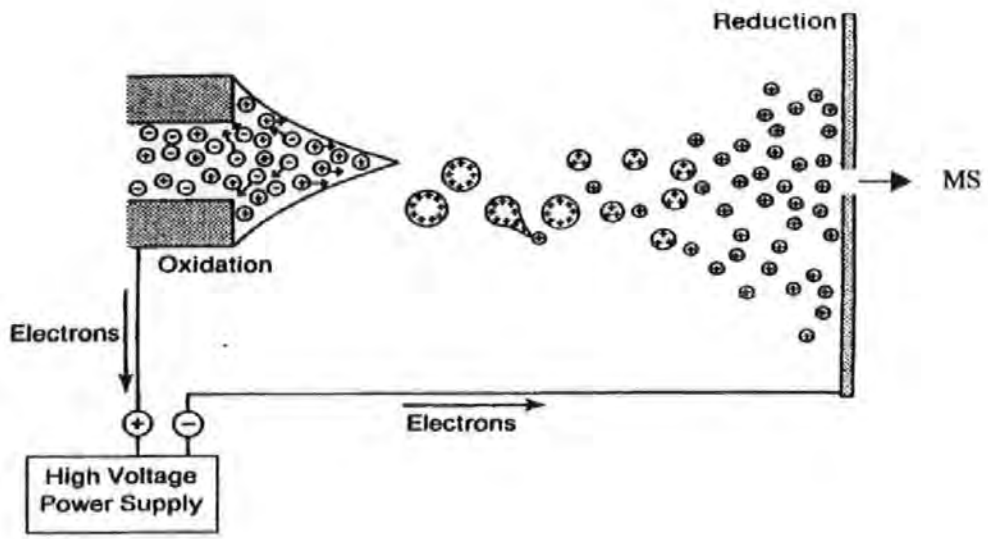


Figure 1-7 Schematic of major processes occurring in electrospray [77].

The gas phase ions produced during the electrospray process drift towards the counter electrode (e.g. the mass spectrometer sampling orifice) and enter the mass spectrometer where they are separated according to their mass/charge ratio under reduced pressure.

Limitations of electrospray as a technique for elemental speciation are mainly attributable to the physical processes responsible for the generation of the

electrospray and chemical considerations [78, 79]. A stable electrospray is only generated within a given conductivity range for a given solvent for a fixed set of operating conditions. Outside of this range droplet production, and hence ion current, becomes erratic. The use of electric fields for nebulization leads to some restraints on the solvents employed. Fluids with higher surface tensions require a higher threshold voltage for stable electrospray production and higher dielectric liquids produce higher currents. This can lead to the onset of electrical discharges from the capillary tip. The presence of an electrical discharge degrades the performance of ESMS particularly at high discharge currents. The ES ions are seen at much lower intensities than prior to the discharge and the corresponding discharge-generated ions have much higher intensities. It is likely that the discharge reduces the electric field near the capillary tip, which in turn interferes with the charged droplet formation. Ideally, the solvent flow rate, surface tension and electrolyte concentration will be low to avoid the use of higher electric fields for droplet formation and droplet charging. Amenable solvents for ESMS are volatile (aqueous NH_4HCO_3, MeOH, CH_3CN) and at a suitable pH for ion formation in solution: acidic for operation in the positive ion mode; and basic for operation in the negative ion mode.

Although ESMS is primarily a qualitative technique it can be used for quantitation but the linear relationship of analyte concentration to signal is limited due to the complex and competitive nature of ion production. When a matrix ion is present in excess, signal suppression can be severe.

1.5 The speciation of arsenic and selenium

Speciation analysis of elemental compounds and their oxidation states has become more frequent over the past decades as it is now known that toxicity and bioavailability, for example, of an element can be several orders of magnitude different depending on the chemical form [28]. One of the key issues surrounding speciation analysis is to preserve the integrity of the species in a sample. Many elements of interest are present in trace amounts and the species a fraction of this. This requires that analytical techniques and methodology are capable of sensitive and selective separation and detection.

1.5.1 The use of HPLC-ICP-MS for speciation analysis

ICP-MS has fast become the detector of choice for the determination of elements in a wide range of samples at concentrations in the ng l^{-1} to μg l^{-1} range. The versatility and reliability of the technique in terms of element specificity and sensitivity and the ease in which HPLC systems can be hyphenated to it make it ideally suited for use as a chromatographic detector. Due to its multi-element capability arsenic and selenium can be determined simultaneously with the correct chromatographic conditions. The advantages of ICP-MS as a detector include a wide linear dynamic range, low limits of detection and high speed of analysis. Liquid chromatography with ICP-MS detection is principally used for speciation analysis and a review of the literature reflects the versatility of the technique [80].

The molecular forms of arsenic commonly encountered in the environment are anions, e.g. arsenate, MMA and DMA, cations, eg. the quaternary arsonium compounds AsBet, AsC and tetramethyl-arsonium ion (TeMA) or uncharged compounds at neutral pH, e.g. arsenite and trimethylarsine oxide (TMAO). Arsenosugars present in marine algae are another commonly encountered group of arsenic-containing compounds. Their chromatographic behavior is dependent on the size of the molecule and the functional groups present in given solvent conditions. Similarly with selenium compounds, the inorganic selenate and selenite form anions at neutral pH whilst the amino acids SeMet, SeCys and selenocysteine are zwitterions. Because of this the most common types of liquid chromatography used for the speciation of arsenic and selenium include ion exchange (anion and cation), reversed phase and ion-pairing.

The simultaneous separation of 17 arsenic species has been achieved using HPLC-ICP-MS with an anion exchange column and a gradient mobile phase within 15 mins [81]. More commonly, fewer species are reported relying on the use of both anion and cation exchange chromatography for the separation of neutral, anionic and cationic arsenic compounds independently [82, 83, 84, 66, 85]. Cation and anion exchange chromatography has also been reported for the speciation of selenium compounds [86, 87]. Simultaneous use of the two forms of ion-exchange has allowed the separation of a mixture of 12 inorganic and organic selenium compounds [36]. The use of both types of ion exchange for the analysis of either arsenic or selenium (or both) in the same sample ensures the separation of compounds that would otherwise be eluted in the dead volume of a column.

Arsenic and selenium have some common chemical properties. The predominant inorganic forms appear as oxyacids and/or ions and in pH conditions that ionize organic forms of arsenic the organic forms of selenium are also ionized and therefore conditions can be applied for simultaneous separation [88, 89]. Simultaneous separation conditions often rely on gradient elution programs whereas isocratic mobile phases are the preferred choice as they provide greater stability [90]. Isocratic mobile phases may reduce analysis times, as no equilibration step is required between analyses although late eluting compounds in isocratic conditions will hinder the overall times.

Many of the ion exchange column packing materials are based on the co-polymerization of styrene with divinylbenzene to produce degrees of cross-linking with the Hamilton PRP X100 (strong anion exchange) being the most frequently employed. These columns are resistant to a pH range of 1-13 as opposed to silica based columns that dissolve at a pH above 8. This allows for a greater variety of eluents to be employed when establishing optimum separation conditions. High salt eluents such as sulphates and phosphates are very good at providing ion-exchange chromatographic separation. However, when coupling HPLC to ICP-MS several precautions must be taken. The salt content in the mobile phase needs to be maintained at less than 2% to reduce the risk of blocking the nebulizer and eroding the sampler and skimmer cones in the MS detector. Ion exchange chromatography generally utilizes low concentrations of buffer, which help to reduce these problems and of those related to matrix

interference effects. Although much work is based on salt buffers, nitric acid has been used successfully with less clogging of the sampler cone being reported [91].

Reversed-phase and ion-pairing reversed-phase chromatographic methods have been applied successfully to the separation of arsenic and selenium compounds. Two reviews, the first by Sutton and Caruso [92] and the second by Guerin et *al.* [80] discuss the merits of these techniques with a comprehensive reference list of speciation methods employed. However, work by Larsen [36] suggests that full optimization of ion-exchange techniques provide superior results to those obtained by reversed-phase techniques. This appears to be due to the direct interaction of analyte ions with the stationary phase of the chromatographic column making the analyte ion less prone to interferences from co-eluting matrix constituents and due to the lower amounts, if any at all, of organic solvents used in the mobile phase which can de-stabilize the ICP-MS system. Gilon et *al.* [93] tested ion- pairing with ion-exchange chromatography for the separation of selenium and also found ion-exchange to be the superior mode of analysis.

Modifications to the chromatographic and ICP-MS system have been reported that can improve the sensitivity of detection. Matrix removal and pre-concentration of samples by solid phase extraction (SPE) techniques have been applied to the speciation of arsenic [94, 95] and selenium [96, 97] compounds. However, these techniques are often limited in that only cationic or anionic species can be observed at any one time due to the nature of the SPE cartridge. Pre-concentration does not directly affect sensitivity or limits of detection but is

frequently used to improve the analyte signal. On-column pre-concentration work with selenium speciation [98] has demonstrated a signal improvement by 1-2 orders of magnitude and techniques for arsenic have reported similar efficiencies [94].

Modifications to the ICP-MS instrumentation have included the use of various types of nebulizers, spray chambers and the introduction of alternative gases to the plasma. Crossflow nebulizers are most frequently used for samples that contain a heavy matrix or have small amounts of undissolved matter. Although they are much less likely to block, they are generally not as efficient as concentric nebulizers at creating a fine aerosol of droplets required for ICP-MS. Low-flow nebulizers are also available for work with micro-bore HPLC columns where eluent flow rates are typically at 0.2 ml min^{-1}. Two designs of spray chamber are used in commercial ICP-MS instrumentation - the double-pass and cyclonic. Research carried out into the performance of spray chambers [99] demonstrates that the transport efficiency and washout times for the cyclonic spray chambers are much more effective thereby improving signal:noise ratios and the overall performance of analyte detection. Cooling jackets around the spray chamber can also improve performance by reducing the solvent load to the plasma, particularly where organic solvents are employed in the mobile phase. The introduction of N_2 into the plasma [70] is a well-documented technique for reducing polyatomic interferences in the analysis of arsenic. The polyatomic ion $^{40}Ar^{35}Cl^+$ is a particular problem as arsenic is monoistopic at mass 75. The addition of N_2 has

been effective for samples containing in excess of 1% chloride. Without modification results for arsenic can be up to 30% higher [100].

HPLC with ICP-MS as a detector is now well established with an extensive number of publications supporting its popularity. Much work has been directed towards the separation of a greater numbers of species per analysis [101, 102] with high-speed separation [103] and improving on limits of detection. For arsenic speciation detection limits as low as 0.005 ng as arsenic have been reported [104, 105]. For selenium speciation limits in the region of 0.8 ng as selenium are reported [93]. Simultaneous separations give similar limits which implies there is no deterioration in analytical capability between individual determinations and when performed concurrently [106, 107]. The choice of system depends primarily on the research objective. There are a number of excellent reviews published that cover this area of research [80, 108, 109] providing an overview of the many systems used and results obtained.

Although HPLC is undeniably an excellent separation technique it relies on the identification of species by matching retention times with that of known standards. However, the complexity of samples may lead to errors in identification due to co-elution of species, matrix constituents altering retention times and the lack of commercially available standards for peak identification. This has led analytical science towards the use of multi-dimensional approaches for the speciation of compounds. As previously stated, anion and cation exchange chromatographic techniques have been employed simultaneously for the

determination of arsenic and selenium species. Size exclusion chromatography has also been used together with ion-exchange and reversed phase chromatography allowing unambiguous peak assignments to be made where standards are available [110, 111]. This is brought about by matrix simplification and by distinction of compounds against standards according to their elution times that remain independent of the type of chromatography used. This type of approach is usually successful for speciation of elemental compounds but is still limited by the availability of standards. When performing complex biochemical speciation analysis standards are usually unavailable since the majority of species remain unidentified and, as in the case of arsenosugars, compounds with similar chemical structures may persistently co-elute despite the use of multi-dimensional chromatographic techniques. The lack of convenient methods for structural determination and confirmation remains a major barrier to the mechanistic understanding of function. Mass spectroscopic analyses that retain molecular information of the species have become powerful tools for the identification of compounds particularly when used in conjunction with HPLC-ICP-MS instrumentation.

1.5.2 The use of HPLC-ESMS in speciation analysis

ESMS is based on a 'soft' ionization approach of the sample components and, therefore, can retain the molecular structure of a compound. By studying spectra obtained from samples it is possible to deduce molecular masses and, by fragmentation patterns of the parent molecule, their molecular structure. In 1996

Corr and Larsen [112] obtained spectra of four arsenosugars by HPLC coupled with ESMS demonstrating the possibility of structural characterization for these species. Since this time, an increasing amount of work has been carried out using HPLC-ESMS with successful results in verifying compound structures and in the characterization of previously unidentified species [57, 113]. However, some of the limitations in ESMS result from the matrix components swamping the signal from the analytes of interest and subsequently affecting the detection limit. This has resulted in the use of multi-dimensional chromatographic techniques for matrix removal and sample pre-concentration by fraction collection to obtain the best results. McSheehy et *al.* [110] employed three-dimensional chromatography (size exclusion, anion exchange and cation exchange) and was able to confirm the presence of four previously unidentified arsenic compounds in the kidney of the clam *Tridacna derasa*. Similar success has been reported for selenium speciation [114, 115] again with the use of multi-dimensional chromatography. HPLC-ESMS has also been used as a complementary technique to that of HPLC-ICP-MS [116]. HPLC-ICP-MS provides information of where arsenic-containing species elute and then by HPLC-ESMS their molecular mass and structure can be confirmed.

ESMS precludes the use of crude extracts as it can suffer from matrix suppression of the analyte signal. Another drawback is that the mass spectra obtained can be highly complex and the possibility of matrix constituents having coincident masses to that of the analytes under investigation must also be considered. However, the use of chromatographic techniques together with ESMS provides

an attractive alternative to that of the so far exclusively used NMR, which requires labour-intensive isolation and purification techniques [117].

ESMS allows an accurate determination of the molecular mass of a compound with structural characterization provided by tandem MS techniques. It is becoming a valuable tool for the identification and characterization of species in real samples detected by HPLC-ICP-MS where lack of available standards or co-elution of species may cause ambiguity in peak assignment. However, at the present stage of development the sensitivity of ESMS is approximately 2-3 orders of magnitude higher than that of ICP-MS. Improvements in purification of sample extracts and choice of eluents compatible with ESMS is required.

The paucity of ESMS applications in the literature to environmentally relevant samples may be as a direct impact of its poor sensitivity in comparison to techniques such as ICP-MS. However, the application of ICP-MS, with its element selectivity and sensitivity, and ESMS, with its molecular specificity, as complementary to one another has proved a powerful analytical tool with the subsequent detection and characterization of previously unidentified compounds [110].

Further research and development to provide methods that are HPLC-ESMS/ICP-MS compatible and easy to use is required in promoting elemental speciation analysis of real environmentally complex samples.

1.6 Aims of the study

Research in the field of elemental speciation has utilized instrumental techniques where elegant separation techniques can be coupled with sophisticated detectors according to requirements. When investigating samples for toxicological purposes, in-depth speciation analysis may be unnecessarily time-consuming and expensive. For initial environmental pollution screening purposes the separation of relatively toxic from relatively non-toxic species may be all that is required. Should high levels of toxic species be encountered then further more rigorous analytical procedures can be employed. The aims of this work are to develop a simple, yet effective, system for the separation of relatively toxic from relatively non-toxic arsenic and selenium species. This may be achieved by the development of methodology based on low-pressure chromatographic systems for separation coupled with ICP-MS detection. By selection of suitable chromatographic conditions, which utilize the pKa values of the various species under investigation, optimum conditions may be achieved to target the level of analytical performance required. The use of ICP-MS instrumentation, which offers multi-element 'simultaneous' capabilities of high sensitivity should extend the analytical performance of the chromatographic systems, developed.

Analytical data on individual molecular species of an element present in a sample can provide fundamental information regarding its toxicity, bioavailability, metabolism or biogeochemical cycling. However, for quality control and quality assurance purposes all analytical measurements must be comparable and

traceable. The basic purpose of certified reference materials (CRMs) is to improve the comparability of results [118]. They can be used at two different stages of the analytical process: as a tool to demonstrate traceability; and as a tool for method validation. One of the main obstacles in the progress of speciation analysis is the paucity of reference materials that are certified for species as well as for total elemental concentrations. The aims of the following work include that of using conventional and novel analytical approaches for the determination of arsenic and selenium species present in a variety of environmentally important samples for future inclusion as new certified reference materials. This will be achieved by participation in two European collaborative feasibility and pre-certification trials.

Further aims of this work are to use HPLC-ICP-MS together with ESMS to develop and improve methodologies available for speciation analysis. Initial studies will be carried out using HPLC-ICP-MS with multi-dimensional chromatography employed where appropriate. ESMS will be used as a harmonizing technique for species identification and confirmation, particularly in cases where commercial standards are unavailable for matching peak retention times. Development of an HPLC system that is compatible with ICP-MS and ESMS will be undertaken so that comparisons between the two techniques can be made.

When performing speciation analysis it is of vital importance that species are extracted from the matrix unchanged. Investigation of extraction procedures to broaden techniques available for arsenic and selenium speciation will be pursued.

Chapter two

2 Development of a novel LC-ICP-MS system for the simultaneous separation and determination of arsenic and selenium species based upon toxicity

2.1 Introduction

The elemental speciation of arsenic and selenium has become an area subject to increasing attention due to their toxicological and biological significance in living organisms. A range of toxicities is exhibited for both elements dependent on the chemical form. Inorganic forms of arsenic exhibit high toxicities with As^{III} being the most toxic and a suspected human carcinogen. Organic species such as arsenobetaine are considered to be essentially non-toxic. Current permitted or allowable levels for arsenic in drinking water stand at 50 $\mu g\ l^{-1}$ (EU and USA) and 10 $\mu g\ l^{-1}$ (WHO) and levels in foodstuffs stand at 1 $mg\ kg^{-1}$ total arsenic, under review as inorganic arsenic [119].

Selenium is an essential trace element in the human diet possessing a narrow therapeutic range; too low an intake can lead to various deficiency syndromes whereas too high an intake can be toxic or even lethal [120]. Its nutritional bioavailability, toxicity and anti-carcinogenic properties have been found to be species dependent. It is thought that inorganic selenium, particularly as selenite, is the most likely form to induce toxic symptoms [41]. The UK Reference Nutrient Intake has been given as 75 μg daily for men and as 60 μg daily for women [2].

Symptoms of toxicity are thought to occur at an intake of approximately 1000 μg daily. However, in susceptible individuals this level may be as low as 600 μg [121].

Determination of distinct chemical species, often referred to as speciation analysis, of arsenic and selenium in a variety of foodstuffs has provided essential information for elucidation of, for example, absorption, bioavailability, metabolic pathways of the compounds under investigation and the nature of their toxicities [10]. Research in this area has utilized instrumental techniques that include HPLC for separation purposes coupled with element-specific detectors such as ICP-MS [101, 103], atomic absorption spectrometry (AAS) [122, 123], atomic emission spectrometry (AES) [124, 125] and atomic fluorescence spectrometry (AFS) [126, 127]. However, when investigating samples for toxicological purposes, in-depth speciation analysis may be unnecessarily time-consuming and expensive. For screening purposes an estimate of the toxicology of a sample may suffice, i.e. separation of inorganic from organic species of arsenic and selenium, whereby an overall simplification of the instrumentation and methodology for the analysis can be implemented. A protocol showing the progression through a speciation analysis is shown in Figure 2-1.

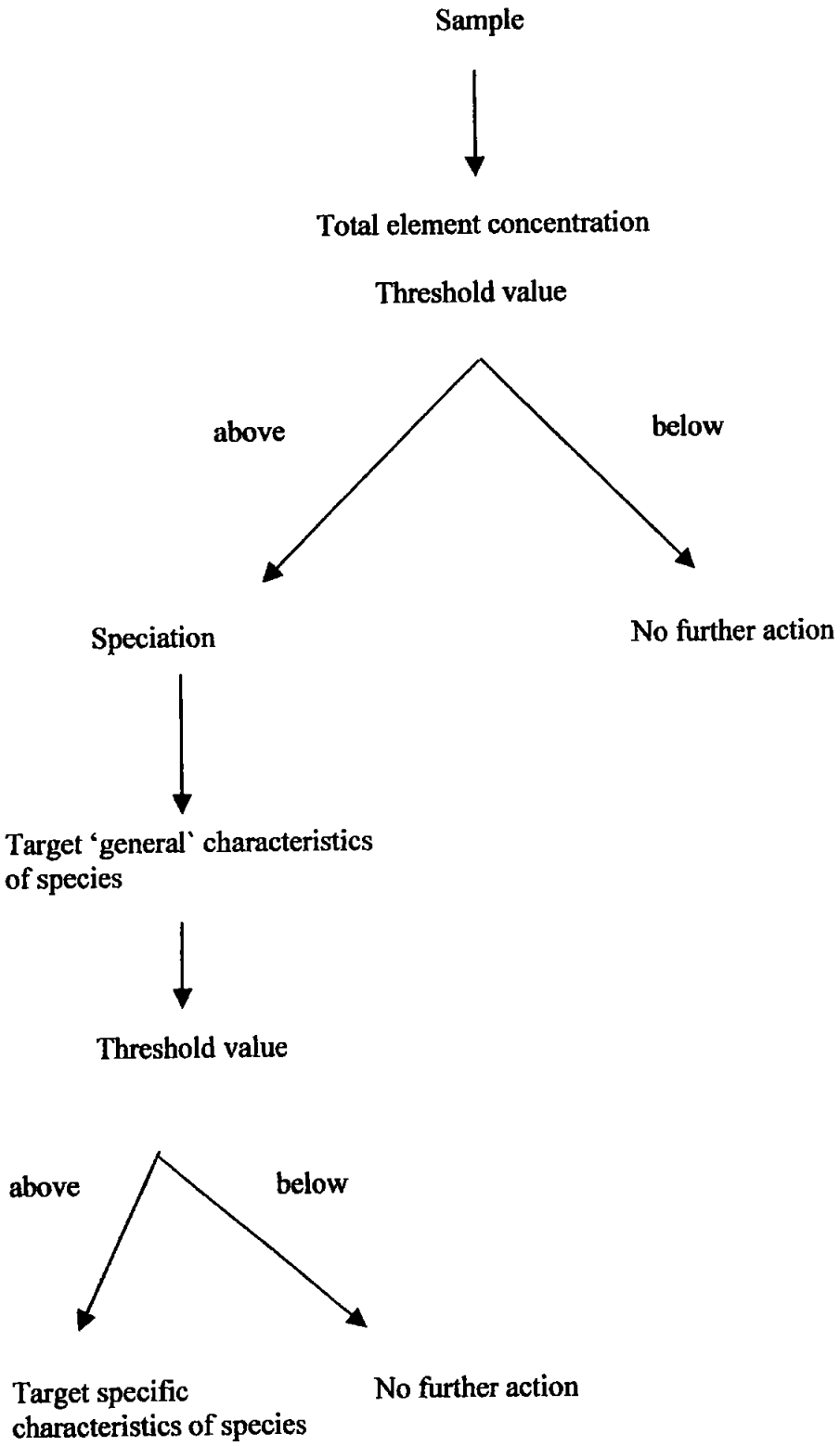


Figure 2-1 Protocol showing progress through a speciation analysis, which demonstrates inherent screening steps

The aim of this study was to investigate and develop a simple, yet effective, system for the simultaneous separation and detection of inorganic (high toxicity) from organic (relatively lower toxicity) arsenic and selenium species present in a variety of environmentally and biologically important samples. The samples chosen for investigation were a variety of food types known to contain high levels of arsenic (fish) and selenium (selenized yeast dietary supplement) which have obvious health implications in the human diet. In terms of toxicity the LD_{50} values for the species under investigation in this work are shown in Table 2-1.

Table 2-1 LD_{50} values for arsenic [28] and selenium [128, 41] species under investigation

Species	*LD_{50} (rat) oral dose – mg kg^{-1} body weight*
Arsenite	1.5
Arsenate	5.0
MMA	50
DMA	600
Selenite	7
Selenate	53
SeMet*	< toxic than selenite
SeCys*	≡ to selenite

* variable data, therefore given in relation to selenite [41].

2.2 Experimental

2.2.1 Instrumentation

ICP-MS measurements were performed using a VG Plasmaquad, 2+ (TJA Solutions, Winsford, Cheshire, UK), using the operating conditions described in Table 2-2. A Perkin Elmer series 410 high-pressure pump (Perkin Elmer, CT, USA) and a Gilson peristaltic pump were used for control of chromatographic eluent flow rates. A Rheodyne 7152 injection valve (Rheodyne, Cotati, CA, USA) was used for column loading. pH readings were taken using a 3010 pH meter (Jenway, Ltd., Essex, UK).

Arsenic was measured using m/z 75. 4% (v/v) N_2, for the reduction of $ArCl^+$ interferences on m/z 75, was added via a Signal series 850 gas blender (Signal, Camberley, Surrey) to the nebulizer gas flow.

Selenium was measured using isotpes at m/z 77, 78 and 82.

Table 2-2 ICP-MS operating conditions used for the determination of arsenic and selenium in all samples.

ICP-MS	Plasma Quad 2+	
	V groove nebulizer	
	Double-pass, water cooled Scott type spray chamber	
	Fassel torch – 1.5 mm bore injector	
Parameters	Argon plasma – 4% N_2 added for direct total As determination to the nebulizer gas flow	
	Nebulizer flow rate	0.81 l min^{-1}
	Coolant gas flow rate	13.1 l min^{-1}
	Auxiliary gas flow rate	0.8 l min^{-1}
	Forward power	1350 W
	Dwell time	500 ms

Two types of anion-exchange mini-column (Benson AX10, 7 – 10 μm, and Hamilton PRP X100, 12 – 20 μm, both polystyrene divinylbenzene-based resins with quaternary ammonium functional groups) were prepared. A wet slurry eluent technique was used to pack the different columns at low pressure. The experimental parameters under consideration are given in Table 2-3 and conditions were optimized for both resin type mini-columns.

Table 2-3 Range of experimental chromatographic conditions used for the separation of inorganic from organic arsenic and selenium species.

Parameters	*Experimental conditions*
Column dimensions	25, 50, 100 x 3 mm
Column packing materials	Benson AX10, 7-10 μm
	Hamilton PRP X100, 12-20 μm
Eluent flow rate	0.75 – 1.4 ml min^{-1}
Sample loop	100 – 250 μl volume
pH	4 – 10.2
Competitive counter ion	Sulphate, phthalate, phosphate
Concentration of eluents	Sulphate - 0.1, 1.0, 2.5, 5.0 and 10 mM
	Phthalate – 0.0, 0.1, 50, 100 mM
	Phosphate – 5.0, 7.5, 10 mM

2.2.2 Chemicals and Reagents

All commercial chemicals were of analytical grade and used without further purification. Sodium selenate, sodium selenite, selenomethionine, arsenous acid, arsenic acid, dimethylarsinic acid (Sigma-Aldrich Chem. Co., Poole, Dorset, UK) and arsenobetaine (BCR, Retieseweg, Belgium) were used as stock solutions of 1000 μg ml^{-1} in terms of the element. They were stored in the dark at 4°C. Solutions of the compounds for daily use were prepared by appropriate dilution from the stock solutions using Milli-Q water (Milli-pore, Bedford, MA, USA).

Eluent solutions of potassium sulphate (Sigma-Aldrich), potassium hydrogen phthalate and di-ammonium hydrogen orthophosphate (Merck, Poole, Dorset, UK) were prepared as required using Milli-Q water and the pH adjusted using a solution of 0.91 sp. gr. NH_3 (Merck). Bovine trypsin and type XIV protease (Sigma-Aldrich) were used for enzymolysis digestion of samples. Hydrogen peroxide, 37% v/v (Sigma Aldrich), stored in the dark at 4°C, and nitric acid, 69% v/v, (Primer, Fisons, Loughborough, UK) were used for microwave digestion procedures.

2.2.3 Reference materials and samples

The certified reference materials, DORM-2 (Dogfish muscle) and TORT-2 (Lobster hepatopancreas) (National Research Council, Ottowa, Canada) were used to validate the methods. Oyster samples were obtained from a European inter-laboratory pre-certification trial. 'Selenoprecise'® tablets are available commercially (Pharma Nord, Vejle, Denmark). Samples of plaice, intended for domestic consumption, were obtained locally. They were dried at –60°C and 1 x 10^{-2} Torr in a freeze drier (Edwards Super Modulyo, Edwards High Vacuum, Crawley, Sussex) and ground to fine powder using an Optiblend 2000 electrical blender (Moulinex, France) prior to use.

2.2.4 Sample digestion procedures

In the determination of total element concentrations it is necessary to utilize a sample decomposition technique that will ensure that the analyte of interest

remains in solution, is stable and any chemicals used do not cause instrumental interferences that may increase the limits of detection, particularly in cases where trace or ultra-trace elemental levels are expected. The practice of microwave digestion has been comprehensively reviewed [129] and in the dissolution of biological matrices, it has been shown that the three primary components of carbohydrates, proteins and lipids completely decompose in nitric acid ($\geq$ 2 M) at temperatures of between 145 - 165^{O}C [130]. Nitric acid (69% - azeotropic) has a boiling point of 122^{O}C and in order to adjust the oxidizing potential of HNO_3, by means of elevating the temperature, closed vessel microwave conditions are used [130]. The overall decomposition process is further assisted by the addition of hydrogen peroxide as the oxidizing power of HNO_3 increases with higher acidity. Once complete digestion has been obtained, the elements of interest remain in solution and can be determined by the chosen method of detection.

Acidic microwave digestions can destroy speciation information. Where speciation analysis is to be performed digestion procedures that retain the chemical form of the compound must be employed. The choice of a suitable enzyme for the sample matrix where the cell contents can be released into solution unchanged is one way in which this can be achieved. Enzymatic digestions are widely reported in the literature with effective extraction of the species under consideration [65, 131, 132]. Optimum conditions of pH and temperature must be employed, as enzyme activity is sensitive to these parameters.

- **HNO_3 digestion for the measurement of 'total' arsenic and selenium**

Microwave bombs (Savillex, Minetonka, Minnesota, USA) were cleaned with 3 ml HNO_3 (69%, v/v) in a Perfecto 800 W microwave oven (DeLonghi, Italy) on medium power for 2 mins. Samples of approximately 0.25 g were accurately weighed into the bombs and 4 ml HNO_3 (69%, v/v) and 1 ml H_2O_2 (37%, v/v) were added. The bombs were loosely capped and left overnight to allow easily oxidised material to be destroyed. After pre-digestion, the bombs were sealed tightly and microwaved on medium power for 1 - 2 mins, or until the sample was a clear colour (indicating a completed digest). The samples were transferred quantitatively into volumetric flasks and made up to volume with deionized water. The samples and standards were spiked to give a final concentration of 100 $\mu g\ l^{-1}$ Indium (In) which acted as an internal standard prior to analysis by ICP-MS using the conditions previously described. The internal standard was used to correct for instrumental drift (sample viscosity effects, mass transport, etc.) over the analysis period

- **Extraction procedure for the speciation of materials**

Freeze-dried samples (plaice, oyster, DORM-2 and TORT-2) of approximately 0.25 g of tissue were accurately weighed together with 0.025 g trypsin (Sigma-Aldrich, Dorset, UK) and approximately 20 ml NH_4HCO_3 (0.1 M, pH 8) [132]. The solutions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. The samples were centrifuged at 2500 rpm for 40 min, the

supernatant transferred quantitatively to 25 ml volumetric flasks and made up to volume with the NH_4HCO_3 buffer. In this case, samples and standards were spiked to give a final concentration of 100 μg l^{-1} caesium (Cs) that acted as an internal standard prior to analysis. Due to the change in pH conditions Cs was used in preference to In as In is not very soluble at pH 8 and can precipitate out of solution onto surfaces of the container or any particles present thereby being lost to the analysis.

The Selenoprecise® yeast samples were prepared by accurately weighing approximately 0.25 g of the pre-ground material together with 0.025 g of protease (Type XIV) and approximately 20 ml NH_4HCO_3 (0.1 M, pH 8) [133]. The solutions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. The samples were centrifuged at 2500 rpm for 40 min, the supernatant transferred quantitatively to volumetric flasks and made up to volume with the NH_4HCO_3 buffer. Samples and standards were spiked to give a final concentration of 100 μg l^{-1} caesium (Cs) that acted as an internal standard prior to analysis.

2.3 Results and Discussion

2.3.1 Choice of chromatographic conditions

To achieve the separation of highly toxic from less toxic arsenic and selenium species in the chosen samples a number of experimental parameters were manipulated until optimum conditions were attained. The species targeted were inorganic As^{III}, As^{V}, Se^{IV} and Se^{VI} which are considered to be the most toxic and the organic forms of arsenobetaine (AsBet), dimethylarsinic acid (DMA) and selenomethionine (SeMet) which have a much lower toxicity. These species, being weak acids or zwitterions, for which the structure of AsBet is shown in Figure 2-2, appear in a number of ionic forms dependent upon the pH and redox conditions used. The pK_a values for these species, shown in Table 2-4, were used to provide a basis for the work.

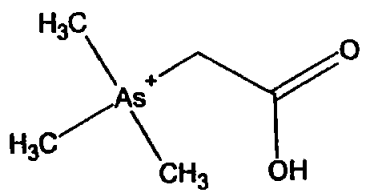


Figure 2-2 Structure of AsBet [14]

Table 2-4 pK_a values for arsenic [134] and selenium [135] compounds

Compound	*pK_a*	*Species present*
Arsenite	9.23	$HAsO_2 \rightarrow AsO_2^-$
Arsenate	2.20, 6.67, 11.53	$H_3AsO_4 \rightarrow AsO_4^{3-}$
AsBet	2.18	$(CH_3)_3As^+CH_2COOH$
DMA	1.28, 6.2	$(CH_3)_2As(O)OH$
Selenite	2.46, 7.31	$H_2SeO_3 \rightarrow SeO_3^{2-}$
Selenate	1.92	$HSeO_4^- \rightarrow SeO_4^{2-}$
SeMet	2.6, 8.9	$CH_3SeCH_2CH_2CH(COOH)NH_2$

The degree of ionization of the species is pH dependent. At pH 10.2 the arsenate present will be in the form of $HAsO_4^{2-}$ whereas at pH 5 the predominant species is $H_2AsO_4^-$. A diagram of As^V as a function of pH is shown in Figure 2-3. At pH 10.2 AsBet, being a quaternary arsonium compound, exists as a zwitterion. DMA and As^{III} species are similarly dependent on the pH of solution.

Ionization of selenium compounds is also pH dependent and a diagram of Se^{IV} as a function of this is shown in Figure 2-4. Both the Se^{IV} and the Se^{VI} have doubly negative charges at pH 10.2, existing as the anions SeO_3^{2-} and SeO_4^{2-}, respectively. SeMet, a zwitterion at neutral pH, will possess a single negative charge in alkaline conditions.

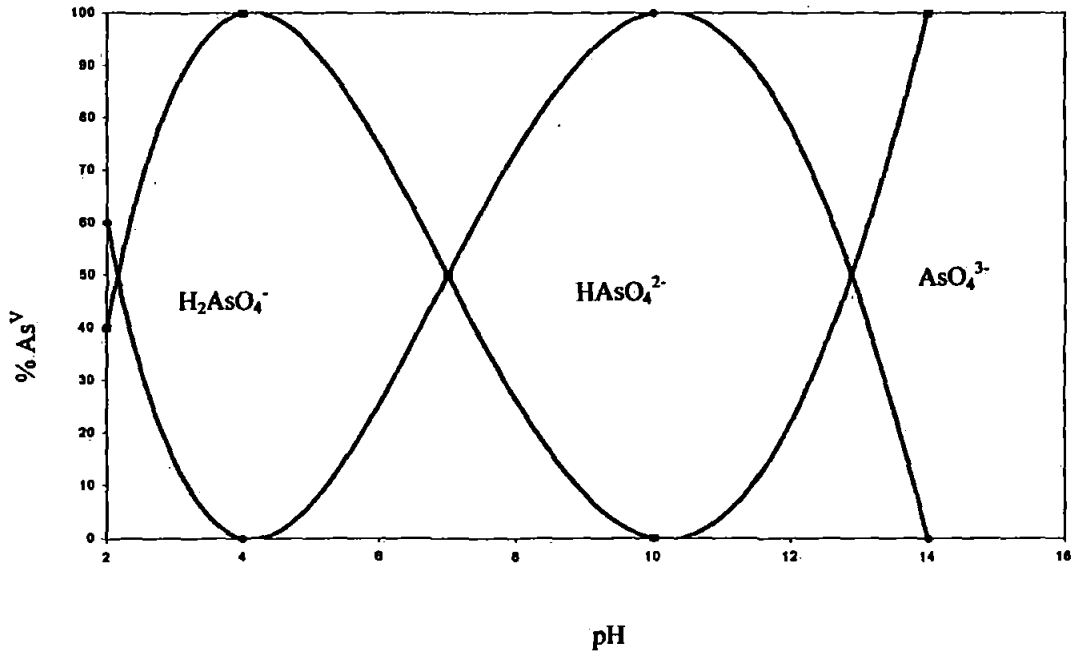


Figure 2-3 Predominance diagram of As^V as a function of pH [3]

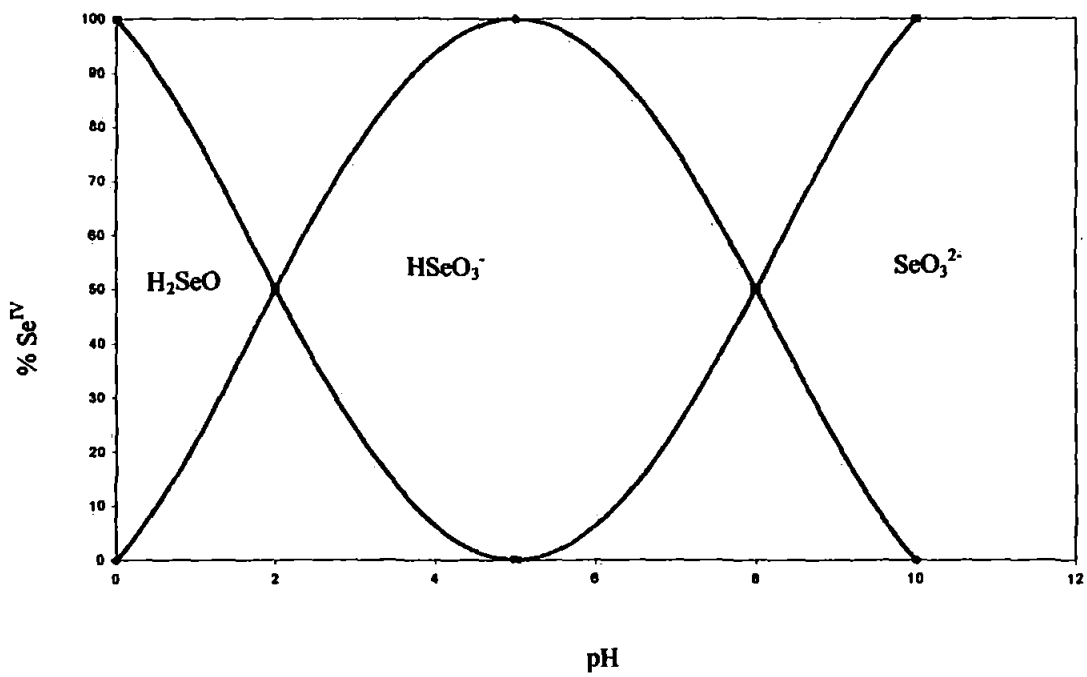


Figure 2-4 Predominance diagram of Se^{IV} as a function of pH [135]

The ionic character of the various arsenic and selenium species under particular pH and oxidation conditions determined that greater flexibility and suitable separation would be obtained using anion-exchange chromatography and this was employed throughout. The parameters considered were: column length; type and particle size of anion exchange resin; eluent flow rate; pH of mobile phase; concentration of mobile phase; and type of competitive counter ion. The range of experimental conditions used for each of the parameters is shown in Table 2-3.

The chromatogram shown in Figure 2-5 demonstrates the elution patterns of the four arsenic species under consideration using the optimum conditions derived experimentally for the Hamilton PRP X100 column. It demonstrates the co-elution of the organic species followed by the co-elution of the inorganic species under investigation. Optimum conditions were a column of 100 x 3 mm dimensions, with a 10 mM K_2SO_4, pH 10.2 and a mobile phase at a flow rate of 1 ml min^{-1} with a sample injection loop volume of 100 µl. The larger particle size of the Hamilton PRP X100 resin (12-20 µm) as opposed to the Benson AX10 resin (7-10 µm) reduced the back-pressure experienced allowing the use of a simple peristaltic pump for control of the mobile phase flow-rate.

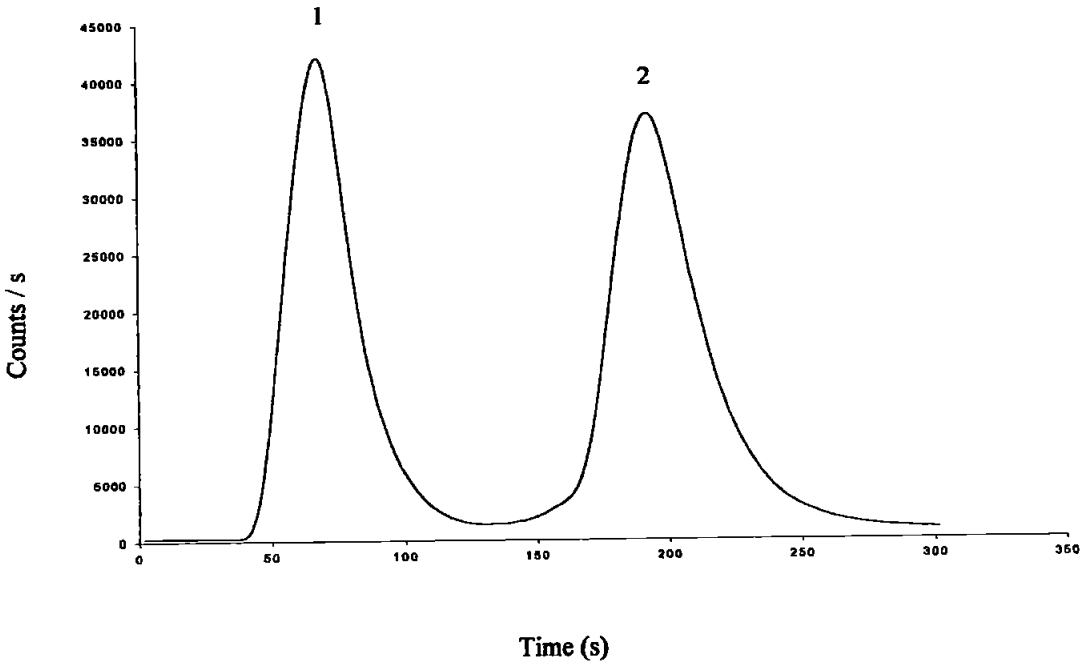


Figure 2-5 Chromatogram of 4 arsenic standards (250 μg l^{-1}) obtained using a Hamilton PRP X100 column, 100 x 3 mm dimensions, with a 10 mM K_2SO_4, pH 10.2, mobile phase demonstrating the separation of organic from inorganic species. Peak 1, AsBet and DMA; Peak 2, As^{III} (oxidized to As^{V}) and As^{V}.

The co-elution of the organic species followed by the co-elution of the inorganic species under investigation was expected when taking into account theoretical considerations of pK_a values, pH used and column chromatography. The degree of ionization of the species is pH dependent. At pH 10.2 the arsenate present will be in the form of $HAsO_4^{2-}$. This species has a doubly negative charge and thus a greater affinity for the anionic stationary phase than the other arsenic species under investigation and would be expected to elute last. At pH 10.2 AsBet, being a quaternary arsonium compound, exists as a zwitterion and elutes with the solvent front. DMA and arsenite, the latter being present in the form AsO_2^-, at this pH will exist as singly charged anionic species and will also elute early due to limited interaction with the stationary phase. Due to the shorter column length

and larger particle size than would be used for HPLC there are fewer theoretical plates and, therefore, less ability of the column to separate and resolve species having similar chemical properties. By experiment it was found that only arsenate, possessing two negative charges at pH 10.2, had any affinity for the stationary phase and that all the other species co-eluted. The intention of this work was to separate organic arsenic from inorganic arsenic species. To overcome the co-elution of As^{III} with the organic species, As^{III} was oxidized to As^{V}. This was possible by the addition of 37% v/v H_2O_2 (0.25 ml) to 25 ml of sample (ratio of 1:100 H_2O_2: sample). The standard potential of the reduction half-reaction of $H_2O_2 \leftrightarrow H_2O$ is more positive than that of $As^{V} \leftrightarrow As^{III}$. The stability of the other arsenic and selenium species under investigation was studied with the addition of 37% v/v H_2O_2. It was experimentally determined that the addition of H_2O_2 had no adverse effect since there were no conversions of the other concomitant arsenic and selenium species.

Investigation of alternative column dimensions indicated that smaller column dimensions gave rise to poor resolution between peaks as would be expected from fewer theoretical plates being present [136]. Higher and lower flow rates than the optimum gave poor peak shapes due to the effects of longitudinal diffusion and mass-transfer effects.

The use of phosphate as a mobile phase eluent is often reported by workers [137, 138] for the separation of arsenic species by HPLC, with pH conditions frequently being within the range of pH 5.75 to 6.5. Investigation of di-ammonium hydrogen

phosphate providing the competitive counter ion in the mobile phase for this study, although providing the necessary resolution, appeared to be fairly unstable with shifting peak retention times. This may be accounted for by greater equilibrium effects for HPO_4^{2-} at pH 10.2 than for the SO_4^{2-} ion, which was fully ionized under these pH conditions. The retention times were also longer suggesting that 10 mM phosphate provides a weaker counter ion than does 10 mM sulphate. To negate the lengthened retention times a higher concentration would be required. An increase in ionic strength is known to have a deleterious effect on the ionization processes in the plasma and the mass spectrometric detection of ions [139]. It was therefore, decided to proceed no further with the investigation of phosphate as a mobile phase counter ion.

Experimental conditions to elute the species in a similar way to that of the Hamilton column were also found when using the Benson resin. However, a step gradient elution of 0 M potassium hydrogen phthalate (Milli-Q water), pH 7 and 0.1 M potassium hydrogen phthalate, pH 4 (2 mins then 5 mins, respectively), at an eluent flow rate of 1.4 ml min^{-1} was required with a column length of 25 x 3 mm. A range of pH values for the mobile phase were analyzed. It was shown that the higher concentration of the competitive ion in the mobile phase solution was required to elute the inorganic arsenic anions. By lowering the pH the number of charges associated with each species would have also been reduced (see pK_a values – Table 2-4) giving them less affinity for the stationary phase. Mobile phase solutions of sulphate and phosphate also required a step gradient. However, 0.1 M potassium hydrogen phthalate is naturally at pH 4 and it was therefore

decided that this solution was the most convenient as no adjustment of pH was required. The optimum conditions required could be accounted for by the smaller particle size and greater surface area of the resin and it possessing a higher capacity (1.5 meq ml^{-1}) than that of the Hamilton resin (0.19 meq ml^{-1}).

The Benson AX 10 system was considered to be less practicable due to the step gradient requirement, in concentration as well as pH, which necessitated a re-equilibration stage, thereby lengthening the analysis time. Calibration results with standards also showed greater standard deviation than those obtained with the Hamilton column. Further work was performed using the optimum conditions derived from the Hamilton PRP X100 column system only.

During investigations for experimental conditions to achieve the required separation of organic from inorganic arsenic species, the simultaneous separation of selenium species in a similar manner was also considered. Using the conditions applied for the separation of arsenic species (using either the Hamilton PRP X100 column with 10 mM potassium sulphate, pH 10.2 or the Benson AX 10 column with the step gradient elution of 0 M potassium hydrogen phthalate, pH 7 to 0.1 M potassium hydrogen phthalate, pH 4, as the mobile phase) it was possible to separate the three selenium species under investigation. A chromatogram of this separation is shown in Figure 2-6.

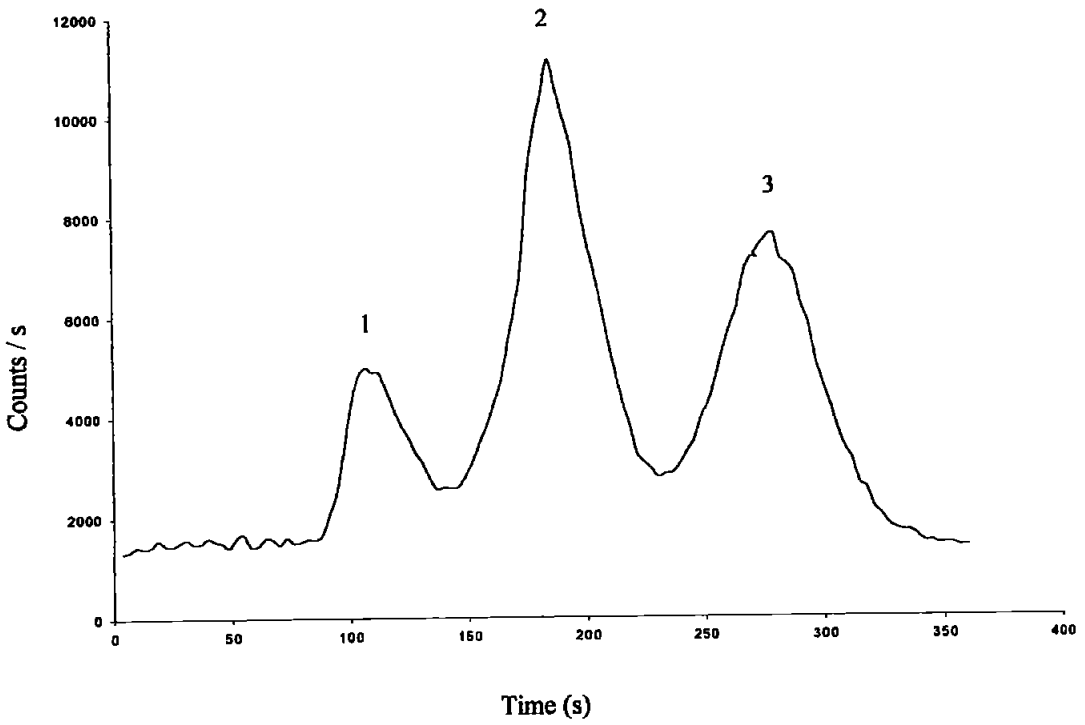


Figure 2-6 Chromatogram of three selenium standards (250 μg l^{-1}) obtained using a Hamilton PRP X100 column, 100 x 3 mm dimensions, with a 10 mM K_2SO_4 mobile phase at pH 10.2 demonstrating the separation of all species. Peak 1, SeMet; Peak 2, Se^{IV}; Peak 3, Se^{VI}.

Both the Se^{IV} and the Se^{VI} have doubly negative charges at pH 10.2, existing as the anions SeO_3^{2-} and SeO_4^{2-}, respectively. SeMet, a zwitterion at neutral pH, will possess a single negative charge in alkaline conditions. SeMet, possessing only one negative charge would have the least affinity for the stationary phase and consequently elute in the shortest time, which was found to be the case. The separation of Se^{IV} from Se^{VI}, although they possess similar charges, would be possible due to the differences in the hydrated radius of each and the resulting charge density. Conditions to either oxidize Se^{IV} or to reduce Se^{VI} were not attainable experimentally to induce co-elution that would have simplified the chromatograms.

The optimum conditions for simultaneous separation of arsenic and selenium species, with the best peak shape and shortest chromatographic run times were found to be the use of a 100 x 3 mm column packed with Hamilton PRP X100 resin, 12-20 μm particle size, a 10 mM K_2SO_4 mobile phase at pH 10.2 and an eluent flow rate of 1.25 ml min^{-1} with an injection loop volume of 250 μl. The arsenic species eluted within 4 mins and the selenium species eluted in less than 6 mins. A chromatogram demonstrating simultaneous separation of arsenic and selenium compounds is shown in Figure 2-7.

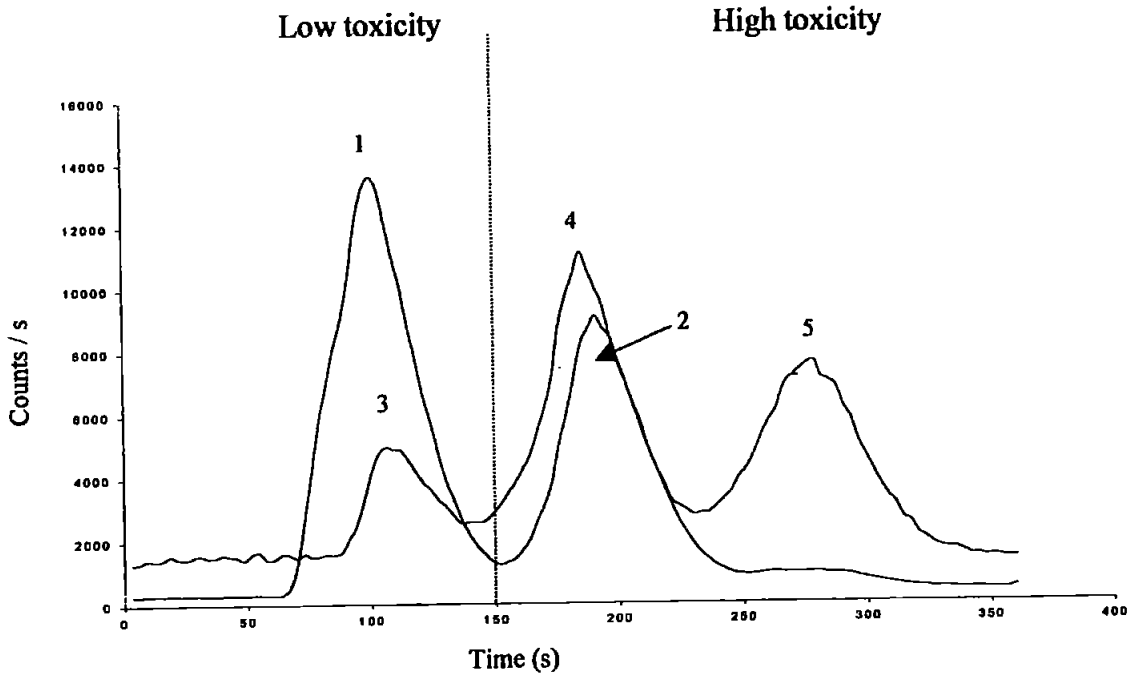


Figure 2-7 Chromatogram demonstrating simultaneous separation of four arsenic and three selenium species, into their relative toxicity classification, under the same experimental conditions – Hamilton PRP X100 (100 x 3 mm) column, 10 mM K_2SO_4 mobile phase at pH 10.2, eluent flow rate of 1.25 ml min^{-1}. Peaks: 1, AsBet and DMA; 2, As^{III} and As^{V}; 3, SeMet; 4, Se^{IV}; 5, Se^{VI}. This chromatogram clearly shows the separation of low toxicity from high toxicity species obtained.

The linearity of results obtained using the column system was determined in the range of 50 – 500 μg l^{-1} for each of the analytes. Graphs demonstrating the linearity obtained for the selenium and arsenic species are shown in Figures 2-8 and 2-9, respectively. Correlation coefficients are shown on the graphs and are all close to 1 demonstrating a linear relationship between concentration and signal response in the form of peak area.

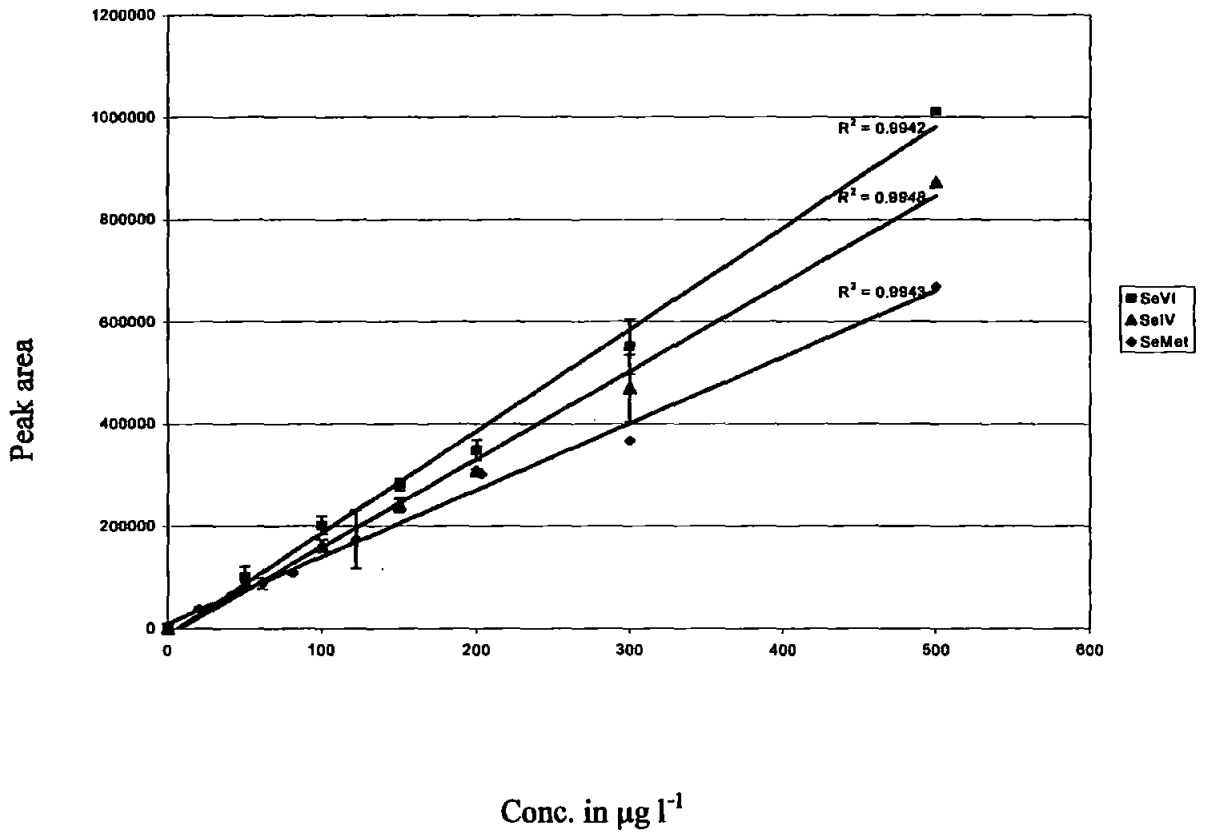


Figure 2-8 Graph demonstrating the linearity obtained for the selenium species on simultaneous separation together with arsenic species using a Hamilton PRP X100 mini-column (100 x 3 mm I.D.). Error bars are derived from 95% confidence intervals where n=3.

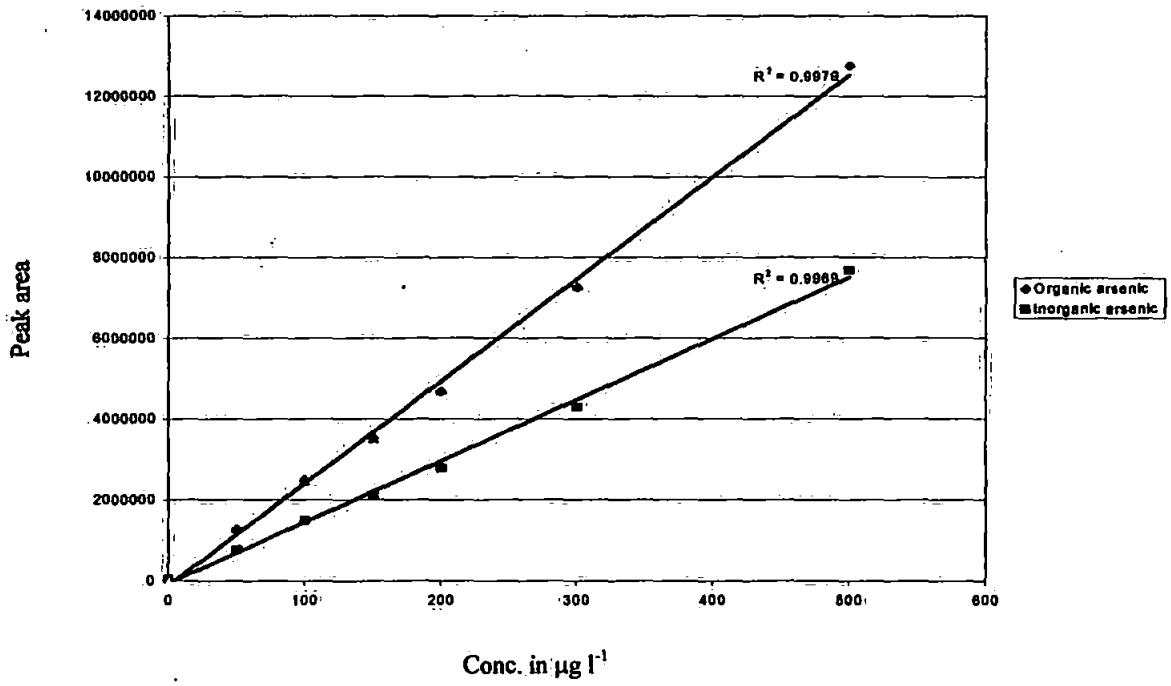


Figure 2-9 Graph demonstrating the linearity obtained for the arsenic species on simultaneous separation together with selenium species using a Hamilton PRP X100 mini-column (100 x 3 mm I.D.).

2.3.2 Speciation of CRMs and real samples using LC-ICP-MS

Having identified the optimum chromatographic conditions followed by assessment of the linearity for the eluting species using commercially available standards, the methodology was applied to that of real samples. Certified reference materials (TORT-2 and DORM-2) were included for quality control and method validation purposes. The sample of TORT-2 is known to contain both arsenic and selenium as certified reference values are given for both elements. DORM-2 was also chosen for analysis as certified values are given for total

arsenic and for that of AsBet. Samples were prepared using a HNO_3 digest for total elemental determinations and using an enzymatic extraction for speciation studies (as described in section 2.2.4). The results are shown in Table 2-5.

Table 2-5 Results of samples analyzed using LC-ICP-MS. All values are given in mg kg^{-1}, as the element.

Sample	*Total As*	*Total Se*	*Organic As*	*SeMet*
Plaice	39.36 ± 1.72		37.6 ± 3.8	
Oyster	10.02 ± 1.1		9.13 ± 1.4	
Selenoprecise™		522 ± 24.8		571 ± 50.2
TORT-2	20.7 ± 1.9	5.23 ± 0.78	23.5 ± 3.1	4.1 ± 0.54
DORM-2	17.8 ± 0.91		15.4 ± 1.68	
Limits of detection	0.001	0.008	0.002	0.010

Certified values: TORT-2 – Total As 21.6 ± 1.8 mg kg^{-1}, Total Se 5.63 ± 0.67 mg kg^{-1}
DORM-2 - Total As 18.0 ± 1.1 mg kg^{-1}, AsBet 16.4 ± 1.1 mg kg^{-1}

The results for the samples demonstrate that all species present were found as the organic forms of the element. No inorganic compounds were detected in any sample. The values obtained by LC-ICP-MS closely match the total values obtained by direct ICP-MS suggesting that all species are accounted for. However, it can be seen that the confidence intervals obtained by direct ICP-MS are much narrower than those obtained by LC-ICP-MS. It is worth noting that inorganic arsenic, organic arsenic and the three selenium species used as standards demonstrate differing sensitivities when determined by ICP-MS. This

is evident when examining Figures 2-8 and 2-9. The gradient of the slopes is different for all species although the same concentrations, based upon the element, have been measured. As the system is used to separate organic from inorganic arsenic and selenium species the fact that the detection is species-sensitive will affect calculations. Where there are two or more co-eluting species, or in a situation where an unidentified arsenic or selenium compound is present, errors will inevitably occur as signal response varies for individual compounds. The results obtained in this experiment, particularly for the organic arsenic species where co-elution of species occurs, appear to be satisfactory probably because the samples analyzed predominantly contain AsBet and AsBet was used as a standard for quantification. The standard addition technique was used for quantification purposes as this is known to minimize the effects of non-spectral interferences caused by the sample matrix. Inaccuracies in original weights of samples and fluctuations in temperature and pressure on the column are other factors that must be considered.

The use of CRMs provided useful data for method validation. A chromatogram of the TORT-2 sample demonstrating the simultaneous separation of organic from inorganic arsenic and selenium species is shown in Figure 2-10. Mass 75 was monitored for arsenic as it is mono-isotopic, and mass 82 was monitored for selenium as this isotope suffers the least from spectroscopic interferences. At mass 75, for direct ICP-MS analysis, 4% N_2 was added to reduce the spectroscopic interference of $ArCl^+$. However, this measure was not necessary when using the mini-column as Cl^- ions are retained on the resin. This was

verified by the introduction of a 2% NaCl solution onto the column and no subsequent peak being seen when recording at mass 75.

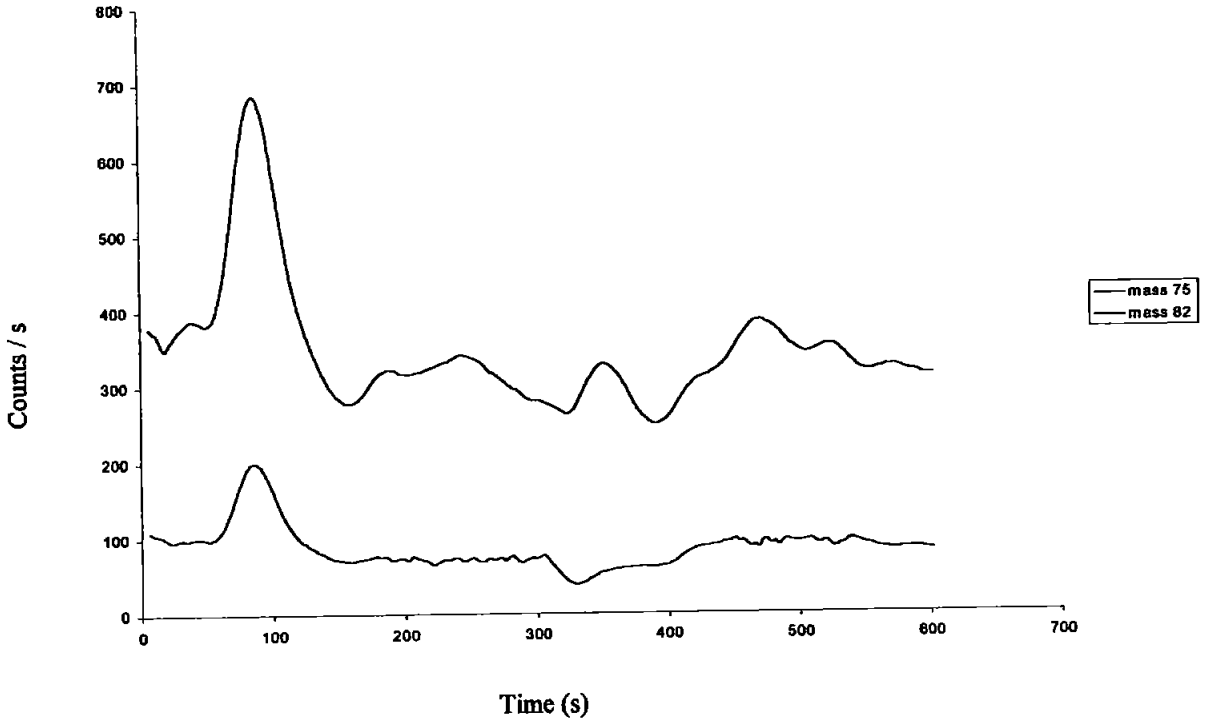


Figure 2-10 Chromatogram of TORT-2 demonstrating the simultaneous separation of organic arsenic (mass 75) and SeMet (mass 82) using optimum mini-column LC-ICP-MS conditions.

2.4 Conclusions

The implementation of multi-element detection using ICP-MS coupled to a low-pressure mini-column liquid chromatographic system provided a useful method for the simultaneous separation of organic from inorganic arsenic and selenium species. It was successfully applied for the separation and detection of standards as well as real samples that were of environmental importance. As stated, the method can be employed for initial, rapid species monitoring for samples of environmental and biological importance pollution. If high levels of toxic species are found then further, more rigorous analytical techniques can be used as required.

Low-pressure systems were obtained by the use of mini-columns with column dimensions no greater than 3 x 100 mm. Conditions suitable for the chromatographic separation of inorganic from organic species utilizing the pK_a values of the species were investigated together with choice of competitive ion eluent, its concentration and flow rate. ICP-MS was used as the detector due to its multi-element detection capability, although less expensive element-specific detectors can be used. Optimum conditions to achieve simultaneous separation of relatively toxic from relatively non-toxic arsenic and selenium species were found to be the use of a Hamilton PRP X100, 12-20 μm, resin packed in a column of 100 x 3 mm I.D. The eluent competitive counter ion was K_2SO_4 at 10 mM concentration and pH 10.2. The optimum eluent flow rate was 1.25 ml min^{-1}.

An injection volume of 100 µl was found to provide optimum sample loading onto the column.

Under the optimum conditions employed, the results obtained for the CRMs DORM-2 and TORT-2, the selenoprecise® and two marine samples of plaice and oyster compare favourably with the expected and reference values. Limits of detection were determined to be in the range of 2 – 10 µg kg^{-1} for organic and inorganic arsenic and selenium species.

The validity of this simple procedure for screening biota samples in terms of their arsenic and selenium toxicity was, therefore, demonstrated. This rapid screening technique allows a suitable estimate of the implications to health to be made for samples containing arsenic and selenium without resorting to a full speciation procedure.

Chapter three

3 Feasibility study for the speciation of arsenic and selenium in candidate reference materials

3.1 Introduction

Speciation analysis focuses on the clear identification of specific chemical species or forms of an element and its quantification [140]. Sample preparation and extraction techniques for the analyte of interest together with hyphenated techniques commonly used for speciation separation and detection will all possess their own sources of errors. Measurements by laboratories, even when applying the same method may differ significantly from each other [141]. However, results are only of use where they are considered to be accurate and precise. Reliable measurements are vital for quality control and assurance in areas of trade, agriculture, food and nutrition science, health, environment, toxicological studies and in legal requirements for monitoring and consumer protection [142].

Traceability and comparability of measurements have long been acknowledged as holding an important place in analytical science [143]. Results can only be accurate and comparable worldwide if they are traceable. An unbroken chain of calibrations connecting the measurement process to fundamental units achieves traceability of a measurement [144]. It must be demonstrable that no loss or contamination has occurred and that chemical species have been preserved [145]. It is possible to verify analytical procedures in a simple manner by the use of certified reference materials and by the use of mass balance calculations.

Analysts involved in method development require suitable validation procedures, which ideally involves the use of a CRM of the same or similar matrix type and similar analyte concentration to that of the sample. Reference materials for arsenic and selenium speciation are very limited. More are urgently required to expand the base of CRM sample matrices available to analysts, in order to meet the growing demands of society [143].

Certified reference materials are costly to produce and therefore feasibility studies are generally undertaken to determine whether a 'candidate' material will produce a viable CRM. A reference material suitable for control purposes will usually be a 'real world' material so that it behaves as similarly as possible to the samples being measured with the method being controlled. Preference is given to materials where there are naturally occurring higher levels of particular species or to naturally contaminated materials rather than having materials spiked with the substance of interest during the preparation stage. The requirements of a material suitable for entering into a feasibility study depends on a number of criteria: the analytes of interest need to be extractable and in concentrations that are representative of the sample type; the prepared material must be homogenous and stable in storage conditions, i.e. absence of degradation of species over time in a range of acceptable moisture, light and temperature conditions [146].

The work presented here comprises part of a feasibility study into the production and certification of six new reference materials, analyzed for species concentrations of the element arsenic (in chicken, fish, rice and soil) and

selenium (in wheat and yeast). The project was funded by the European Community under the 'Competitive and Sustainable Growth' programme (1998-2002) [147].

The project was set out in three stages. The role of the University of Plymouth in the first stage of this project was to conduct a survey on a variety of fish types to identify a particular sample that would be suitable as a candidate reference material. The purpose of the survey was to determine total arsenic concentrations in the fish and the number of arsenic-containing species present in measurable quantities with the best extraction efficiency obtainable. The plaice sample appeared to be most fit for purpose and a quantity of this material was obtained and forwarded to the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). Here, the materials underwent sample preparation processes to produce 100 bottles of homogenous candidate reference material. These were dispatched back to the laboratory for homogeneity and stability studies. Each of the six participating laboratories in this part of the feasibility study underwent a similar process for their chosen material.

The second stage of the study encompassed the homogeneity and stability studies undertaken by each partner for their chosen candidate material. Following this, each partner laboratory sent two bottles of their candidate material to all other participating laboratories. This allowed the third and final stage of the project to be carried out which involved the inter-laboratory comparison of all sample types for the species of interest. There were nine participating laboratories for this stage

of analysis [147]. The data obtained from this stage of the project was used to calculate the uncertainties in the measurements obtained and hence the suitability of the material to go forward for full certification. Ideally, a CRM should possess a narrow confidence range for a specified substance with no, or minimal, operationally defined limits [144].

3.2 Preliminary survey of a variety of possible fish types with subsequent homogeneity and stability studies using plaice

3.2.1 Instrumentation

ICP-MS measurements were performed using a VG Plasmaquad (2+, TJA Solutions, Winsford, Cheshire, UK), using the conditions described in Table 3-1. A Perkin Elmer series 410 high-pressure pump (Perkin Elmer, CT, USA) was used for control of the chromatographic eluent flow rate. A Rheodyne 7125 injection valve (Rheodyne, CA, USA) with a 20 µl loop volume was used for column loading of sample digests. pH readings were taken using a 3010 pH meter (Jenway, Ltd., Essex, UK).

Table 3-1 ICP-MS operating conditions used for the determination of total and arsenic species in fish sample extracts.

ICP-MS	Plasma Quad 2+	
	Concentric pneumatic nebulizer (Meinhard)	
	Water-cooled cyclonic spray chamber – 50 ml vol	
	Fassel torch – 1.5 mm bore injector	
Parameters	Argon plasma – 4% N_2 added for direct total As determination to the nebulizer gas flow	
	Nebulizer flow rate	0.8 l min^{-1}
	Coolant gas flow rate	13.0 l min^{-1}
	Auxiliary gas flow rate	0.8 l min^{-1}
	Forward power	1350 W
	Dwell time	500 ms

Arsenic was measured using m/z 75. 4% (v/v) N_2, for the reduction of $ArCl^+$ interferences on m/z 75, was added via a Signal series 850 gas blender (Signal, Camberley, Surrey) to the nebulizer gas flow.

Hydride generation atomic absorption spectroscopy (HG-AAS) was performed using a continuous flow hydride generator (PS Analytical, Sevenoaks, Kent, UK) and a PYE Unicam SP9 AAS instrument (Philips Scientific, Cambridge, UK). Operating conditions are shown in Table 3-2.

Table 3-2 Operating conditions for determination of reducible arsenic in the plaice sample using HG-AAS

Hydride generation	Rise time – 10 s Decay time – 10 s Argon purge – 250 ml min^{-1} Reducing agent – 1% $NaBH_4$ in 0.1 M NaOH at 3 ml min^{-1} Acid – 3 M HCl at 8 ml min^{-1} Sample flow rate – 5 ml min^{-1}
Atomic absorption Spectrometer – Unicam SP9	Wavelength – 193.7 nm (As) Lamp current – 10 mA Band pass – 2 nm Air – 5.0 l min^{-1} Acetylene – 1.5 l min^{-1}

3.2.2 Chemicals and reagents

All commercial chemicals were of analytical grade and used without further purification. Arsenous acid (assay – 99.95-100.05 % purity), arsenic acid, dimethylarsinic acid, (Sigma-Aldrich Chem. Co., Poole, Dorset, UK), arsenobetaine (BCR, Retieseweg, Belgium), monomethylarsonous acid (kindly donated by Dr. A. Moreda-Pineiro, University Santiago de Compostela, Spain) and trimethylarsenic oxide (TMAO, Argus chemicals, Vernio, Italy) were used as stock solutions of 1000 μg ml^{-1} in terms of the element. They were stored in the dark at 4°C. Solutions of the compounds for daily use were prepared by

appropriate dilution from the stock solutions using Milli-Q water (Milli-pore, Bedford, MA, USA). The CRM used in the preliminary study was TORT-2 (National Research Council). Eluent solutions were prepared using solid Na_2SO_4, liquid pyridine, 0.91 sp. gr. NH_3 solution and 98% HCOOH (Sigma-Aldrich). Samples for HG-AAS were prepared using L-cysteine (Sigma-Aldrich) and 69% HNO_3.

All plastic/glassware was soaked in HNO_3 (10%, v/v) for a minimum of 24 hours and rinsed thoroughly with de-ionized water prior to use.

3.2.3 Chromatographic conditions for the determination of arsenic species

All mobile phase solutions were degassed by ultra-sonication for 15 mins prior to use. Anion-exchange HPLC, for the speciation of arsenic, was carried out using a column (250 x 4.6 mm) packed with Hamilton PRPX100 10 μm resin (Phenomenex, UK) with a guard column (50 x 4.6 mm) of the same material. The mobile phase employed a step gradient elution using a solution of 5 mM and 50 mM Na_2SO_4 at pH 10.2, adjusted with NH_3 solution (0.91sp. gr.). The programme for elution is given in Table 3-3. An eluent flow rate of 1.2 ml min^{-1} was used throughout.

Table 3-3 Elution programme for anion-exchange HPLC of arsenic species using a Hamilton PRP X100 column.

Concentration	*0 –5 mins*	*5 – 9 mins*	*9 – 15 mins*
5 mM Na_2SO_4 (adjusted to pH 10.2)	100 %	0 %	100 %
50mM Na_2SO_4 (adjusted to pH 10.2)	0 %	100 %	0 %

Cation-exchange HPLC was carried out using a Partisil SCX 10 column (250 x 4.6 mm, Phenomenex) packed with a silica gel of 10 μm particle size with a guard column (50 x 4.6 mm) of the same material. The mobile phase employed an isocratic elution using 20 mM pyridine solution adjusted to pH 3 with HCOOH (98%, v/v) [148] with an eluent flow rate of 1.2 ml min^{-1} used throughout.

3.2.4 Mass balance calculation

To obtain data for mass balance calculations determination of total element concentrations in the sample, a sum of the species in the extracts together with total element concentrations in any residues must be accounted for. A flow diagram of mass balance analysis can be seen in Figure 3-1.

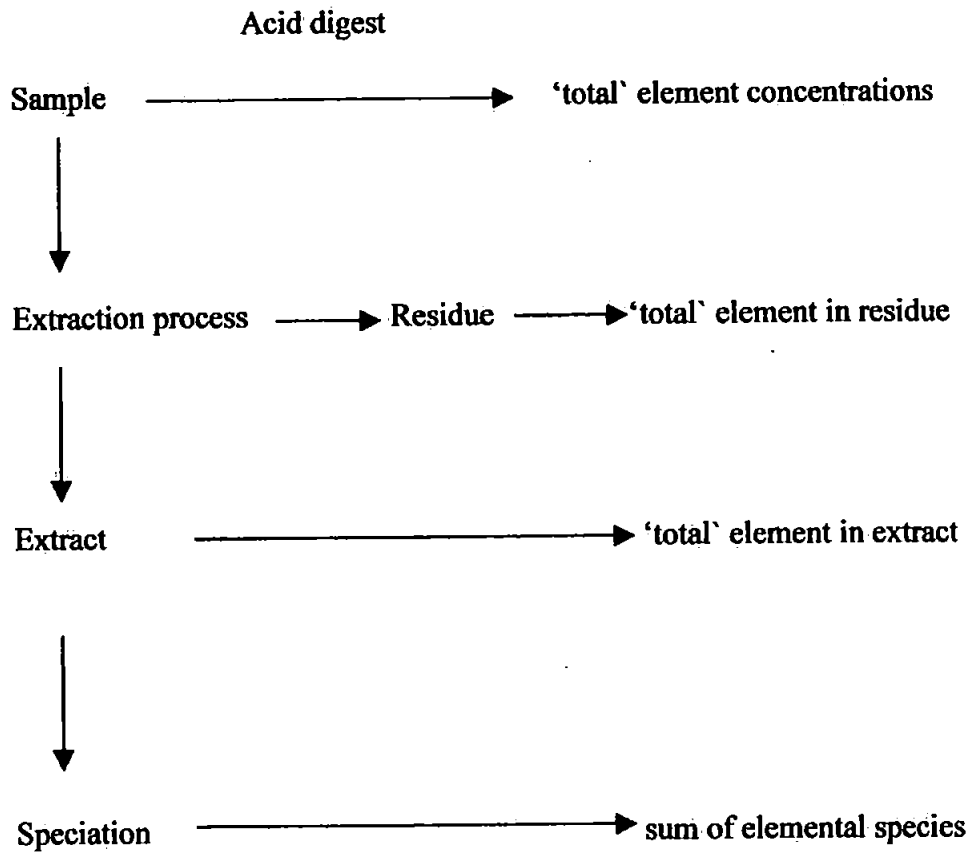


Figure 3-1 Flow diagram for acquisition of mass balance data

3.2.5 Sample preparation procedures for determination using ICP-MS and HPLC-ICP-MS

Eight fish samples (plaice, monk, hake, haddock, cod, coley, pollack and whiting) were purchased locally from the Plymouth fish market where they were beheaded, gutted and filleted. The fish were further prepared in the laboratory by removing the skin and ensuring that all bony material was removed. They were then cut into small pieces and freeze dried (Edwards Super Modulyo, Edwards High Vacuum, Crawley, Sussex, UK) at $-60^{O}C$ and 1×10^{-2} Torr (or until a stable pressure was maintained) for approximately 48 hours, at which point a constant weight had been achieved. The dried fish were blended gently in an Optiblend 2000 electrical blender (Moulinex, France) until fine powder was obtained. A microwave digestion for total arsenic determination and an enzymolysis extraction for the determination of arsenic species were both performed using the following methods,

HNO_3/H_2O_2 digestion for 'total' arsenic concentrations in fish samples and residues

Microwave bombs were pre-cleaned using 3 ml 69% v/v HNO_3 (Aristar, Fisons) on medium power in a Perfecto 800 W microwave oven (DeLonghi, Italy) for 2 mins. Samples of approximately 0.5 g (all fish types and TORT-2 as CRM) were accurately weighed into the bombs and 3 ml HNO_3 (69% v/v) together with 1 ml H_2O_2 (37% v/v) were added [132]. The bombs were loosely capped and left overnight to allow easily oxidizable material to be destroyed. After predigestion,

the bombs were gently swirled, sealed tightly and microwaved on medium power for 1 - 2 mins, or until the digest was a clear colour with no residue (indicating a completed digest). The samples were transferred quantitatively into volumetric flasks and made up to volume with deionized water. The digested samples and matrix matched standards were spiked with indium to give a final concentration of 100 μg l^{-1} In, which acted as an internal standard prior to analysis by N_2-ICP-MS using the conditions described in Table 3-1.

Enzymolysis Extraction of Arsenic Species

An enzymatic extraction procedure adopted by Branch *et al.* [132] was employed. Samples (all fish types and TORT-2 as the CRM) of approximately 1.0 g of tissue were accurately weighed together with 0.1 g trypsin (Sigma-Aldrich, Dorset, UK) and approximately 20 ml NH_4HCO_3 (0.1 M, pH 8) buffer. The solutions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. The samples were centrifuged at 2500 rpm for 20 min, the supernatant transferred quantitatively to volumetric flasks and made up to volume with the NH_4HCO_3 buffer. The samples and standards were spiked with caesium to give a final concentration of 100 μg l^{-1} Cs, which acted as an internal standard prior to analysis by direct ICP-MS using the conditions described in Table 3-1. Determination of arsenic species was carried out using the chromatographic conditions described in Section 3.2.3.

Mass balance

To obtain further mass balance data, total arsenic concentrations were determined in extract solutions and the residues from the trypsin extraction procedure. The residues were digested using the HNO_3 digestion method described above.

3.2.6 Sample preparation procedures for determination of reducible arsenic in plaice samples using HG-AAS

Enzymatic sample digest solutions, prepared as above, were used for the determination of reducible arsenic species in the plaice samples using HG-AAS. Sample volumes of 25 ml were added to 4 ml of 8.75% L-cysteine in 50 ml volumetric flasks and made up to volume with 0.05 M HNO_3 [149]. Standards and blanks were prepared using the same concentrations of L-cysteine and HNO_3. The solutions were allowed to stand for approximately 1 hour to allow complete pre-reduction of any arsenate to arsenite by L-cysteine prior to analysis using the conditions described in Table 3-2.

3.2.7 Preparation of plaice samples for homogeneity and stability studies

The most suitable fish type, plaice, was taken forward for the homogeneity and stability studies, after sample processing by IRMM as shown in Figure 3-2. It was analyzed for total element concentrations and speciation of arsenic compounds according to the microwave and enzymolysis digestion techniques described in Section 3.2.5.

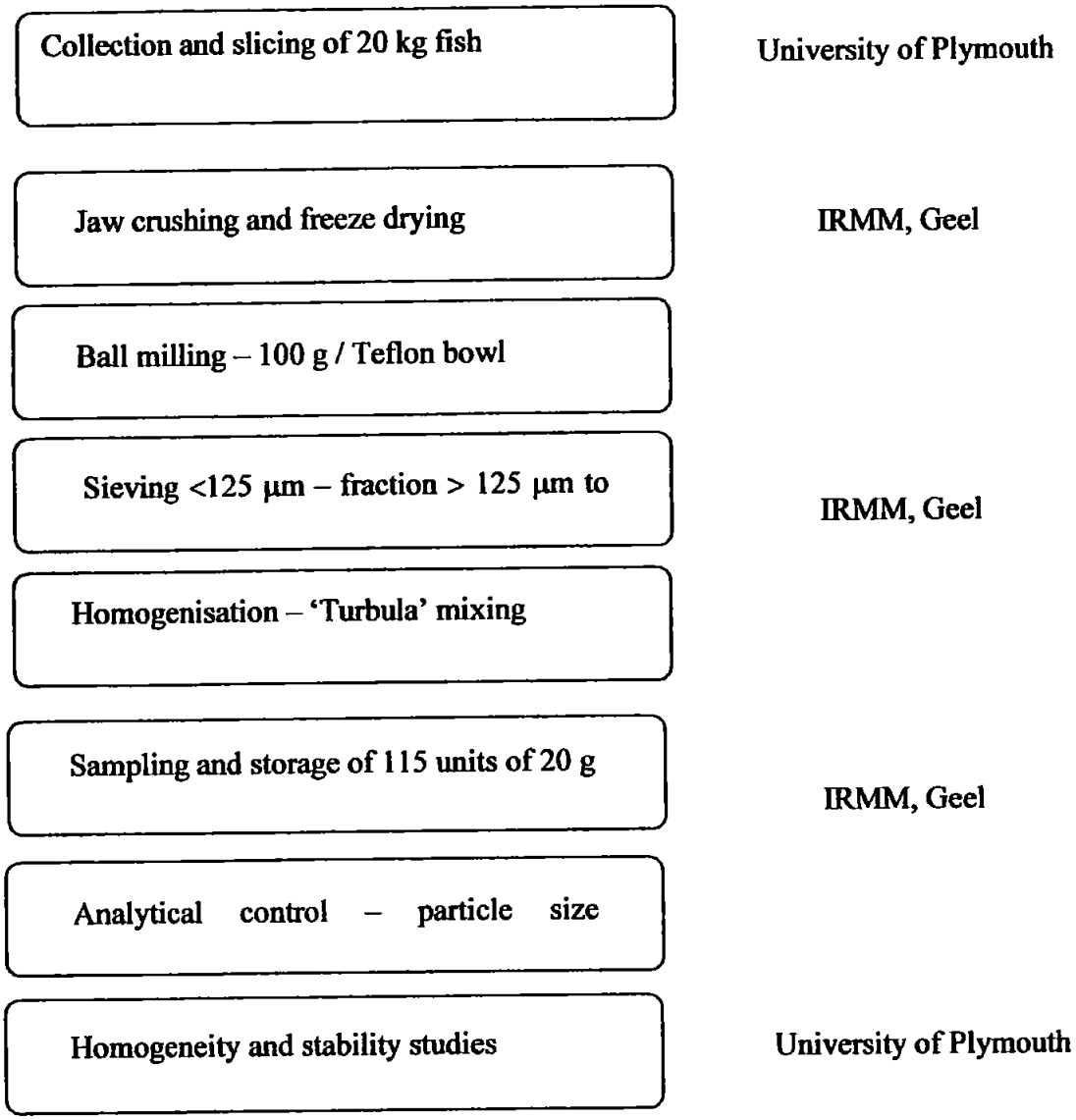


Figure 3-2 Flowchart for the preparation of fish samples by the University of Plymouth and IRMM.

When carrying out the homogeneity studies sample weights of 0.25, 0.5 and 1.0 g were taken. To ensure complete digestion of all the material the amount of trypsin added was varied in a ratio of 10:1 for sample:trypsin, which is considered to be the optimum ratio [93]. The stability studies performed were based on the results from 1 g sample weights using HNO_3 microwave and enzymolysis digestion techniques. The stability of total arsenic and AsBet was determined under conditions of temperature and time, ranging from 4 to 40^{O}C with material at –20^{O}C as a reference and 0 to 7 months, respectively.

3.3 Results and discussion

3.3.1 Preliminary fish survey

The preliminary survey was carried out on a number of fish types as shown in Table 3-4. The enzyme extraction efficiency given in the experimental data varied from 37-98%. This may be explained in terms of the overall extraction efficiency for arsenic from various types of fish which possess different lipophilic properties [132]. Trypsin, a protease, attacks proteins and can destroy cell walls whereas lipase may be the preferred enzyme for releasing arsenicals that may be lipid-bound. A previous inter-laboratory exercise for the certification of arsenic species in tuna fish [150] reported extraction efficiencies (the sum of arsenic in various chemical forms compared to total arsenic determination) ranging from 50 to 90% depending on the extraction method used (e.g. MeOH/H_2O, H_2O, MeOH/CH_3Cl, trypsin, etc.). Trypsin appeared to be the most efficient in this study [150] and was subsequently used here. The trypsin extraction proved most successful for plaice and whiting giving 98 and 96% efficiencies, respectively. However, the deficit of arsenic following enzymatic extraction compared with the total arsenic values obtained for the other fish in the survey should present itself in the residue. This was not the case for all the fish samples and was most noticeable for hake, haddock and cod. The reasons for this are not clear but may include instrumental matrix effects and loss of analyte to container surfaces.

Table 3-4 Arsenic speciation for all fish samples. Results given as mg kg^{-1} in terms of the element at 95 % confidence interval. n = 3

Sample	*Total As - HNO_3*	*Total As - digest*[+]	*Total As residue*	*AsBet - digest*[+]	*Inorganic As*	*Extraction efficiency*
Plaice	39.5 ± 1.7	38.7 ± 1.9	0.78 ± 0.034	38.7 ± 2.7	1.95 ± 0.21	98%
Monk	48.1 ± 2.4	32.6 ± 2.0	3.33 ± 0.12	12.6 ± 4.9	1.33 ± 0.19	68%
Hake	17.5 ± 27	6.6 ± 1.5	1.83 ± 0.15	7.6 ± 1.9	1.09 ± 0.18	37%
Haddock	34.6 ± 2.6	22.1 ± 1.7	4.20 ± 0.28	21.7 ± 0.8	1.54 ± 0.13	65%
Cod	122.1 ± 4.3	92.3 ± 6.1	5.36 ± 0.22	91.0 ± 0.9	1.46 ± 0.18	75%
Coley	9.4 ± 0.7	7.9 ± 0.9	1.09 ± 0.053	8.7 ± 0.5	0.91 ± 0.11	85%
Pollack	17.1 ± 0.7	10.7 ± 1.3	1.70 ± 0.19	8.3 ± 1.4	1.48 ± 1.9	63%
Whiting	11.9 ± 0.7	11.6 ± 0.4	0.42 ± 0.014	11.5 ± 2.0	1.23 ± 0.11	96%
TORT-2[•]	21.8 ± 0.8	17.8 ± 1.1	2.41 ± 0.087	16.6 ± 1.2	< LOD	80%

[•] certified value of TORT-2 = 21.6 ± 1.8 mg kg^{-1} for 'total' arsenic
[+] 'digest' refers to trypsin enzymolysis digestion solutions

The purpose of this survey was to screen for the most suitable material to go forward into a feasibility study for the production of a CRM. As outlined in the introduction, an ideal candidate reference material would contain a number of species at easily extractable and measurable concentrations. Work by Francesconi and Edmonds [14] suggests that while AsBet is the major form of arsenic found in marine animals with inorganic forms constituting < 2% of the total, other organoarsenic compounds may be present. These most important species include methylated forms such as MMA and DMA (which are known to be formed in the detoxification process [14]), tetramethylarsonium ion (TeMA), arsenocholine (AsChol) and trimethylarsine oxide (TMAO) all of which have been reported in various types of marine fauna. AsChol is thought to be a precursor of AsBet, although the metabolic pathway of AsBet has yet to be fully elucidated. TeMA is usually present as a degradation product caused by microbial breakdown of AsBet [151] and its presence has been reported in catfish [152]. It is conjectured to be an intermediate of the methylation of arsenate. TMAO is also considered to be an important organoarsenical accounting for up to 50% of the total arsenic in some marine fauna for example, the sea anemone, *Parasicyonis actinostoloides* [153].The results for the fish samples under investigation in this survey, shown in Table 3-4, show that AsBet was the predominant form of organic arsenic found. Chromatograms of the arsenic standards and plaice sample using anion-exchange HPLC-ICP-MS are shown in Figures 3-3 and 3-4. Although Francesconi and Edmond's work [14] suggests that a number of organic arsenic species exist in nature and is supported in a review by Cullen and Reimer [10] summarizing this, the results are

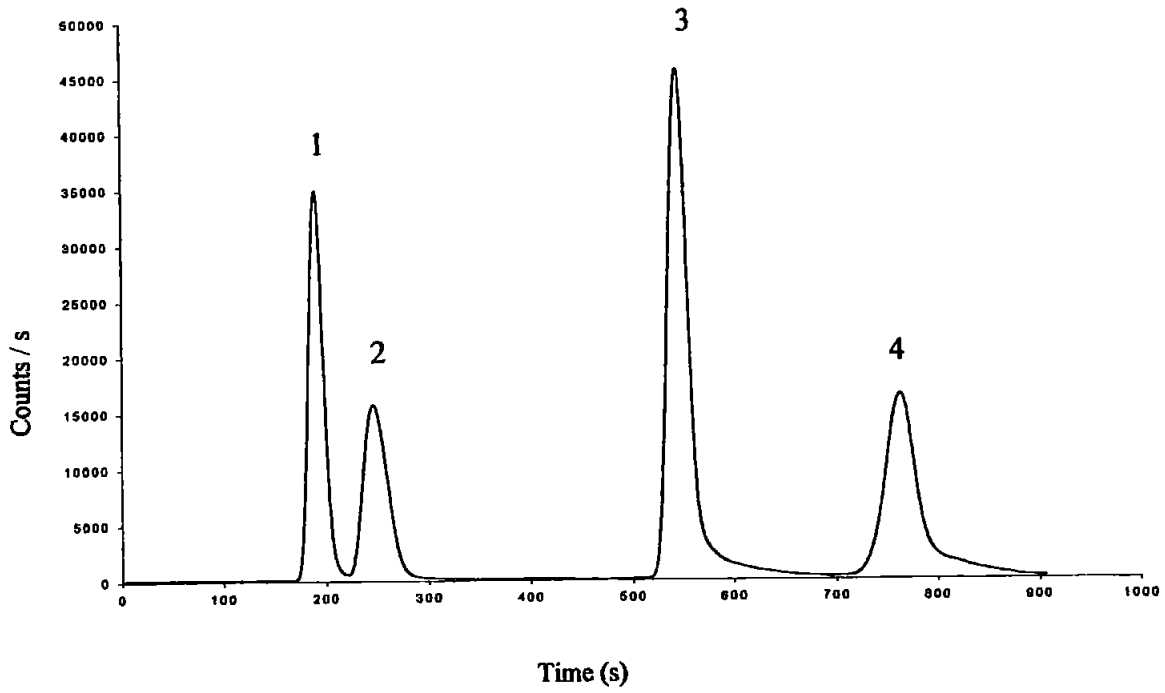


Figure 3-3 Chromatogram obtained using anion-exchange HPLC-ICP-MS of arsenic standards at 250 μg l^{-1}, 1=AsBet; 2= DMA; 3=MMA; 4=As^V. Conditions as shown in Table 3-3.

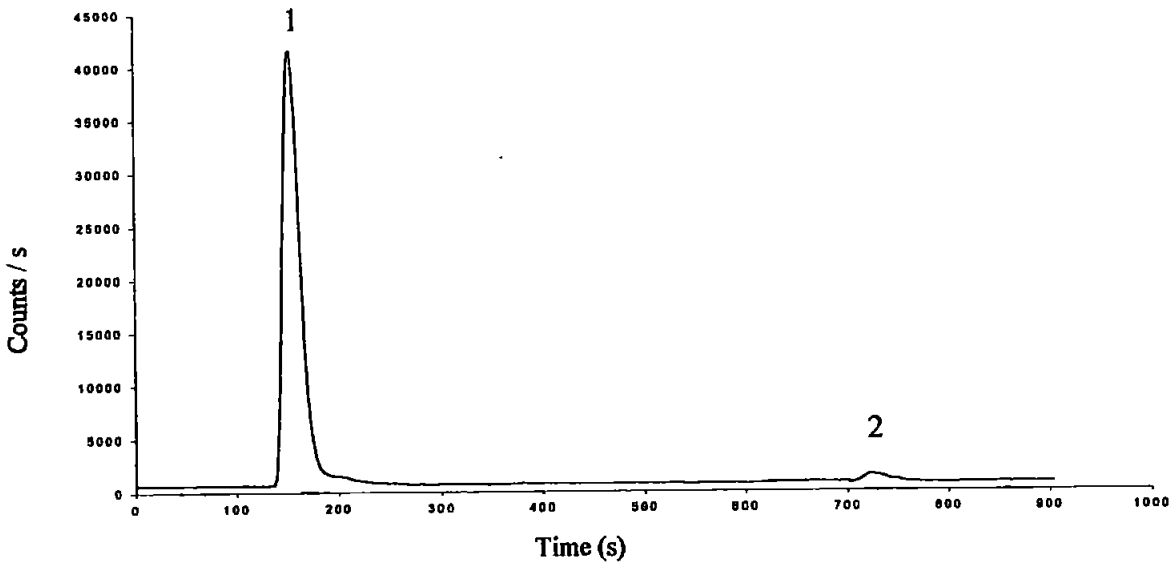


Figure 3-4 Chromatogram of plaice using anion-exchange HPLC-ICP-MS, using conditions shown in Table 3-3. Peaks: 1, AsBet; 2, As^V/ artifact.

comparable to those reported by other workers [132, 148] where AsBet is the major compound found in fish.

Identification of species based on matching chromatographic peak retention times with that of known standards can lead to ambiguity in peak assignment due to co-elution of chemically related compounds. TMAO is an arsenical that can be present in significant amounts in seafood. TMAO, being a cation, is likely to co-elute with AsBet under the anion exchange chromatographic conditions employed. Experimental analysis with the use of standards showed this to be the case. To minimize the risk of misidentification of TMAO and AsBet, a cation exchange chromatographic system was employed using a Partisil SCX 10 column with a pyridine mobile phase (see Section 3.2.3). Figures 3-5 and 3-6 provided experimental evidence that the major peak in the plaice sample was due to the presence of AsBet and not TMAO. There is also no evidence of an early eluting peak in Figure 3-6, which may be caused by Cl- ions indicating the lack of spectroscopic interference from this ion.

No simple methylated forms of arsenic were found in the fish studied in this report although small amounts of inorganic arsenic were found to be present. It is thought that in the presence of amines, inorganic arsenic is displaced from the detoxification pathways of methylation [154], reducing the number of species likely to be found. However, the levels of amines in white fish are generally low and methylated species have been detected by other workers [112, 155].

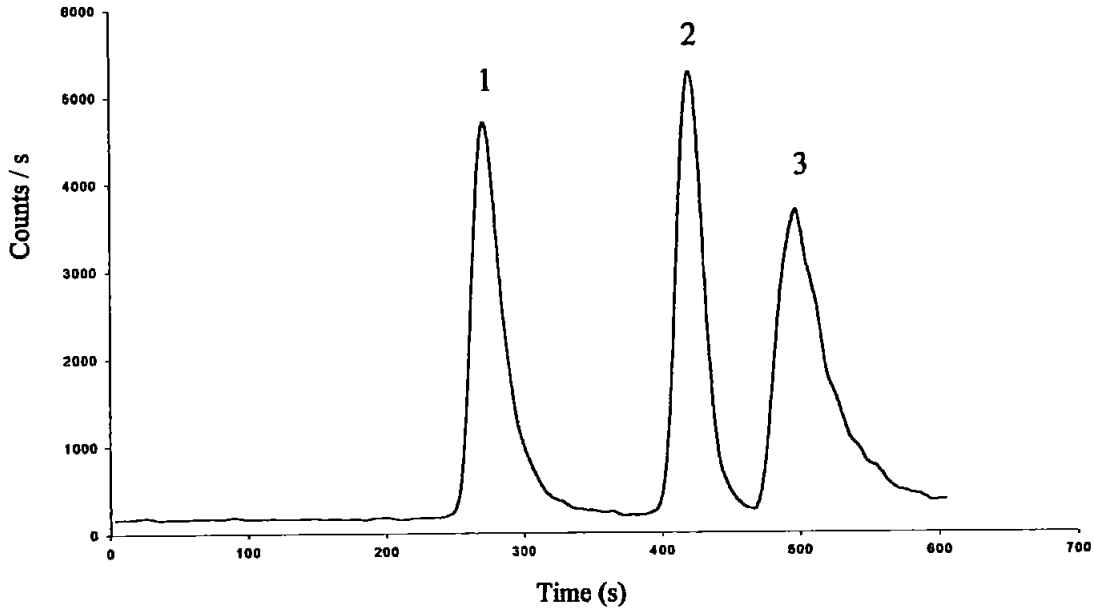


Figure 3-5 Chromatogram obtained using cation-exchange HPLC-ICP-MS, using a Partisil SCX 10 column with 20 mM pyridine at pH 3, of arsenic standards at 250 μg l^{-1} of: 1,DMA; 2, AsBet; 3, TMAO.

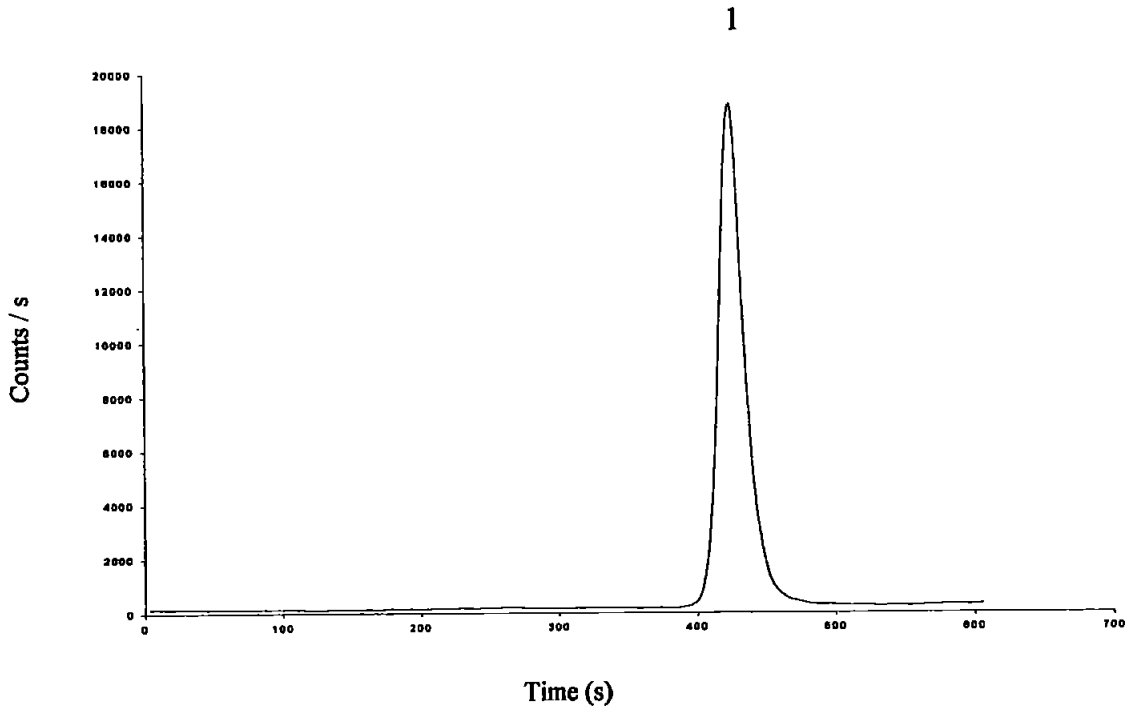


Figure 3-6 Chromatogram of plaice sample using cation-exchange HPLC-ICP-MS, using a Partisil SCX 10 column and eluent of 20 mM pyridine, pH 3. Peak 1 = AsBet.

The amount of inorganic As found was unusually high when compared with other research [156]. Inorganic arsenic is not commonly found at levels higher than approximately 6% of that of the total arsenic determined although levels as high as 25% have been recorded for some fish [132]. It is almost always found as As^{V} as opposed to As^{III}, as was the case in this study. This species may be considered, in the light of the predominance of As^{V} in seawater, to be relatively abundant due to prevailing redox conditions [157]. All the fish in this survey were found to have similar levels of As^{V} regardless of the total levels of As found. This appears to be an anomalous finding when taking into account work published by other researchers. Their work suggests that levels of inorganic arsenic in marine animals will vary according to the type of animal and its habitat [158].

Although all the fish in this study were caught locally and therefore subjected to similar environmental pollution levels, it is possible that the similar levels of As^{V} found may be accounted for by contamination of samples prior to purchase and during preparation. It is important to note that no contamination was seen in the blank samples. Also, results from the CRM, Tort-2 (lobster hepatopancreas), showed that approximately 90% of the extracted As was present as AsBet within the limits of experimental error and no inorganic arsenic was detected; this sample having gone through an identical analytical procedure after the sample preparation stage suggests that contamination post-preparation of samples is unlikely to be the cause for the presence of As^{V} in the other fish samples. A survey using HG-AAS, with an experimental LOD of 11.3 $\mu g\ l^{-1}$, did not detect

any reducible forms of arsenic, confirming the absence of inorganic arsenic forms in the fish samples.

The preliminary study of all fish types was carried out in order to identify one that would be appropriate as a candidate reference material providing a suitably high extraction efficiency for arsenic species in measurable quantities. It was decided that plaice met the criteria of merit based upon it having a relatively high level of arsenic combined with the best enzymatic extraction efficiency seen for all fish types under investigation. The processed plaice samples were, therefore, carried forward to undergo homogeneity and stability studies.

3.3.2 Homogeneity and stability studies

Having identified plaice as being the most suitable fish to go forward for a feasibility study 20 kg of the filleted fish was bought from the Plymouth fish market, frozen and sent to the IRMM for preparation. A flowchart for the preparation of the fish can be seen in Figure 3-2. Homogeneity and stability studies were carried out using instrumental conditions, chemicals and chromatographic conditions as previously described in Section 3.2.1., 3.2.2 and 3.2.3.

The risk of in-homogeneity exists for any material prepared in any manner. There are two types of homogeneity that are of importance. The first is the within-bottle homogeneity, which dictates the minimum sample intake for which the

established uncertainty remains valid. The second is the between-bottle (between-unit) homogeneity. To establish within-bottle homogeneity it is advantageous to take small sample sizes so that between portion effects can be quantified. For between-bottle homogeneity tests it is more practicable to take an optimal sample size to minimize analytical variation [159]. The homogeneity of the plaice was determined using measurements of total arsenic and AsBet. The most relevant quality when analyzing for in-homogeneity is the repeatability of the method rather than the accuracy. A prerequisite for this is that all samples are measured on the same day, with the same instrument and by the same operator.

The plaice samples were prepared using the digestion methods described in section 3.2.5. Sample weights taken for homogeneity studies were in the range of 0.25 – 1.0 g. Within-unit in-homogeneity is more readily detected with lower sample sizes. The results for within-bottle homogeneity comprised 5 sub-samples from 2 bottles at weights of 0.25 g, 0.5 g and 1.0 g (30 samples in total). For between-bottle homogeneity samples of 0.5 g and 1.0 g were taken in triplicate from 10 bottles. Using STATGRAPHICS Plus 2.1, the ANOVA table decomposed the variance of the two components, a between-group component and a within-group component. The F-ratio is a ratio of the between-group estimate to the within-group estimate. Since the p-value of the F-test was greater than 0.05 there was not a significant difference between the means of the variables at the 95% confidence interval. This confirmed the absence of measurable in-homogeneity. The results are presented in table 3-5. The lowest sample size tested for homogeneity was 0.25 g and this must be stipulated, should

the material go forward for full certification. This will become the lowest recommended sample size for analysis, as homogeneity cannot be guaranteed below this amount.

Table 3-5Within and between - bottle homogeneity for total As and AsBet in plaice

	Within bottle – CV% *Bottle 1*	*Within bottle – CV%* *Bottle 2*	*Between bottle – CV%* *0.5 g / 1.0 g*
Total As	**2.4**	**2.3**	**1.7 / 1.9**
F-ratio	2.3	0.28	1.1 / 1.4
p-value	0.14	0.76	0.39 / 0.25
Total AsBet	**2.5**	**2.1**	**2.0 / 1.7**
F-ratio	2.2	0.92	1.7 / 1.7
p-value	0.16	0.43	0.17 / 0.15

The purpose of stability studies is to check that the value of a certified property does not change significantly during transportation, storage conditions or over the time scale in which the CRM is likely to be used. In order to detect stability problems it is usual to check the material using small batches prepared in the same manner as is intended for the actual CRM and test over a time scale, usually of up to three years. However, in feasibility studies it is not practicable to wait this length of time before proceeding with full certification exercises. Therefore, if no significant change in the material is detected by stability studies carried out

for an initial six-month period then full certification may be approved. Whilst assessing the material for stability over time it is also necessary to assess the stability of the material in likely storage conditions. Conditions that are usually regarded as having the potential to cause instability in biological materials are light, temperature and the presence of moisture leading to degradation of the material by microbial action. It is, therefore, necessary to design a study that tests the material over time and in various storage conditions, taking into account temperature, exposure to light and moisture content. In this study, material was stored in hermetically sealed dark brown glass bottles negating the need to study effects of light degradation.

The samples were assessed for an initial period of 0 – 7 months, at intervals of two weeks for the first two months and monthly for the last five. Any degradation was thought most likely to occur within the first few weeks. The time frame reflects that assumption with more frequent assessment in the initial period in order to provide a fairly accurate life-time of the material should any degradation become apparent. The studies were carried out at three temperatures: + 4^{O}C, + 20^{O}C and + 40^{O}C with samples stored at – 20^{O}C serving as a reference [144]. These temperatures were chosen as it was thought that microbial action (most critical temperature being 40^{O}C) and thermal degradation would be the most likely mechanisms for alteration and destabilization of the biological material.

The stability study was designed to test for total arsenic and AsBet content. Spiking experiments with standard species were performed in order to identify

potential losses or transformation of species during the study. Moisture content was determined by placing pre-weighed material in an oven at 85^{O}C until a stable weight was obtained. All data reported was corrected for moisture content. The 'total' arsenic content was measured directly by ICP-MS and the determination of AsBet (this being the major species identified in the plaice) was performed by anion exchange HPLC-ICP-MS. Three sub-samples were taken at each temperature.

The results for the stability of arsenic are given in graphic and tabular form in Figure 3-7 and Table 3-6. They were calculated from the data obtained using the standard equations of [160]:

$$R_T = \bar{X}_T / \bar{X}_{-20^{o}C}$$
$$U_T = \{(CV_T^2 + CV_{-20^{o}C}^2)^{1/2} . R_T\} / 100$$

R_T represents the mean $\bar{X}_T$ of three replicates at temperature T (+4^{O}C, +20^{O}C and +40^{O}C)divided by the mean $\bar{X}_{-20^{o}C}$ of the three replicates at –20^{O}C.U_T represents the uncertainty on R_T. CV is the coefficient of variance. In the case of ideal stability the ratios R_T should be 1.0. However, in practice, there will be random variations due to the variation on the measurement. The results obtained demonstrate this. However, in all cases the expected value of 1.0 is obtained within $R_T \pm U_T$ and on this basis it can be concluded that there is no instability over 7 months of time or at temperatures of +4^{O}C, +20^{O}C or +40^{O}C.

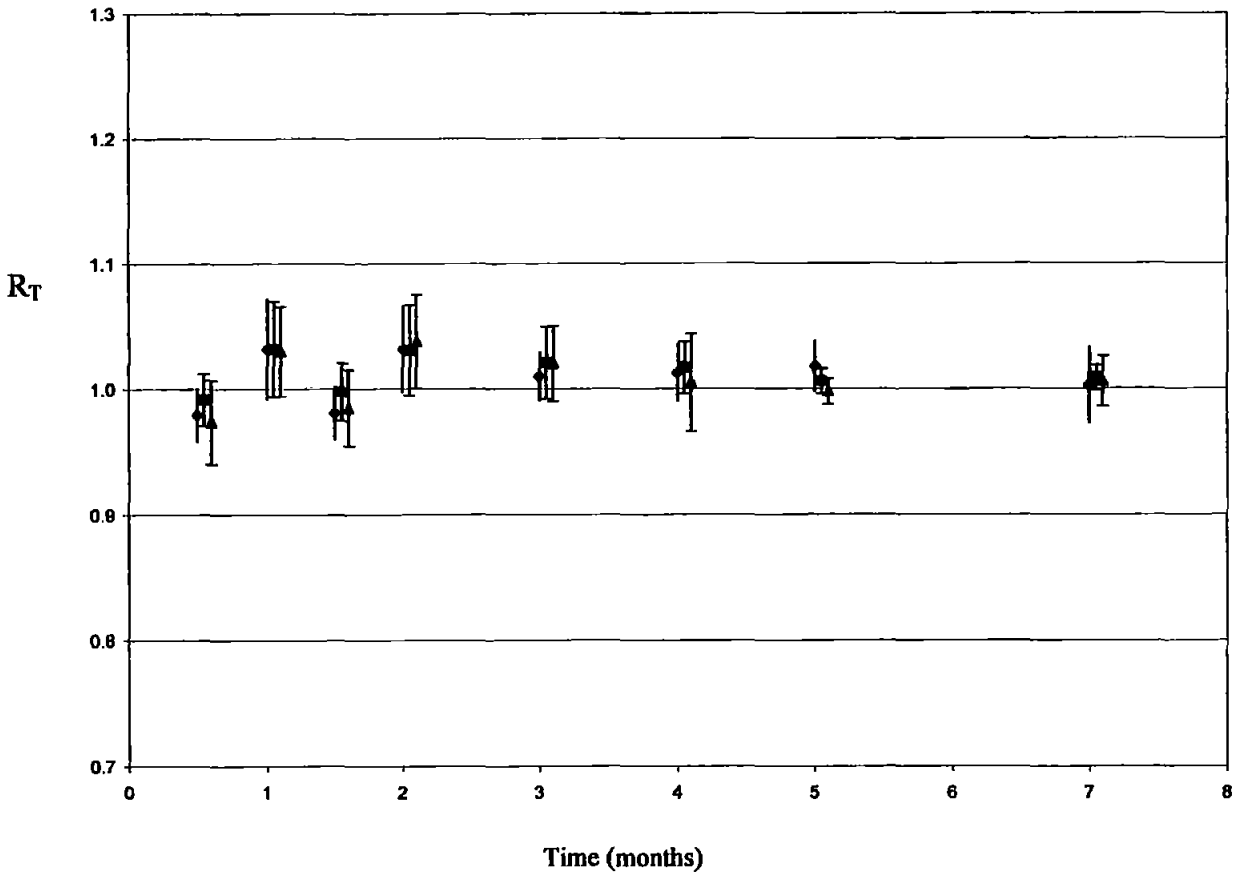


Figure 3-7 Stability study of 'total' As in plaice at temperatures set at 4 ^{O}C, 20 ^{O}C and 40 ^{O}C (baseline set at -20 ^{O}C values) to identify any changes over time.

Table 3-6 Stability study of total As in plaice. Values of $R_T \pm U_T$ over a 7-month period at temperatures of 4 °C, 20 °C and 40 °C (baseline set at -20 °C values).

	Time in months	*Temp °C*	$R_T \pm U_T$
As	0.5		0.980 ± 0.02
	1		1.032 ± 0.04
	1.5		0.981 ± 0.02
	2	+ 4	1.032 ± 0.03
	3		1.010 ± 0.02
	4		1.013 ± 0.02
	5		1.018 ± 0.02
	7		1.003 ± 0.03
	0.5		0.992 ± 0.02
	1		1.032 ± 0.04
	1.5		0.998 ± 0.02
	2	+ 20	1.031 ± 0.04
	3		1.021 ± 0.03
	4		1.017 ± 0.02
	5		1.006 ± 0.01
	7		1.009 ± 0.01
	0.5		0.974 ± 0.03
	1		1.030 ± 0.04
	1.5		0.985 ± 0.03
	2	+ 40	1.038 ± 0.04
	3		1.020 ± 0.03
	4		1.005 ± 0.04
	5		0.998 ± 0.01
	7		1.006 ± 0.02

Stability studies were also carried out on AsBet in plaice in a similar manner to that of the stability studies for total arsenic. The results are summarized in Figure 3-8 and Table 3-7. Stability is confirmed in that the value of 1.0 is obtained within the uncertainty range. The uncertainty obtained for total arsenic is, in general, less than that obtained for AsBet. This may be accounted for by greater precision in calculations on data obtained by direct ICP-MS measurement compared with the data collected for AsBet which was via HPLC-ICP-MS.

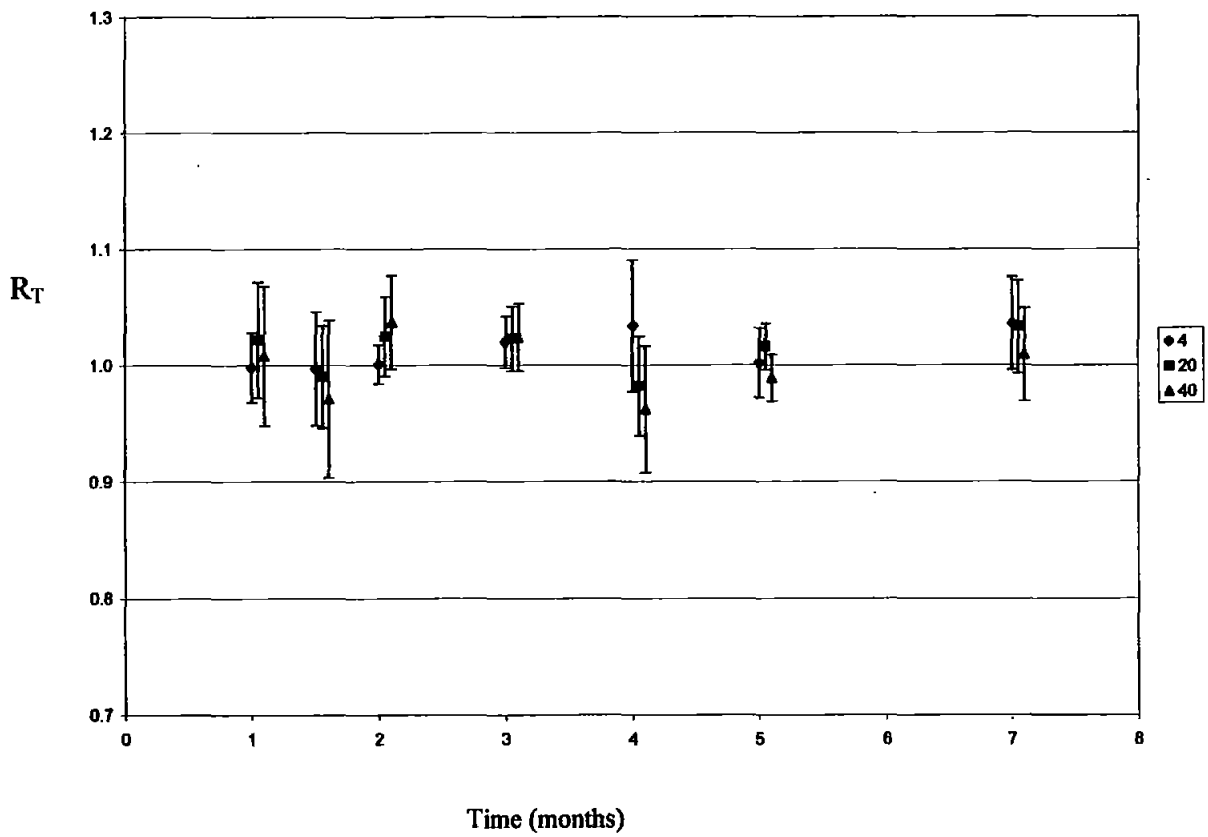


Figure 3-8 Stability study of AsBet in plaice at temperatures set at 4 °C, 20 °C and 40 °C (baseline set at -20 °C values) to identify any changes over time.

Table 3-7 Stability study of AsBet in plaice. Values of $R_T \pm U_T$ over a 7-month period at temperatures of 4 °C, 20 °C and 40 °C (baseline set at -20 °C values).

	Time in months	*Temp °C*	$R_T \pm U_T$
AsBet	1		0.998 ± 0.03
	1.5		0.997 ± 0.05
	2	+ 4	1.001 ± 0.01
	3		1.020 ± 0.02
	4		1.034 ± 0.06
	5		1.002 ± 0.03
	7		1.036 ± 0.04
	1		1.022 ± 0.05
	1.5		0.990 ± 0.04
	2		1.025 ± 0.03
	3	+ 20	1.023 ± 0.03
	4		0.982 ± 0.04
	5		1.016 ± 0.02
	7		1.03 ± 0.04
	1		1.008 ± 0.06
	1.5		0.971 ± 0.07
	2		1.037 ± 0.04
	3		1.024 ± 0.03
	4	+ 40	0.962 ± 0.05
	5		0.989 ± 0.02
	7		1.009 ± 0.04

The plaice material was found to meet the criteria required for a sample to go forward as part of an inter-laboratory arsenic speciation comparison for the production of a series of certified reference materials.

3.4 Inter-laboratory comparison of arsenic and selenium species in all candidate reference materials

3.4.1 Instrumentation

ICP-MS measurements were performed using a VG Plasmaquad (2+, TJA Solutions, Winsford, Cheshire, UK), using the conditions described in Table 3-1, Section 3.2.1. Isotopes of mass 75 for arsenic and 77, 78 and 82 for selenium were used for recording measurements. A Perkin Elmer series 410 high pressure pump (Perkin Elmer, CT, USA) was used for control of eluent flow rates. A Rheodyne 7125 injection valve (Rheodyne, CA, USA) was used for column loading of sample digests. pH readings were taken using a 3010 pH meter (Jenway, Ltd., Essex, UK). Chemical and Reagents

3.4.2 Chemicals and reagents

All commercial chemicals were of analytical grade and used without further purification. Sodium selenate, sodium selenite, D,L-selenomethionine, D,L-selenocystine, arsenous acid (assay – 99.95-100.05 % purity), arsenic acid,

dimethylarsinic acid, (Sigma-Aldrich Chem. Co., Poole, Dorset, UK), arsenobetaine (BCR, Retieseweg, Belgium) monomethylarsonous acid (kindly donated by Dr. A. Moreda-Pineiro, University Santiago de Compostela, Spain) were used as stock solutions of 1000 μg ml^{-1} in terms of the element. They were stored in the dark at 4°C. Solutions of the compounds for daily use were prepared by appropriate dilution from the stock solutions using Milli-Q water (Milli-pore, Bedford, MA, USA).

The CRMs used in this study were DORM-2 (National Research Council), Rice flour NIES 10 c (National Institute for Environmental Studies, Ibaraki, Japan) Wheat flour 1567 a (NIES), and soil 'Montana' 2710 (NIES). A selenium-enriched yeast sample (Pharma Nord, Denmark) was used as a reference material for selenium determination.

Eluent solutions were prepared using Na_2SO_4, H_3PO_4, pyridine, and NH_4HCO_3, methanol, NH_3 (0.91 sp. gr.), HCOOH and CH_3COOH (Sigma-Aldrich).

All plastic/glassware was soaked in HNO_3 (10%, v/v) for a minimum of 24 hours and rinsed thoroughly with de-ionized water prior to use.

3.4.3 Chromatographic conditions

Arsenic speciation of candidate material fish, rice and chicken

Anion exchange HPLC was carried out using a column (250 x 4.6 mm) packed with Hamilton PRPX100 10 μm resin (Phenomenex, UK) with a guard column (50 x 4.6 mm) of the same material. The mobile phase employed a step gradient elution using a solution of 5 mM and 50 mM Na_2SO_4 at pH 10.2, adjusted with conc. NH_3. The programme for elution is given in Table 3-3, Section 3.2.3. An eluent flow rate of 1.2 ml min^{-1} was used throughout. A loop volume of 20 μl was used for sample loading of fish and rice and a loop volume of 100 μl was used for the chicken samples.

Cation-exchange HPLC was carried out using a Partisil column SCX 10 (250 x 4.6 mm, Phenomenex) packed with a silica gel of 10 μm particle size with a guard column (50 x 4.6 mm) of the same material. The mobile phase employed a 20 mM pyridine solution adjusted to pH 3 with HCOOH (98%, v/v) as an isocratic elution [148] with a flow rate of 1.2 ml min^{-1} used throughout.

Selenium speciation of candidate reference material yeast and wheat

Anion exchange HPLC was carried out using an IC Sep AN 1 column (250 x 4.6 mm, Phenomenex) packed with a 5 μm styrene – divinylbezene polymer resin with quaternary ammonium functional groups together with a guard column (50 x 4.6 mm) of the same material. The mobile phase, modified from work by Madsen

et al. [116] employed was a step gradient elution using a solution of 10 mM and 50 mM NH_4HCO_3 + 10% MeOH at pH 5, adjusted with glacial CH_3COOH. The program for elution is given in Table 3-8. A flow rate of 1.0 ml min^{-1} was used throughout. A loop volume of 100 µl was used for sample loading of yeast and wheat candidate reference materials.

Table 3-8 Elution programme for anion-exchange HPLC of selenium species in yeast and wheat candidate reference materials using a IC Sep AN1 column.

Concentration	*0 –3 mins*	*3 – 10 mins*	*10 – 15 mins*
10 mM NH_4HCO_3 + 10% MeOH adjusted to pH 5	100 %	0 %	100 %
50 mM NH_4HCO_3 + 10% MeOH adjusted to pH 5	0 %	100 %	0 %

Arsenic speciation for candidate reference material 'soil'

For the speciation of arsenic in soil, anion exchange HPLC was carried out using a column (250 x 4.6 mm) packed with Hamilton PRPX100 10 µm resin (Phenomenex, UK) with a guard column (50 x 4.6 mm) of the same material. The mobile phase employed a step gradient elution, modified from a method developed by Thomas *et al.* [161] using a solution of 2 mM and 50 mM H_3PO_4 at pH 6, adjusted with conc. NH_3. The programme for elution is given in Table 3-9.

A flow rate of 1.2 ml min^{-1} was used throughout. A loop volume of 20 µl was used for sample loading of the soil candidate reference material.

Table 3-9 Elution programme for anion exchange HPLC of arsenic species in soil using a Hamilton PRP X100 column.

Concentration	*0 – 3 mins*	*3 – 6 mins*	*6 – 15 mins*
2 mM H_3PO_4 (adjusted to pH 6)	100 %	0 %	100 %
50mM H_3PO_4 (adjusted to pH 6)	0 %	100 %	0 %

3.4.4 Sample preparation procedures

3.4.4.1 Microwave digestion for 'total' elemental concentration of arsenic in fish, rice, chicken and soil and selenium in yeast and wheat

Microwave bombs were pre-cleaned with 3 ml 69% v/v HNO_3 (Aristar, Fisons) on medium power for 2 mins. Samples of approximately 0.5 g (all sample types) were accurately weighed into the bombs and HNO_3 (conc., 3ml) and H_2O_2 (37% v/v, 1ml) were added. The bombs were loosely capped and left overnight to allow easily oxidizable material to be destroyed. After predigestion, the bombs were swirled gently, sealed tightly and microwaved on medium power for 1 - 2 mins, or until the sample was a clear colour with no residue (indicating a completed digest). The samples were transferred quantitatively into volumetric flasks and

made up to volume with deionized water. The samples and matrix matched standards were spiked with indium to give a final concentration of 100 μg l^{-1} In which acted as an internal standard prior to analysis by ICP-MS using the conditions described in Table 3-1.

3.4.4.2 Enzymolysis extraction of arsenic (in fish, rice and chicken) and selenium (in yeast and wheat) species

Fish and chicken

Samples of approximately 1.0 g of tissue were accurately weighed together with 0.1 g trypsin (Sigma-Aldrich, Dorset, UK) and approximately 20 ml NH_4HCO_3 (0.1 M, pH 8). The solutions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. The samples were centrifuged at 2500 rpm (fish) and14500 rpm (chicken) for 20 min and the supernatant transferred quantitatively to volumetric flasks made up to volume with the NH_4HCO_3 buffer. The samples and standards were spiked with caesium to give a final concentration of 100 μg l^{-1} Cs that acted as an internal standard prior to analysis by ICP-MS.

Rice

Samples of approximately 1.0 g of tissue were accurately weighed together with 0.1 g cellulase (Sigma-Aldrich, Dorset, UK) and approximately 20 ml CH_3COONH_4 (0.1 M, pH 5). The solutions were homogenized in a 'Potter'

homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. The samples were centrifuged at 2500 rpm for 20 min and the supernatant transferred quantitatively to volumetric flasks made up to volume with the CH_3COONH_4 buffer. The samples and standards were spiked with caesium to give a final concentration of 100 μg l^{-1} Cs that acted as an internal standard prior to analysis by ICP-MS.

Yeast

Samples of approximately 0.25 g of tissue were accurately weighed together with 0.025 g protease (Sigma-Aldrich, Dorset, UK) and approximately 20 ml NH_4HCO_3 (0.1 M, pH 8) [133]. The solutions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. The samples were centrifuged at 2500 rpm for 20 min and the supernatant transferred quantitatively to volumetric flasks made up to volume with the NH_4HCO_3 buffer. The samples and standards were spiked with caesium to give a final concentration of 100 μg l^{-1} Cs which acted as an internal standard prior to analysis by ICP-MS.

Wheat

Samples of approximately 1.0 g of tissue were accurately weighed together with 0.1 g trypsin (Sigma-Aldrich, Dorset, UK) and approximately 20 ml NH_4HCO_3 (0.1 M, pH 8). The solutions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at

37°C for a minimum of 4 hours. The samples were centrifuged at 14500 rpm for 20 min and the supernatant transferred quantitatively to volumetric flasks made up to volume with the NH_4HCO_3 buffer. The samples and standards were spiked with caesium to give a final concentration of 100 μg l^{-1} Cs which acted as an internal standard prior to analysis by ICP-MS.

To obtain mass balance data the residues from the enzyme extraction procedures were prepared for 'total' arsenic or selenium determination by the microwave digestion method described above.

3.4.4.3 H_3PO_4 microwave extraction for arsenic speciation in soil

For the determination of arsenic species in the soil samples an H_3PO_4 microwave modified extraction procedure, originally developed by Thomas *et al.* [161] was used. A Synthewave 402 focused microwave system (Prolabo, Fontenay-sous-Bois, France) was used for the extraction procedure. Samples of 0.25 g were accurately weighed into the digester flask and 25 ml of 1 M H_3PO_4 was added. A borosilicate glass rod stirrer was inserted which was computer controlled. The flask was placed in the cavity of the microwave digester and processed at a power of 45 W (equivalent to 120°C) for 20 mins. The solution was allowed to cool and transferred into a polyethylene centrifuge tube. The procedure was repeated as a washing cycle and added to the sample solution. The samples were then centrifuged at 2500 rpm for 15 min and the supernatant transferred quantitatively

to 50 ml volumetric flasks and made up to volume with 1 M H_3PO_4. From this solution, 2.5 ml was transferred into a 25 ml volumetric flask and made up to volume with Milli-Q water (overall x2000 dilution) for chromatographic and direct ICP-MS analysis. The samples and matrix matched standards were spiked with indium to give a final concentration of 100 μg l^{-1} In which acted as an internal standard prior to analysis.

3.5 Results and Discussion

When participating in inter-laboratory comparisons one of the most important analytical features is the traceability of the calibration solutions. A primary As_2O_3 standard, available commercially with a purity of 99.9%, was prepared and used throughout to calibrate results obtained when determining arsenic content. Where standards of the species, eg. AsBet, were used they were calibrated against the primary standard prior to use to confirm their concentrations. For quality control purposes method validation was confirmed using CRMs. A variety of CRMs have been used in order to match as closely as possible the matrix under investigation.

3.5.1 Results for total arsenic and arsenic species in fish, rice, chicken and soil candidate reference materials

The results, obtained for different sample matrices are summarized in Table 3-10. The results for 'total' elemental levels were determined directly using ICP-MS, with the introduction of 4% N_2 into the nebulizer gas flow when analyzing for arsenic. The results for the speciation of the elements were determined by anion exchange HPLC-ICP-MS, followed by integration of the peaks obtained using Mass Lynx computer software. Table 3-11 presents the results obtained for the CRMs used for each sample type under investigation for arsenic and arsenic species. The recoveries, shown in Table 3-12, were determined by spiking experiments with x2 and x4 the expected amount of the species present in the original sample. This was achieved by the drop-wise addition of the spiking solution in 2 ml of solvent for 1 g of sample allowing a minimum of 16 h contact time in controlled conditions for temperature and light. Following this, any remaining solvent was evaporated to dryness under a gentle stream of nitrogen. The spiked samples were then prepared in an identical manner to that of the non-spiked samples. Table 3-12 also gives the LODs for each of the species detected in the various samples, which were dependent on the method employed.

Table 3-10 Results for sample types in determination of total arsenic and species. Results given in mg kg^{-1} as the element.

	Total As HNO_3	Total As Extract	As^{III}	As^{V}	MMA	DMA	AsBet
Fish	38.9 ± 0.66 (2.6%)	39.7 ± 0.42 (1.6%)					39.0 ± 0.81 (3.2%)
Rice	0.185 ± 0.0041 (2.3%)	0.177 ± 0.0074 (3.9%)	0.0934 ± 0.0089 (9.0%)	0.0342 ± 0.0022 (6.1%)	0.0344 ± 0.0026 (7.2%)	0.0124 ± 0.0074 (5.6%)	
Chicken	0.157 ± 0.0066 (4.1%)	0.158 ± 0.0054 (3.2%)			0.0768 ± 0.0039 (4.8%)	0.0697 ± 0.0027 (3.6%)	0.0293 ± 0.0020 (6.6%)
Soil IPL 1	634 ± 36 (5.3%)	646 ± 14 (2.1%)	5.11 ± 0.19 (3.4%)	515 ± 15 (2.8%)	32.8 ± 1.5 (4.6%)	33.3 ± 1.0 (2.9%)	
Soil Mix 2	2274 ± 55 (2.3%)	2178 ± 67 (2.9%)	49.8 ± 2.2 (4.2%)	2068 ± 86 (3.9)			

* RSD in parentheses

Table 3-11 CRMS used for total element and species for sample types under investigation for arsenic. Results given in mg kg^{-1} as the element.

	Total HNO_3	Total extract	As^V	As^{III}	MMA	DMA	AsBet	Certified value
DORM-2 (fish samples)	18.78 ± 0.84 (1.8)	17.9 ± 0.82 (1.8)					16.44 ± 0.87 (2.1)	18.0 ± 1.1 As 16.4 ± 1.1 AsBet
DORM-2 (chicken samples)	18.1 ± 0.79 (1.7)						15.36 ± 1.5 (4.1)	18.0 ± 1.1 As 16.4 ± 1.1 AsBet
NIES 10 c (rice samples)	0.149 ± 0.0031 (0.8)	0.149 ± 0.0068 (4.6)	0.0383 ± 0.0056 (5.8)	0.0662 ± 0.0056 (3.4)	0.0315 ± 0.0028 (3.5)	0.0250 ± 0.0035 (5.6)		0.15
Montana 2710 (soil samples)	587 ± 7.9 (0.5)	516 ± 16 (1.4)	482 ± 32 (2.6)	3.06 ± 0.26 (3.4)				626 ± 38

* RSD in parentheses

Table 3-12 LODs for all samples under investigation for arsenic speciation. Results in $\mu g\ kg^{-1}$ as the element. Recoveries calculated from spiking experiments with x2 and x4 expected species concentrations.

	AsBet	*DMA*	*MMA*	As^{III}	As^{V}	*Recoveries*
Fish	0.56	0.42	0.89	0.96	0.64	100 ± 4 %
Chicken	0.92	0.69	1.78	1.01	0.82	100 ± 5 %
Rice	0.86	0.49	0.22	0.54	0.37	97 ± 5 %
Soil	-	0.13	0.23	0.20	0.27	95 ± 4 %
DORM-2	0.46	0.32	0.69	0.71	0.53	100 ± 5 %

The results obtained for the plaice sample are summarized in Table 3-10 with those from the CRM, DORM-2, being given in Table 3-11. The plaice used for this analysis comprised material that had been processed by IRMM. The total arsenic content was determined to be 38.9 ± 0.66 mg kg^{-1}, dry weight. The moisture content was less than 1%. Enzyme digestion of the material gave a complete recovery of the arsenic present (100%). AsBet was determined to be the only arsenic compound present giving a statistically comparable concentration to that found for the total arsenic. The chromatograms of the arsenic standards and that of the sample obtained by anion exchange HPLC-ICP-MS can be seen in Figures 3-9 and 3-10 (Figure 3-9 has been reproduced from Figure 3-3 for ease of comparison).

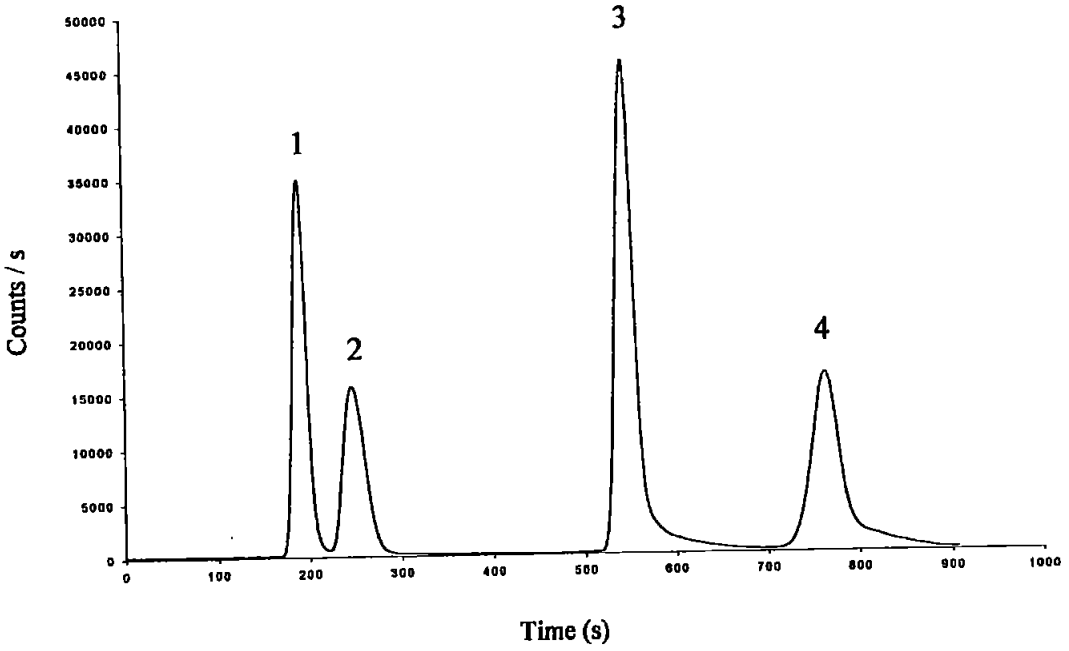


Figure 3-9 Chromatogram obtained using anion-exchange HPLC-ICP-MS of arsenic standards at 250 μg l^{-1}, 1=AsBet; 2= DMA; 3=MMA; 4=As^{V}. Conditions shown in Table 3-3.

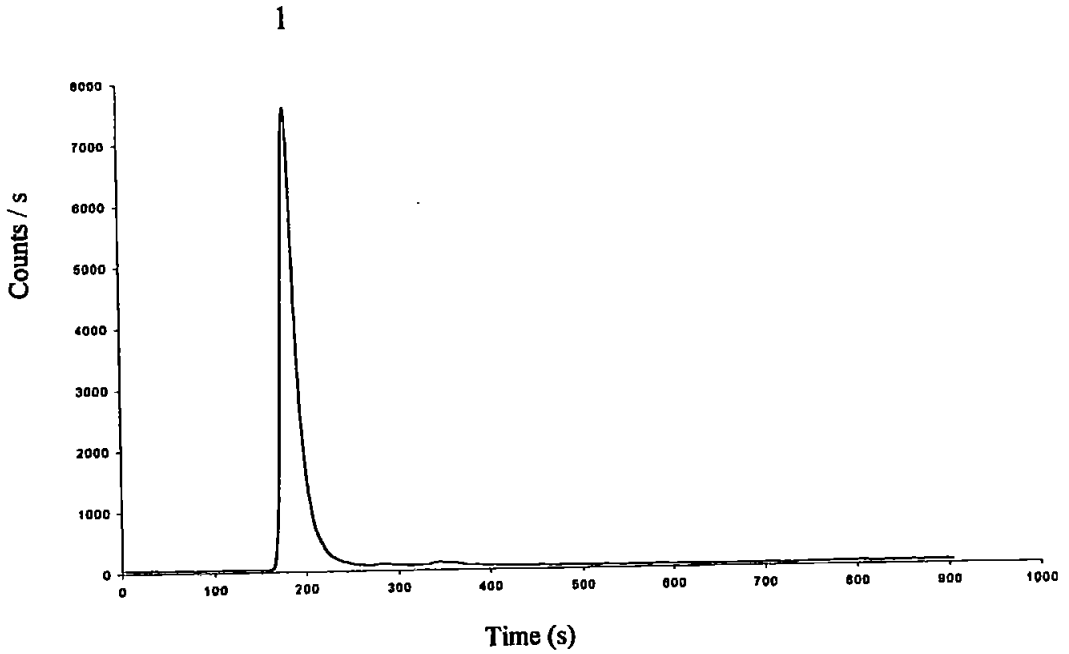


Figure 3-10 Chromatogram of plaice using anion-exchange HPLC-ICP-MS (conditions shown in Table 3-3). Peak 1 =AsBet

The results obtained for total arsenic in the CRM, DORM-2, were 18.78 ± 0.84 mg kg^{-1} and for AsBet were 16.44 ± 0.87 mg kg^{-1}. These results are in agreement with the values for which it is certified. The certified values are given in Table 3-11.

While it is widely acknowledged that much research has been carried out on the chemical forms of arsenic in marine fauna and flora, relatively little is known about the species present in foods of terrestrial origin. Rice was included in this study as it is one of the most important foodstuffs and is consumed daily by millions of people around the planet. Arsenic-contaminated drinking water has been responsible for cases of chronic arsenic poisoning [6]. However, the long-term use of arsenic-contaminated groundwater to irrigate crops, especially paddy fields has resulted in elevated arsenic concentrations in the soil [162]. Measuring the uptake of arsenic by rice plants will give an indication of the biochemical cycling of arsenic and the risks posed for consumers. The use of CRMs by analysts in this area is essential to validate methodology and results.

The result obtained for the total amount of arsenic in the rice sample was 0.185 ± 0.0041 mg kg^{-1}. The levels of arsenic found in terrestrial foodstuffs are commonly found to be within this range [163]. The speciation analysis demonstrated the presence of As^{V}, As^{III}, DMA and MMA. As^{III} accounted for 57% of the total arsenic present. Taking As^{III} and As^{V} together, as the inorganic component, accounted for 70% of the total. A study carried out by Heitkemper *et al.* [164] on a number of different rice types concluded that the inorganic arsenic could account

for between 11 and 91% of the total arsenic found. The higher amount was found in wild rice as opposed to long grain rice.

Chromatograms obtained for the speciation of the rice using anion exchange HPLC-ICP-MS can be seen in Figures 3-11 and 3-12. Figure 3-11 gives the elution pattern of four arsenic standards under consideration demonstrating that good resolution was obtained. Figure 3-12 shows the species present in the rice sample. The chromatogram of the arsenic standards in Figure 3-11 showed a slight decrease in retention times to the standards obtained in Figure 3-9 although the chromatographic conditions were identical. This phenomenon may be accounted for by the loss of functional groups on the stationary phase depending on how often the column has been used and the type of sample matrices introduced. The use of a guard column, with regular replacement, can prolong the life-time of a column. Calibration for each experiment prior to analysis of samples and regular checks throughout, ensured that an alteration of retention times and resolution over the lifetime of the column did not have a deleterious effect on the data collected.

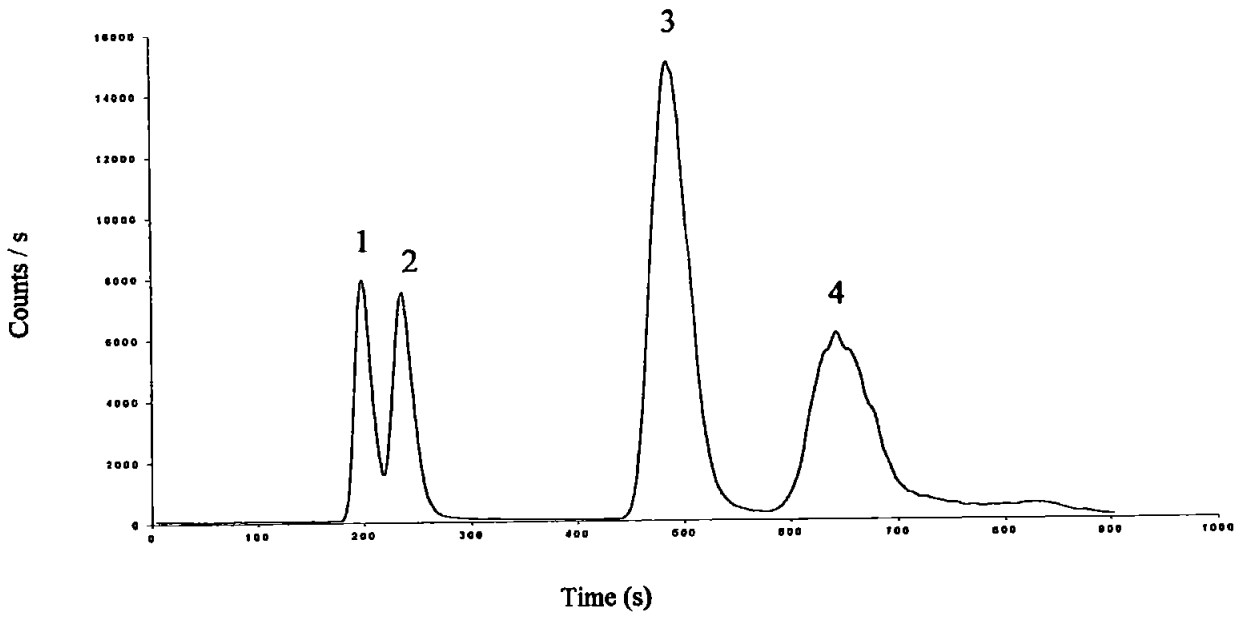


Figure 3-11 Chromatogram obtained using anion-exchange HPLC-ICP-MS of arsenic standards at 250 μg l^{-1}: 1 = AsBet; 2 = DMA; 3 = MMA; 4= As^{V} (conditions shown in Table 3-3).

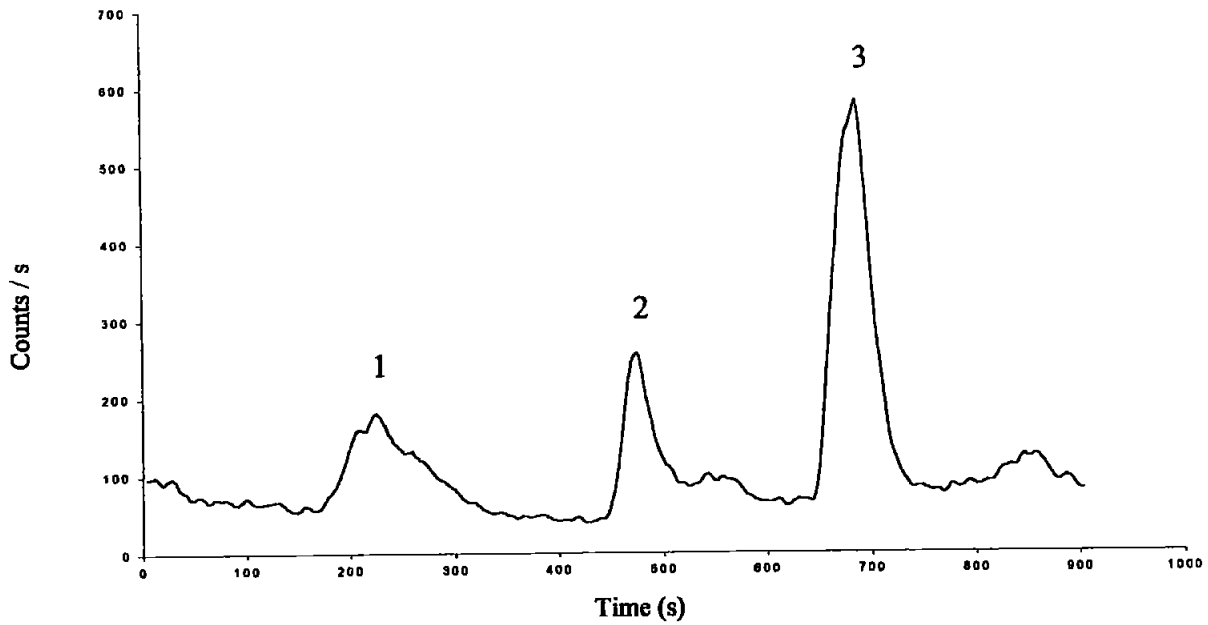


Figure 3-12 Chromatogram of rice sample by anion-exchange HPLC-ICP-MS (conditions shown in Table 3-3). Peaks: 1, DMA; 2, MMA; 3, As^{V}/As^{III}.

As previously discussed, identification was based partly on the matching of retention times of species with that of known standards but also more importantly with spiking experiments to account for matrix effects in shifting of retention times. The first peak in the sample chromatogram (Figure 3-12) showed undeniable signs of broadening and matched the AsBet and DMA standard range. Spiking experiments indicated that the peak was due to DMA. In addition, due to the eluent pH conditions (pH 10.2) employed here for anion-exchange HPLC it was found that As^{III} underwent on-column oxidation and therefore eluted as As^{V}. Due to the uncertainty surrounding the identification and quantification of the species found, cation-exchange HPLC was subsequently employed. The chromatograms of the arsenic standards by cation-exchange HPLC at pH 3 can be seen in Figure 3-13 and the sample in Figure 3-14. Under these conditions it was found that MMA and As^{V} co-eluted with the solvent front, neither having any affinity for the stationary phase due to their anionic character. The peaks assigned to As^{III} and DMA can clearly be seen. By mathematical manipulation of the data from both sets of peak integrations (cation and anion), it was possible to calculate concentrations for the As^{III}, As^{V}, DMA and MMA found. Unfortunately, the confidence limits for rice were larger than for other samples suggesting the introduction of errors, possibly as a result of de-convolution of data. However, it must be considered that where species concentrations are particularly low, as in this case, uncertainty in the data will be magnified.

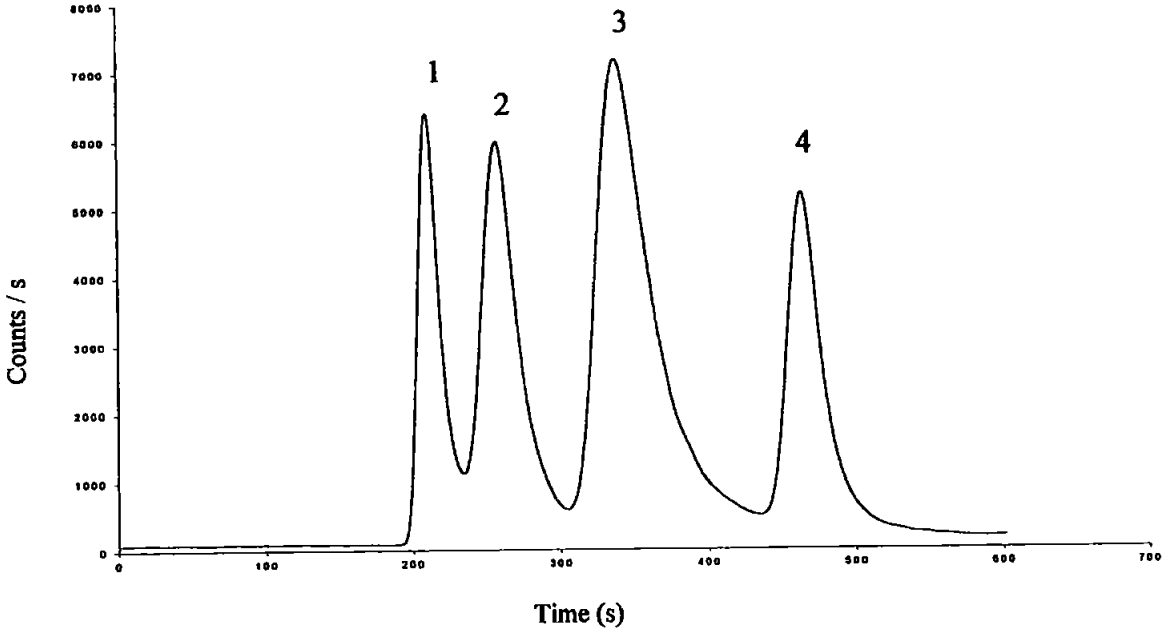


Figure 3-13 Chromatogram of arsenic standards, 250 μg l^{-1}, using cation-exchange HPLC-ICP-MS: 1 = As^{V}; 2 = As^{III}, 3 = DMA; 4 = AsBet.(Partisil SCX 10 column with 20 mM pyridine, pH 3)

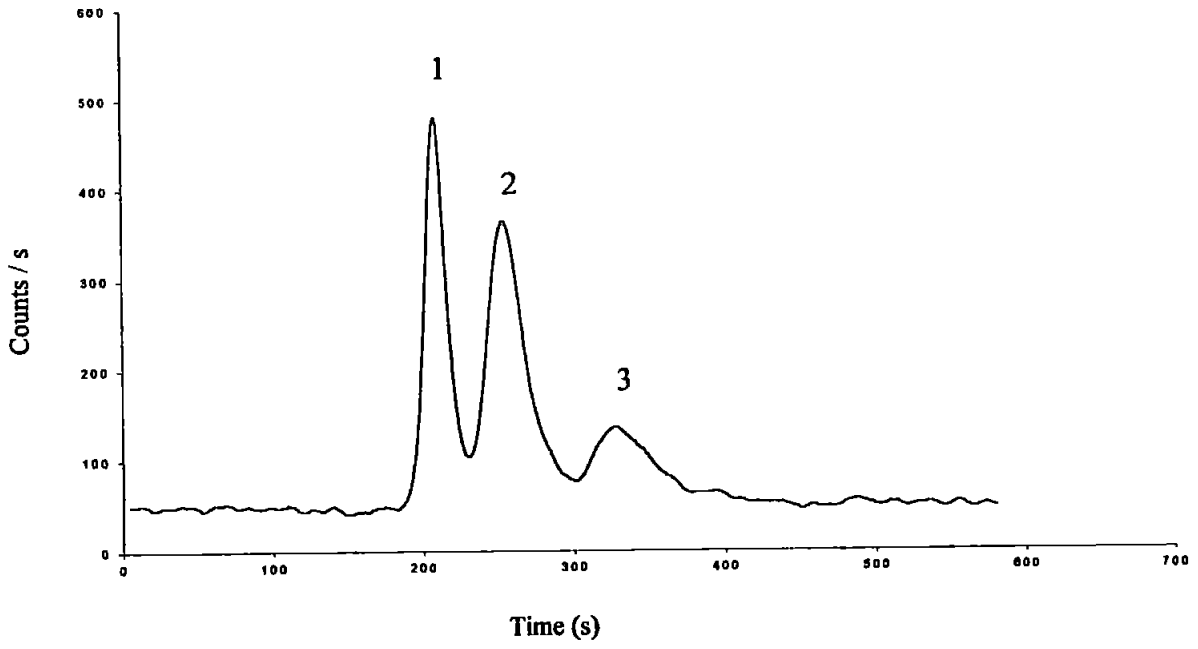


Figure 3-14 Chromatogram of rice sample by cation-exchange HPLC-ICP-MS using a Partisil SCX 10 column and 20 mM pyridine eluent at pH 3. Peaks: 1, As^{V}/MMA; 2, As^{III}; 3, DMA.

The rice flour CRM used to validate the experimental method gave an indicative value for 'total' arsenic and not a certified amount and hence gave no prior indication of the species likely to be present. However, the experimentally obtained value for total arsenic in the CRM was in agreement with the stated value and a mass balance of the species identified and quantified gave a similar amount. This suggested that the mathematical manipulations did not adversely affect the results.

The presence of the four arsenic species in the rice was not unexpected as it is known that vegetation will take up arsenic and speciated arsenic from the surrounding soil and water. It is also known that biomethylation of inorganic arsenic to MMA and DMA is known to occur in terrestrial plants as a detoxification pathway. The distribution of arsenite and arsenate in the rice was found to be in a ratio of approximately 2:1 with MMA at a similar level to arsenate and DMA an order of magnitude lower. Abedin *et al.* [165] have shown that inhibition of arsenate uptake in the presence of phosphate occurs whereas arsenite transport is unaffected. This is indicative of plants having differing uptake mechanisms for the two forms of inorganic arsenic, the result being that plants will take up arsenite and arsenate in amounts that do not necessarily reflect the environmental level of each. It is also known that redox conditions in the soil, the presence of humic substances [166] and other elements together with micro-organism activity will have an effect on the bioavailability and type of arsenic species present. Research by Yang *et al.* [167] has demonstrated that As^V in soils containing a high level of Fe_2O_3 with a concomitant low pH is less bio-available.

The presence of MMA and DMA in the rice may be attributed to its uptake from the surrounding environment or as a metabolite within the rice as a detoxification route for inorganic arsenic. Without information regarding the arsenic species present in the vicinity that the rice was grown in, one can only speculate on the ratios obtained here for the inorganic arsenic available and as to whether the MMA and DMA were exogenously or endogenously derived.

During the rearing of the chicken for this study, the addition of fishmeal to the chicken feed was kept below 1% as it can detract from the overall taste of the chicken on human consumption. Any As^{III} and As^{V} present in the chicken feed or water supply will be absorbed via the gastro-intestinal tract, methylated via s-adenosylmethionine and excreted as MMA and DMA. However, an average urinary metabolite distribution of inorganic arsenic, MMA and DMA is approximately 20 : 15 : 65 (in humans) [168] suggesting that not all inorganic arsenic is transformed. As^{III} was added to the drinking water of the chickens, with the controlled supervision of a veterinary surgeon, in the expectation that it would increase the total amount of arsenic accumulated and give rise to a variety of arsenic species in the chicken sample. It was calculated that the chickens received a total of approximately 1.9 mg As^{III} in this way over their short and happy life-time and ensured that they did not suffer or die from arsenic poisoning!

The results obtained for the chicken material gave total values of 0.157 mg kg^{-1} as dry weight, which lie well within the limit of 1 mg kg^{-1} (dry weight) allowable for arsenic in foodstuffs [169]. The chromatograms using anion exchange HPLC-

ICP-MS of arsenic standards and the chicken sample can be seen in Figures 3-15 and 3-16. Speciation analysis demonstrated the presence of AsBet which accounted for approximately 16% of the total arsenic found. Two other methylated forms of arsenic, DMA and MMA, were also found to be present and accounted for 40 and 44% of the total arsenic, respectively. No inorganic arsenic was detected. Chromatograms of arsenic standards and the chicken sample by cation-exchange HPLC are shown in Figures 3-17 and 3-18 confirming species identification.

The presence of DMA and MMA may be accounted for by the uptake of inorganic arsenic from the water supply being metabolized via a detoxification pathway with subsequent methylation of the arsenic occurring. The presence of AsBet is more difficult to explain. Although AsBet is thought to be the final product in the arsenic cycle, it is thought not to be formed *de novo* from ingested inorganic arsenic [14]. Any production by symbiotic organisms would not be expected to be seen in the muscle tissue of the chicken. In recent years it has been standard practice to administer fishmeal in chicken feed. Fishmeal can contains high levels of AsBet. Although the amount of fishmeal was kept below 1% to avoid the distinctive taste of fish in chicken meat, it is possible that AsBet may still have been accumulated and/or have been present in other feeds administered and given up to the day of slaughter.

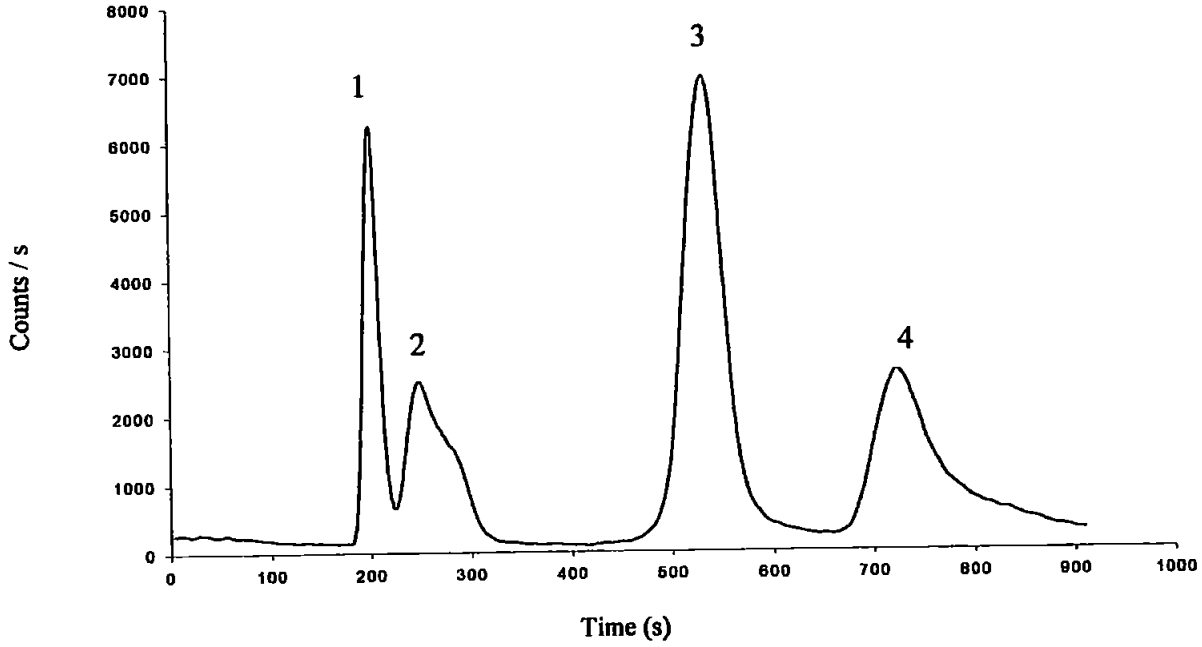


Figure 3-15 Anion-exchange HPLC-ICP-MS of arsenic standards for determination of arsenic in chicken. Peaks 1, AsBet; 2, DMA; 3, MMA; 4, As^{V}, 50 μg l^{-1} each. Conditions shown in Table 3-3.

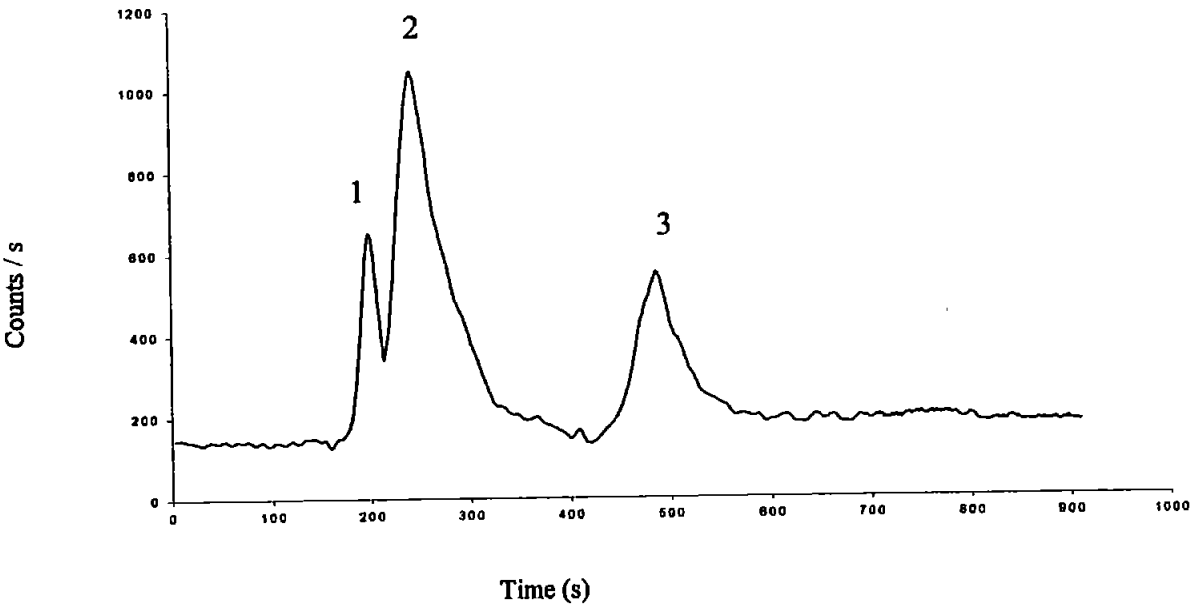


Figure 3-16 Anion-exchange HPLC-ICP-MS, using conditions shown in Table 3-3, of chicken sample demonstrating species present. Peaks: 1, AsBet; 2, DMA; 3, MMA.

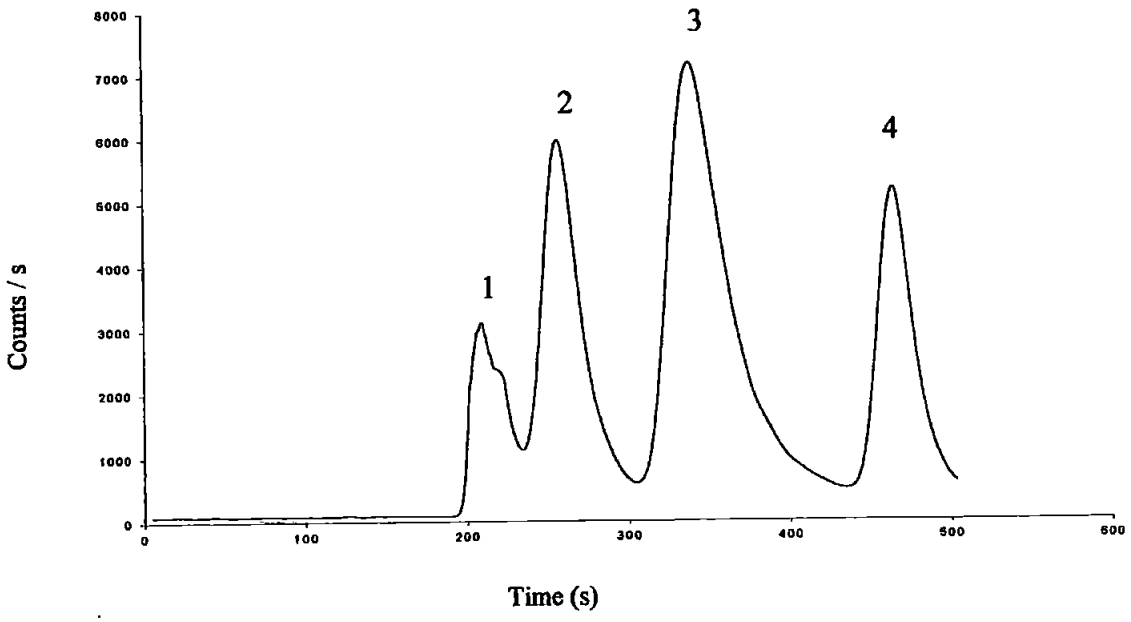


Figure 3-17 Cation-exchange HPLC-ICP-MS, using a Partisil SCX 10 column and 20 mM pyridine eluent at pH 3, of arsenic standards. Peaks: 1, MMA; 2, As^{III}; 3, DMA; 4, AsBet.

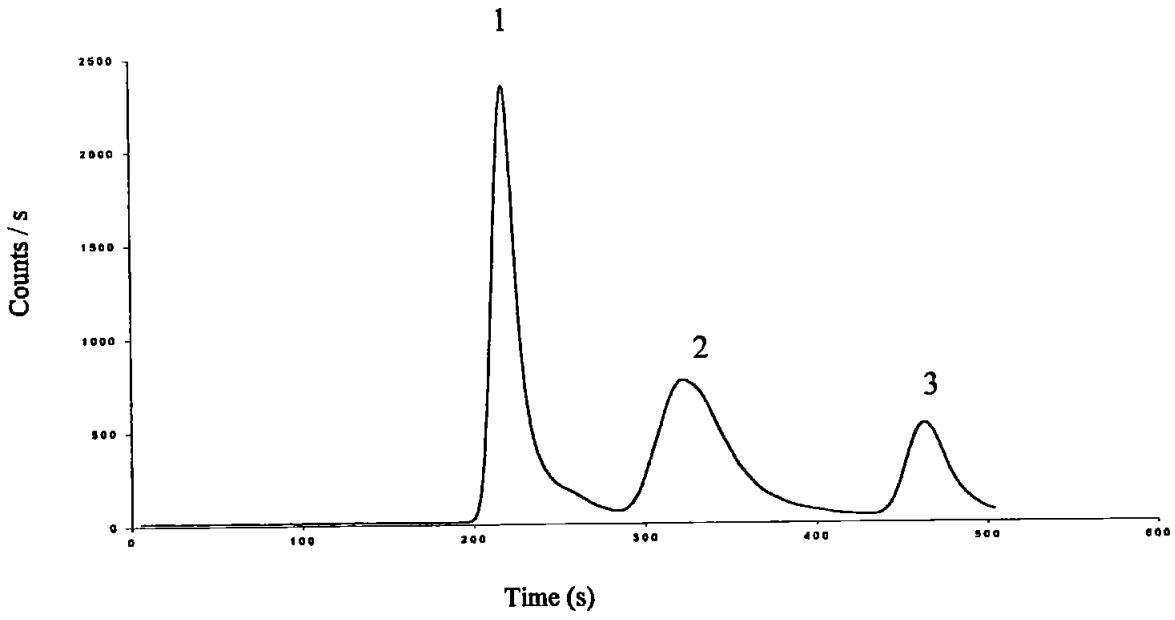


Figure 3-18 Cation-exchange HPLC (conditions as above) of chicken sample. Peaks: 1, MMA; 2, DMA; 3, AsBet.

As it was the most concentrated, the last of the materials tested for total arsenic and speciated arsenic was the soil. Two types of soil were entered for the CRM feasibility study, IPL 1 and IPL mix 2. Various samples of soil were collected from sites in France known to be contaminated with arsenic. It was decided that IPL 1 would contain a artificially polluted mixture of As^{III}, MMA and DMA due to the lack of these species being found in the natural soil samples and IPL mix 2 consisted of a mixture of soils from four different sites.

In the preparation of the soil samples for analysis a closed microwave digestion method utilizing HNO_3 and H_2O_2 was employed for total 'available' arsenic determination. Due to the nature of soil, usually possessing high levels of silicates, HF digestion is a more commonly employed technique. However, the use of HNO_3 in this case was used to obtain 'available' arsenic from a safer chemical mixture which was easier to handle. Experiments on the CRM showed that the results obtained gave satisfactory recoveries of the arsenic spikes and extraction of the original arsenic. The results for the CRM Montana 2710 soil sample gave totals of 587 ± 7.8 mg kg^{-1} which were within the experimental limits of error having a certified value of 626 ± 38 mg kg^{-1}. Using spiking experiments to determine recoveries, a value of 95 % was obtained for the CRM as well as the soil samples under investigation. Determination of extraction efficiency based on the level of arsenic certified to be present in the CRM alone gave a value of 94 % suggesting that using HNO_3 as opposed to HF did not adversely affect the experimental outcome. It also indicated that neither the CRM

nor the soil samples studied contained arsenic intrinsically bound in larger fractions to a silica matrix.

The results obtained for the soils gave total arsenic levels of 634 ± 36 mg kg^{-1} for mix 1 and 2274 ± 55 mg kg^{-1} for mix 2. The speciation of arsenic in these two soil samples by anion exchange HPLC-ICP-MS demonstrated the presence of As^{V}, As^{III}, MMA and DMA in mix 1 and the presence of As^{V} and As^{III} only in mix 2. The chromatograms of the arsenic standards used can be seen in Figure 3-19 and the chromatogram of soil mix 1 and mix 2 can be seen in Figures 3-20 and 3-21, respectively. Baseline resolution of the standard and sample peaks was obtained together with excellent peak shape.

The species identified in soil mix 1 was in keeping with the sample preparation of these soils at the Institute Pasteur de Lille, France and subsequently at IRMM. As mentioned previously, mix 1 was artificially enhanced with As^{III}, DMA and MMA, although these species were found to be at much lower levels than the indigenous As^{V}. Mix 2 had a much higher total level of arsenic with it being predominantly in the form of As^{V}. These results might suggest that most of the As^{III} that may have been originally present had been oxidized to As^{V} during the extraction procedure. However, the modified procedure used, previously developed by Thomas *et al.* [155] was rigorously tested for this phenomenon. The optimum conditions arrived at, which have been recreated here, avoid this problem.

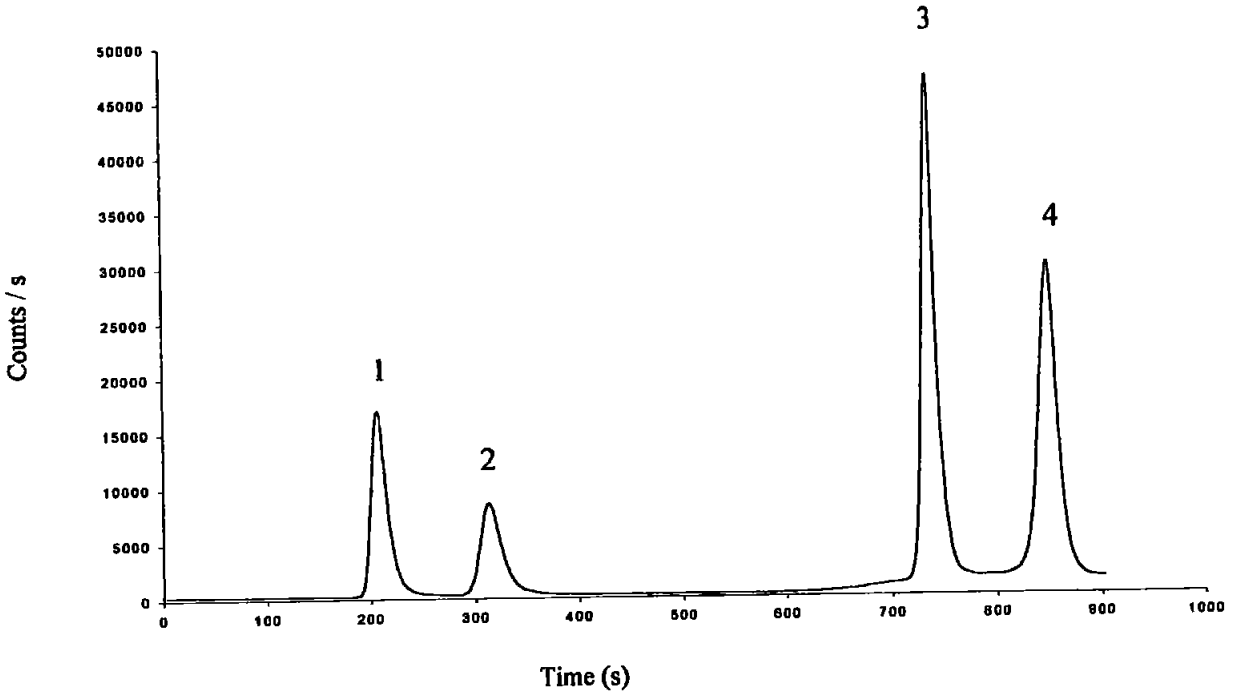


Figure 3-19 Chromatogram of arsenic standards by anion-exchange HPLC with phosphoric acid mobile phase, conditions shown in Table 3-9, for soil analysis: 1 = As^{III}; 2 = DMA; 3 = MMA; 4 = As^{V}, 250 μg l^{-1}.

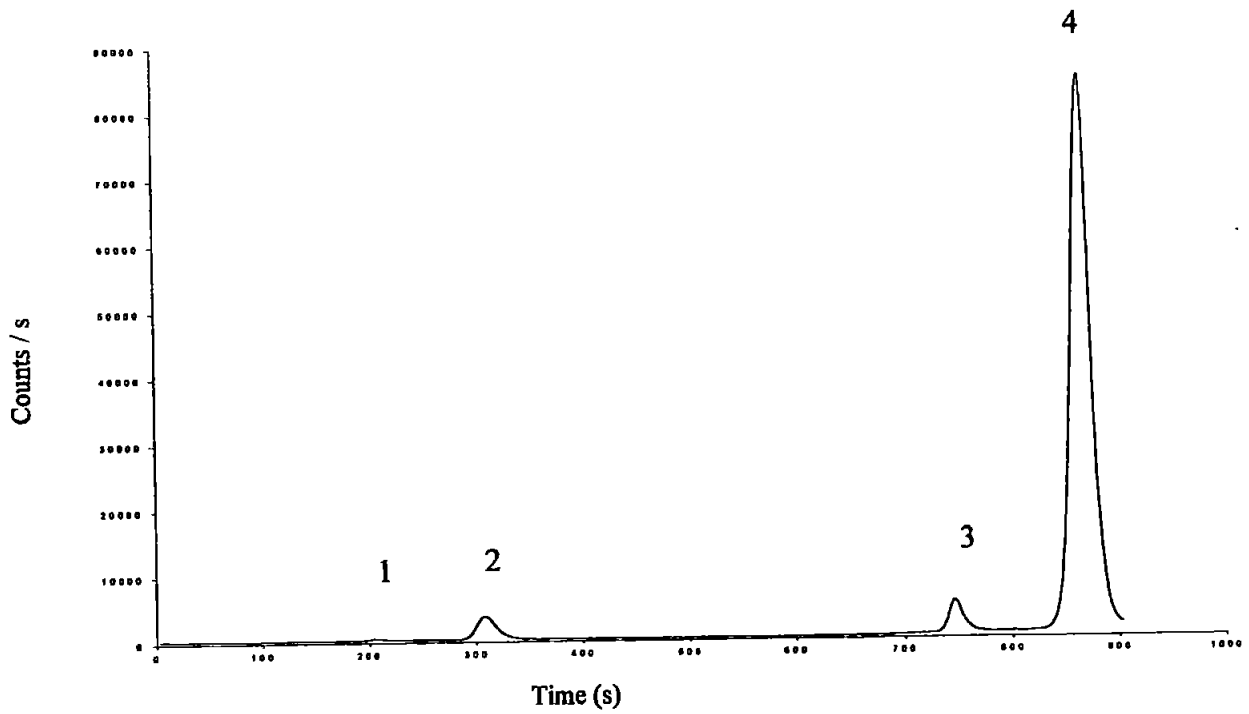


Figure 3-20 Chromatogram by anion-exchange HPLC-ICP-MS (conditions shown in Table 3-9) of soil IPL mix 1. Peaks: 1, As^{III}; 2, DMA; 3, MMA; 4, As^{V}.

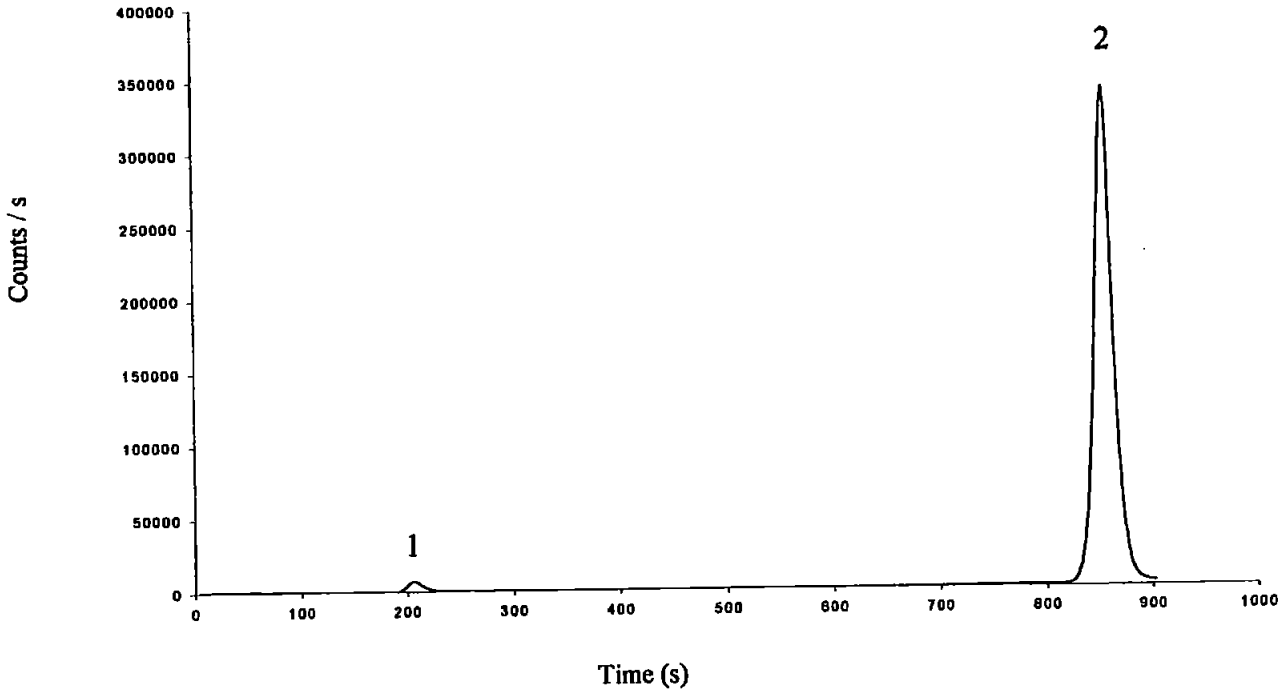


Figure 3-21 Chromatogram by anion-exchange HPLC-ICP-MS, conditions shown in Table 3-9, of soil mix 2. Peaks: 1, As^{III}; 2, As^{V}.

Other, more acceptable reasons for the predominance of arsenate over arsenite include the physical and chemical conditions of the soil, micro-organism activity and the presence of other elements in their various oxidation states. Research carried out by Bohari *et al.* [170] suggested that As^{V} was the predominant arsenic compound found in soil with As^{III} and MMA being minor components and DMA only being found in one soil sample. The varying levels of arsenic found in the two soil mixes is not unprecedented. Background levels of arsenic in soil have, on average, been reported as being in the region of 7 mg kg^{-1} [9] whereas levels in contaminated areas have been reported as high as 3000 mg kg^{-1} [171].

3.5.2 Results for total selenium and selenium species in yeast and wheat candidate reference materials

The remaining samples in this study, wheat and yeast, were analyzed for total selenium and selenium species. Techniques using aqueous extractions have proved to be successful in liberating free or weakly bound inorganic selenium and selenoamino acids [172]. However, where selenium is incorporated into protein structures enzymolysis has proved to be more effective [133]. For the extraction of selenium from wheat, a brief experimentally based comparison between cellulase and trypsin was made. The results demonstrated that trypsin gave the best extraction efficiency and was, therefore, the preferred enzyme. For the yeast, protease is well established as the enzyme of choice. The results for total selenium and selenium species in wheat and yeast are shown in Table 3-13.

Table 3-13 Results for determination of total selenium and species in yeast and wheat. Results given in mg kg^{-1} as the element.

	Total Se HNO_3	*Total Se* *Enzyme extract*	*SeMet in enzyme extract*	*SeCys in enzyme extract*
Wheat	0.652 ± 0.034 (4.9%)	0.674 ± 0.031 (4.4%)	0.413 ± 0.020 (4.6%)	0.202 ± 0.011 (5.1%)
Yeast	1091 ± 69 (6.0%)	1037 ± 58 (5.3%)	988 ± 55 (5.3%)	ND*

RSD given in parentheses
* ND = not detected

Table 3-14 shows the results obtained for total selenium and selenium species in the CRM (NBS 1576 a) for wheat and the yeast reference material. Limits of detection for the species identified in the yeast and wheat samples and CRM NBS 1576a (SeMet and SeCys) are shown in Table 3-15.

Table 3-14 CRMS used for total element and species for sample types under investigation for selenium. Results given im mg kg^{-1} as the element.

	Totals HNO_3	Totals Enzyme	SeMet	SeCys	Recommended value (total Se)
PharmaNord (yeast samples)	1282 ± 24 (1.0)	1158 ± 99 (3.4)	1098 ± 168 (6.2)		1300
NBS 1576 a (wheat samples)	1.091 ± 0.01 (3.7)	1.092 ± 0.078 (2.8)	0.613 ± 0.047 (3.1)	0.293 ± 0.029 (3.9)	1.1

* RSD in parentheses

Table 3-15 LODs for wheat and yeast samples under investigation for selenium species. Results given in μg kg^{-1} as the element. Recoveries calculated from spiking experiments with x2 and x4 expected species concentrations.

	SeMet	*SeCys*	*Recoveries*
Yeast	1.03	3.12	94 ± 4 %
wheat	4.08	4.89	92 ± 5 %
NBS 1576a (CRM – wheat)	3.96	3.54	99 ± 4 %

Various methods for the separation and determination of selenium compounds have been reported in the literature that include ion exchange, reversed phase and ion-pairing chromatography [80, 92], as discussed in Chapter 1. The choice of species studied by these techniques has been predominantly dictated by the availability of commercial standards such as selenate, selenite, SeMet and SeCys. The major selenoamino acid found in humans is selenocysteine [40], of which SeCys is the dimer. Unfortunately, selenocysteine is extremely unstable therefore unavailable commercially and in-house synthesis has also proved to be fruitless.

The separation of selenium compounds in this study has been based on anion-exchange chromatography. A step gradient elution programme was required to elute the Se^{VI} anion, which possesses a doubly negative charge at the pH conditions employed (pH 5). In addition to this, its size and charge density give it a high affinity for the stationary phase. Step gradients are sometimes undesirable

[60] as they may increase the analysis time due to an equilibration stage being required and can adversely affect the baseline stability and in turn the LODs; the latter being of concern when low levels of species are present. The chromatogram of the selenium standards used is shown in Figure 3-22 and that of the wheat sample in Figure 3-23. The baseline instability can be seen quite clearly in Figure 3-23, the wheat sample. Having determined experimentally that no inorganic selenium species were present in the sample it was decided that an isocratic programme using only the lower concentration eluent could safely be employed when analyzing the sample material. This allowed for greater precision when quantifying the data obtained. The RSD calculations on data obtained for speciation by HPLC-ICP-MS are similar to those obtained from direct ICP-MS measurements for total selenium content.

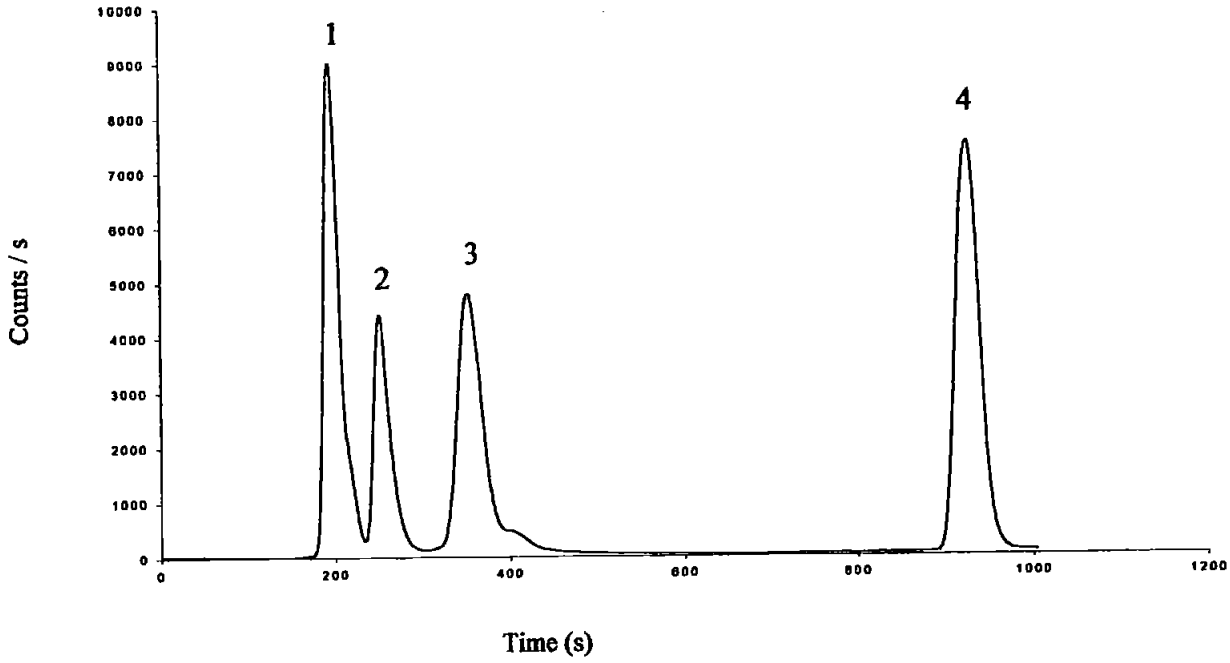


Figure 3-22 Chromatogram of selenium standards, 100 μg l^{-1}, by anion-exchange HPLC, conditions shown in Table 3-8: Peaks: 1, SeCys; 2, SeMet; 3, Se^{IV}; 4, Se^{VI}.

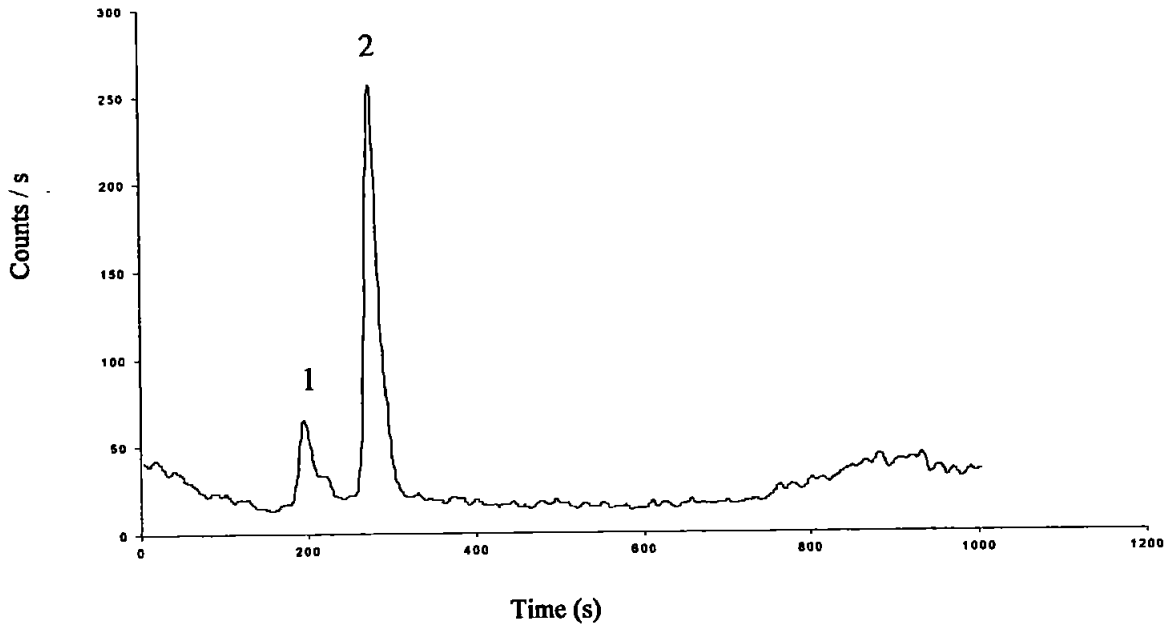


Figure 3-23 Chromatogram of wheat sample by anion-exchange HPLC-ICP-MS, conditions shown in Table 3-8. Peaks: 1, SeCys; 2, SeMet.

The results for wheat, summarized in Table 3-13, gave a total selenium concentration of 0.652 ± 0.034 mg kg^{-1} with an extraction efficiency of 94% using trypsin as the enzyme. The speciation analysis of the wheat flour by anion exchange HPLC-ICP-MS demonstrated the presence of SeMet and SeCys in an approximate ratio of 2:1. The results obtained for the CRM 1576 a (NBS) gave a similar distribution of SeMet and SeCys although the total selenium content was determined to be 1.091 ± 0.010 mg kg^{-1}, which was in agreement with the reference value for this material. The distribution of SeMet and SeCys in the wheat was expected as SeMet is the most abundant selenium compound found in plants.

The discussion in Chapter 1 highlighted the need for selenium-enriched diets to supplement the low naturally occurring levels of selenium in foodstuffs. A particular example of wheat was chosen to demonstrate the effects of human selenium intake when it was grown in seleniferous soils as opposed to soils with low selenium levels. Wheat grown in seleniferous soils is an extremely useful way to remedy low selenium diets as many people rely on wheat, or similar cereals, as a staple food source [173]. Plants differ in their ability to accumulate selenium in their tissues and according to the amounts present it is possible to classify them as hyper-accumulators (accumulation in the range hundreds to several thousands mg kg^{-1}, dry weight), intermediate accumulators (up to 1000 mg kg^{-1}) and non-accumulators (less than 100 mg kg^{-1}) [114]. Hyper-accumulators are thought to protect themselves from the toxic effects of selenium by reducing the intracellular content of SeMet and SeCys, which would

otherwise be incorporated into proteins with a damaging effect on the plant, by accumulating selenium in non-protein selenoamino acids such as Se-methylselenocysteine ($CH_3SeCH_2CH(NH_2)COOH$) and SeCystathionine ($COOH(NH_2)CHCH_2CH_2SeCH_2CH(NH_2)COOH$). The low level of selenium found in this wheat sample suggests that it is a non-accumulator. The presence of the selenium in the wheat as organic selenium is of interest in research regarding the anti-carcinogenic properties attributed to selenium. Although it is not clear in which chemical form, or combination of forms, selenium has its most effective anti-carcinogenic effect, it is known that organic selenium species are less toxic to humans than inorganic forms and hence more suitable for inclusion into the diet with less risk of accidentally induced toxic symptoms.

The final sample in this study was a selenized yeast. The results are summarized in Table 3-13 for the sample, Table 3-14 for the material used as a reference to validate the methodology and LODs are presented in Table 3-15 together with extraction recoveries. Once again, as no inorganic selenium was detected using a step gradient elution programme, analysis of the organic selenium was performed with an isocratic mobile phase of 10 mM NH_4HCO_3 + 10% MeOH at pH 5. The results obtained for the sample of yeast under investigation gave a total selenium content of 1091 ± 69 mg kg^{-1} by HNO_3 microwave digestion and a total of 1037 ± 58 mg kg^{-1} by protease digestion.

The chromatograms of the selenium standards and yeast sample are presented in Figures 3-24 and 3-25, respectively. By the matching of retention times with

known standards, the only compound identified in the speciation of the yeast was SeMet at a concentration of 988 ± 55 mg kg^{-1}. As observed previously, an improvement in the resolution of the standards, SeCys and SeMet, can be seen in Figure 3-24 over that of Figure 3-22. This was attributed to replacement of the column packing material between the wheat and yeast experiments.

The extraction efficiency was determined to be 94%. The Pharma Nord yeast, although not a CRM, used for method validation had a reference value of 1300 mg kg^{-1} for 'total' selenium (manufacturer – Pharma Nord, Vejle, Denmark). Again, SeMet was the only compound identified in this material at a concentration of 1098 ± 168 mg kg^{-1}. This is slightly lower than the mean reference value but does fall within the limits obtained for total selenium analysis when taking into account 95% confidence limits. The results obtained for the yeast sample are in keeping with other research which has demonstrated that SeMet is the most abundant compound found in yeast [174, 36].

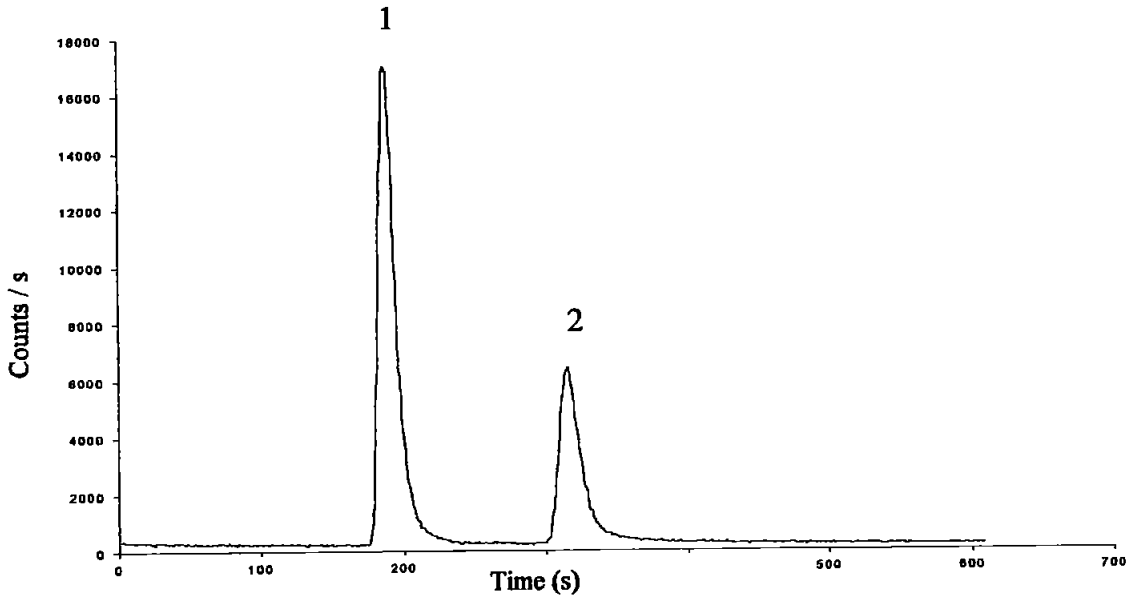


Figure 3-24 Chromatogram of selenium standards, 1 mg l^{-1}, by anion-exchange HPLC-ICP-MS, condition s shown in Table 3-8. Peaks: 1, SeCys; 2, SeMet.

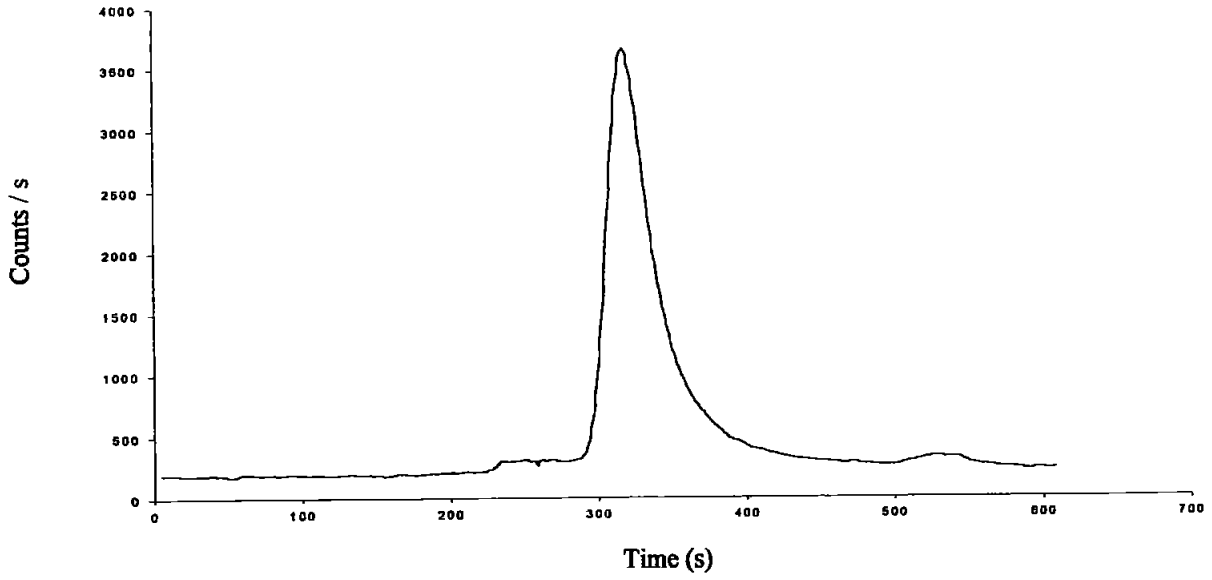


Figure 3-25 Chromatogram of yeast sample by anion-exchange HPLC-ICP-MS, conditions shown in Table 3-8. Peak = SeMet

At the time of writing, the results obtained for 'total' selenium and selenium species in yeast from the other participating laboratories in this feasibility study were available and are shown in Table 3-16.

Table 3-16 Results from participating laboratories for 'total' selenium and SeMet in the yeast sample ^{147} – Lab no. 1 = Plymouth

Lab no.	*Total Se* $Mg\ kg^{-1}$ *as Se*	*STDev*	*SeMet* $Mg\ kg^{-1}$ *as SeMet*	*STDev*
1	1090.077	2.569	2434.655	150.131
2	1101.761	3.117	1455.875	28.461
3	1452.500	3.563	2133.588	7.124
4	1540.167	33.234	-	-
5	1351.375	1.237	-	-
6	1121.683	35.214	2167.883	16.075
7	1266.667	9.428	2318.000	98.524
8	1451.433	0.896	2598.433	106.396
9	1418.667	53.504	-	-
10	596.750	2.475	-	-
11	186.746	2.621	-	-
12	1352.625	10.076	1927.625	8.662
13	1416.833	108.187	204.702	1.336

The results obtained by Lab. 1 (Plymouth) are comparable with the results obtained by other participants leading to greater confidence in the overall analysis for selenium and selenium species in yeast. SeMet was found to be the predominant species present.

Since the work of Clark *et al.* [47] on the use of selenized yeast for cancer chemoprevention, the development of selenium supplements has grown in popularity. In a review by Spallholz [41], it is well-documented that both the bioavailability and toxicity of selenium are closely related to the type of selenium species present . In respect to humans, organic selenium compounds are regarded as being less toxic than inorganic forms. The high levels of organic selenium relative to that of inorganic levels of selenium found in yeast has directed research towards yeast as a dietary supplement.

3.6 Conclusions

The preliminary survey of a variety of fish types was undertaken to identify one sample that would go forward into a feasibility study to assess its suitability in becoming a reference material, certified for arsenic and arsenic species. For this purpose, a fish sample containing a number of species, without having to artificially introduce any, would have been preferred. However, all the fish samples investigated (plaice, monk, hake, haddock, cod, coley, pollack and whiting) demonstrated the presence of AsBet as the major compound. In light of this, the fish chosen was plaice as it contained an appropriate amount of arsenic

as AsBet and the digestion with trypsin proved to be the most successful in terms of extraction efficiency. The 'total' arsenic concentration in the plaice was determined as 39.5 ± 1.7 mg kg^{-1} with 38.7 ± 2.7 mg kg^{-1} being in the form of AsBet. The enyzme extraction efficiency was determined as being 98%.

Homogeneity and stability studies performed on the plaice, after processing by IRMM, indicated that sample units were homogenous, within and between unit, at a sample weight not less than 0.25 mg and that the material was stable within the temperature range of 4°C to 40°C and a time-scale of 0 to 7 months.

An intra-laboratory and inter-laboratory comparison was carried out on six sample matrices (fish, rice, chicken, soil, wheat and yeast) as part of a feasibility study for future production of reference materials certified for arsenic and selenium species. Speciation analyses of all materials in this feasibility study have been presented together with the CRMs employed for method validation. Primary standards were used, where available, for quality control and assurance purposes in the traceability of measurements. These results will be presented, at a later date, in a technical meeting to be organized by the project coordinators with the data from all other participating laboratories for statistical evaluation. However, the results for the speciation of the yeast material from participating laboratories were available at the time of writing and have been presented here. Comparison of the inter-laboratory results for yeast showed that values obtained by the Plymouth partners were in agreement with the majority of participants, with the notable exceptions of laboratories 10 and 11. Overall, the inter-

laboratory comparison of the results for the yeast sample indicate that a high standard of laboratory analysis and practice has been adhered to with excellent method validation and quality assurance.

Results from the fish, rice, chicken, soil, yeast and wheat demonstrated the effective use of HNO_3/H_2O_2 microwave digestion techniques for determination of 'total' elemental concentrations together with efficient enzymolysis extraction procedures for species determination, whilst maintaining the integrity of the species. Extraction efficiencies were between 92-100% for all sample types investigated. The use of ICP-MS for determination of arsenic and selenium was proficient, providing results with excellent precision and, in the case of yeast, excellent accuracy. For speciation analysis, the use of a variety of HPLC methods coupled with ICP-MS provided optimum resolution between species in the same matrix and, in the case of soil, this was exceptional. Corroborative evidence obtained by the use of anion and cation-exchange chromatography for species identification and determination (in fish, rice and chicken) proved highly beneficial.

The use of different methodologies in different laboratories with independent calibration should lead to results for these materials, if in agreement, that will have a low uncertainty and no operationally-defined limits on the subsequent use of the material as a CRM.

As previously stated, speciation analysis can focus on the clear identification of a specific chemical species or form of an element and its quantification. The preparation and certification of a variety of environmentally and biologically relevant materials for total element and species concentrations is required in order to facilitate laboratory analysis where the accuracy of a result for a trace element in a sample may be assessed by the parallel analysis of a certified reference material with a matrix composition and concentration that closely matches that of the sample analyte.

Chapter four

4 The extraction and speciation of selenium compounds in bio-natured nutrients

4.1 Introduction

The essential trace element of selenium, which humans obtain from their diet, largely from cereals, fish, poultry and meat, plays a crucial role in many biological activities. While current research [44] has implicated selenium as an anti-carcinogen, it has also become apparent that dietary intake in some regions of the world is falling. This is predominantly due to the consumption of food grown in areas of low natural abundance of selenium in the soil [173]. It has become increasingly popular for companies to make selenium supplements available for human consumption in order to redress the balance. Current UK dietary reference values are set at 75 μg and 60 μg daily for men and women, respectively [2].

Research studies carried out by Clark *et al.* [175] have shown that the use of supplementation with selenium (as selenized yeast) substantially decreased the incidence of cancers, in particular prostate cancers (63% decrease), colorectal cancers (58% decrease) and lung cancers (46% decrease) with an overall reduction in mortality by 50%. The Bonelli study, presented at the Annual Research Conference of the American Institute for Cancer Research in Washington DC, 1998 [120], reported a statistically significant reduction of metachronous adenomas of the large bowel through intervention with 200 μg

daily of selenium as L-selenomethionine. At present the PRECISE trial (Prevention of Cancer through Intervention with Selenium), a randomized, placebo-controlled, double-blind study using selenized yeast as the active supplement, is in progress [120]. If it confirms the findings of the Clark and Bonelli studies it may have a significant impact for the future of public health policy-making [120]. However, the use of selenium supplements raises issues surrounding which selenium species or combination of species confers anti-carcinogenic properties.

Selenomethionine is the principal form of selenium found in plant-based foods and is thought to be efficiently absorbed and stored in the body [176]. Inorganic selenium is generally excreted more rapidly and is thought to be more toxic than selenomethionine [177]. However, studies of the chemical form of selenium in supplements demonstrates a wide variety of compounds present, ranging from simple inorganic sodium selenite and sodium selenate and organic selenomethionine to the more complex derivatives found in selenium-enriched yeasts [54]. In a study carried out by B'Hymer and Caruso [131], brands were found to have near label values for total selenium content but dramatically different profiles for the actual chemical form of the selenium in the supplement. One brand appeared to contain all inorganic selenium and another, despite claims of being only selenomethionine, contained greater than 50% inorganic selenium. The form of selenium most effective in providing anti-oxidant defense and anti-carcinogenic properties is not specified although it is thought that the absorption and bioavailability conform to the following trend [178]:

selenomethionine > selenium yeast > selenate > selenite

It is known that different types of yeast demonstrate a variety of selenium species [54, 44] and that selenium supplements do not always contain the form specified by the manufacturers [131]. There is also a dearth of information regarding proportions of organic to inorganic selenium in many foodstuffs. Although it is well-documented that selenomethionine is more easily absorbed than inorganic forms of selenium and provides a more stable selenium profile in the body, its bioavailability when compared to other selenium species is still in doubt [49].

Bio-natured nutrients, an alternative to the commercially available selenium supplements and selenized yeast, can be used to combine a particular nutrient with its native food constituents. In this way, each nutrient is matched to an appropriate food and is delivered into the body with the components, or co-nutrients, with which it is associated. By doing this, it is thought that absorption in the gastrointestinal tract will be increased and its bioavailability in the body improved. The latest foods to be developed, based on this technology, are a selenium-enriched yeast and a probiotic bacteria, *Lactobacillus bulgaricus*, for the supplementation of selenium in the diet.

The aim of the following study was to identify and quantify the selenium species present in two samples of novel, previously unstudied, bio-natured nutrients in order to assist in establishing an understanding of the absorption and

bioavailability of the novel selenium compounds; these nutrients being: i) a selenized yeast from a new process and: ii) a probiotic bacteria-based dried milk sample (Biogurt®). Specific interest was directed towards extraction efficiencies involving a number of established and new sample preparation procedures and the need to retain species integrity. Selenium speciation was performed using methodology based upon anion-exchange HPLC coupled with ICP-MS detection. The selenized yeast material, previously validated as part of an inter-laboratory feasibility study for a candidate reference material (Chapter 3), was used as the reference for method validation purposes.

Speciation analysis requires that the endogenous selenium species are extracted without modification of their chemical form or disturbance to the equilibrium existing between the various species present. To achieve this, a number of extraction techniques were compared for overall extraction efficiency and for species stability using recovery values from spiking with selenium standards. Comparison between the techniques and evaluation of results should highlight the most effective system for speciation extraction. From this, separation and detection using bi-dimensional ion-exchange HPLC-ICP-MS will provide qualitative and quantitative information regarding the selenium species present in the yeast and Biogurt® samples.

4.2 Experimental

4.2.1 Instrumentation

ICP-MS measurements were performed using a VG Plasmaquad 2+ (TJA Solutions, Winsford, Cheshire, UK), using the operating conditions described in Table 1-1. A Perkin Elmer series 410 high pressure pump (Perkin Elmer, CT, USA) was used for control of the chromatographic eluent. A Rheodyne 7152 injection valve (Rheodyne, Cotati, CA, USA) together with a 100 µl volume sample loop was used for on-column sample introduction. pH readings were taken using a 3010 pH meter (Jenway, Ltd., Essex, UK).

Table 4-1 ICP-MS operating conditions for the determination of 'total' selenium and selenium species in yeast and Biogurt® by HPLC-ICP-MS, using isotopes 77, 78 and 82.

ICP-MS	Plasma Quad 2+	
	V-groove nebulizer	
	Double-pass, water cooled Scott type spray chamber	
	Fassel torch – 1.5 mm bore injector	
Parameters	Nebulizer flow rate	0.81 l min^{-1}
	Coolant gas flow rate	13.1 l min^{-1}
	Auxiliary gas flow rate	0.8 l min^{-1}
	Forward power	1350 W
	Dwell time	500 ms

4.2.2 Scanning electron microscopy

A JEOL JSM 6100 electron microscope (Oxford Instruments, Oxford, UK) interfaced with an Oxford CT 1500 cryo-trans low temperature station (Oxford instruments) was used to view yeast cell structure.

4.2.3 Chemicals and reagents

All commercial chemicals were of analytical grade and used without further purification. Sodium selenate, sodium selenite, selenomethionine and selenocystine (Sigma Aldrich Chem. Co., Poole, Dorset, UK) were used as stock solutions of 1000 $\mu g\ ml^{-1}$ as the element. They were prepared using Milli-Q water (Milli-pore, Bedford, MA, USA) and stored in the dark at 4°C. Solutions of the compounds for daily use were prepared by appropriate dilution from the stock solutions. Methanol (Fisher Chemicals), 25% tetramethylammonium hydroxide (TMAH) in MeOH and 25% potassium hydroxide in MeOH (Sigma Aldrich) were used in the preparation of solutions for extraction procedures. Cellulase, protease type XIV, trypsin and pancreatin (Sigma-Aldrich) were used for enzymatic extraction procedures. Buffer solutions were prepared from ammonium hydrogen carbonate and ammonium acetate (Sigma-Aldrich). Hydrochloric acid (Fisher Chemicals) was used for digestion procedures and neutralization of KOH solutions.

4.2.4 Chromatographic conditions for selenium speciation

Anion-exchange chromatography

Experiments using anion-exchange HPLC were carried out using a Dionex AS 11 column (250 x 4.1 mm, Dionex) packed with a 10 μm styrene – divinylbezene polymer resin with quarternary ammonium functional groups together with a guard column (50 x 4.1 mm) of the same material. The mobile phase used was a step gradient elution employing a solution of 10 mM and 50 mM NH_4HCO_3 + 10% MeOH at pH 5, adjusted with CH_3COOH. The program for elution is given in Table 4-2. An eluent flow rate of 1.0 ml min^{-1} was used throughout.

Table 4-2 Elution programme for anion-exchange HPLC of selenium species using a Dionex AS 11 column

Concentration	*0 –3 mins*	*3 – 10 mins*	*10 – 15 mins*
10 mM NH_4HCO_3 + 10% MeOH (adjusted to pH 5)	100 %	0 %	100 %
50 mM NH_4HCO_3 + 10% MeOH (adjusted to pH 5)	0 %	100 %	0 %

Cation-exchange chromatography

Cation-exchange HPLC was carried out using a Partisil SCX 10 column (250 x 4.6 mm, Phenomenex) packed with a silica gel of 10 μm particle size with a guard column (50 x 4.6 mm) of the same material. The mobile phase employed

an isocratic elution using 20 mM pyridine solution adjusted to pH 3 with HCOOH (98%, v/v) and with an eluent flow rate of 1.0 ml min^{-1} being used throughout.

4.2.5 Sample preparation procedures

HNO_3 microwave digestion for 'total' selenium determination in samples

Microwave bombs (Savillex, Minetonka, Minnesota, USA) were pre-cleaned with 3 ml 69% v/v HNO_3 (Primer, Fisons, Loughborough, UK) in a Perfecto 800 W microwave (DeLonghi, Italy) oven on medium power for 2 mins. Samples of approximately 0.25 g were accurately weighed into the bombs and 4 ml HNO_3 (69%, v/v) together with 1 ml H_2O_2 (37%, v/v) were added. The bombs were loosely capped and left overnight to allow easily oxidised material to be destroyed. After predigestion, the bombs were swirled gently, sealed tightly and microwaved on medium power for 1 - 2 mins, or until the sample was a clear colour with no residue (indicating a completed digest). The samples were transferred quantitatively to volumetric flasks and made up to volume with 2% HNO_3 giving an overall dilution of x2000. The samples and standards were spiked with indium to give a final concentration of 100 μg l^{-1} Indium (In), which acted as an internal standard, prior to analysis by ICP-MS using the conditions described in Table 4-1. The internal standard was used to correct for instrumental drift (sample viscosity effects, mass transport, etc.) over the analysis period.

Eleven extraction procedures for the determination of selenium species were evaluated using the 'new processed' selenized yeast sample (referred to as yeast A from here onwards) and the Biogurt® sample together with the previously analyzed candidate reference material, selenized yeast from Pharma Nord (Pharma Nord, Vejle, Denmark), acting as a reference material. To date, there are no commercially available CRMs for selenium species in yeasts. The Pharma Nord selenized yeast which has been extensively measured and also has a concentration of selenium similar to that of the yeast sample under investigation, suitably fits the validation requirements for this study.

Enzymolysis extraction procedures

1. Samples of approximately 0.25 g were accurately weighed together with 0.025 g protease XIV (Sigma-Aldrich, Dorset, UK) and approximately 20 ml NH_4HCO_3 (0.1 M, pH 8). The solutions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. The samples were centrifuged at 2500 rpm for 20 min, the supernatant transferred quantitatively to volumetric flasks and made up to volume with the NH_4HCO_3 buffer. Samples and standards were spiked with caesium to give a final concentration of 100 $\mu g\ l^{-1}$ Cs that acted as an internal standard prior to analysis. Cs was used instead of In due to the change in pH conditions and reduced solubility for In at pH 8. Protease type XIV, a non-specific protease which breaks peptide bonds of any protein present in the sample, results in amino acid

information only. The buffer employed was NH_4HCO_3 (0.1 M, pH 8) as the optimum activity of protease type XIV is at pH 8 [133].

2. Samples of approximately 0.25 g were accurately weighed together with 0.025 g cellulase (Sigma-Aldrich, Dorset, UK) and approximately 20 ml CH_3COONH_4 (0.1 M, pH 5). The solutions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. The samples were centrifuged at 2500 rpm for 20 min, the supernatant transferred quantitatively to volumetric flasks and made up to volume with the CH_3COONH_4 buffer giving a final dilution of x2000. Samples and standards were spiked with caesium to give a final concentration of 100 $\mu g\ l^{-1}$ Cs that acted as an internal standard prior to analysis.

3. Samples of approximately 0.25 g were accurately weighed together with 0.025 g pancreatin (Sigma-Aldrich, Dorset, UK) and approximately 20 ml NH_4HCO_3 (0.1 M, pH 8). The solutions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. The samples were centrifuged at 2500 rpm for 20 min, the supernatant transferred quantitatively to volumetric flasks and made up to volume with the NH_4HCO_3 buffer. Samples and standards were spiked with caesium to give a final concentration of 100 $\mu g\ l^{-1}$ Cs that acted as an internal standard prior to analysis. Pancreatin

contains a mixture of enzymes including amylase, trypsin, lipase, ribonuclease and protease.

4. The final enzyme extraction procedure employed trypsin. Samples of approximately 0.25 g were accurately weighed together with 0.025 g trypsin and approximately 20 ml NH_4HCO_3 (0.1 M, pH 8). The solutions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. The samples were centrifuged at 2500 rpm for 20 min, the supernatant transferred quantitatively to volumetric flasks and made up to volume with the NH_4HCO_3 buffer. Samples and standards were spiked with 100 $\mu g\ l^{-1}$ Cs that acted as an internal standard prior to analysis. Trypsin is an enzyme that will break peptide bonds next to specific amino acids, namely arginine and lysine. This results in polypeptides being broken into shorter chains.

MeOH:H_2O extraction procedures

5. Samples of approximately 0.25 g were accurately weighed into polyethylene centrifuge tubes to which 10 ml MeOH:H_2O solution (75:25) was added. The samples were placed in an ultrasonic bath for 40 min, centrifuged at 3000 rpm for 15 min and the supernatant transferred to round-bottomed flasks. The procedure was repeated twice more and the washings were combined in the flasks (total of 30 ml). The samples were taken to dryness by rotary evaporation at 40°C. Sample residues, following rotary evaporation, were re-

dissolved in Milli-Q water and transferred quantitatively into volumetric flasks and made up to volume with an overall dilution of x2000. Samples and standards were spiked with 100 μg l^{-1} Cs that acted as an internal standard prior to analysis.

6. The above MeOH:H_2O extraction technique was repeated using sample material that had been freeze-dried prior to use.

HCl digestion procedure of samples

7. Samples of approximately 0.25 g were accurately weighed into polyethylene centrifuge tubes to which 35 ml 0.01 M HCl (pH 2) was added. Hydrochloric acid at pH 2 was chosen as it closely mimicked the pH found in the human stomach. They were placed in a shaking water bath at 37^{O}C for a minimum of 4 hrs then centrifuged with the supernatant being transferred quantitatively to volumetric flasks and made up to volume in HCl (0.01 M, pH 2). Samples and standards were spiked with 100 μg l^{-1} In that acted as an internal standard prior to analysis.

8. Multi-step extraction procedure using enzymolysis techniques (protease) and MeOH

   The following extraction technique comprised three stages of extraction and analysis

- to accurately weighed samples, of approximately 0.25 g, in polyethylene centrifuge tubes 25 ml 0.1 M NH_4HCO_3 (pH 8) was added. They were placed

in a shaking water bath at 37°C for a minimum of 4 hrs. Following centrifugation at 3000 rpm for 15 min, the supernatants were transferred quantitatively to volumetric flasks and made up to volume with the NH_4HCO_3 buffer with a final dilution of x2000. Samples and standards were spiked with 100 μg l^{-1} Cs that acted as an internal standard prior to analysis.

- to the residues from the first stage, 0.025 g protease was added together with 35 ml 0.1 M NH_4HCO_3 and placed in a shaking water bath at 37°C for a minimum of 4 hrs. Following centrifugation, the supernatants were transferred to volumetric flasks and made up to volume with the NH_4HCO_3 buffer. Samples and standards were spiked with 100 μg l^{-1} Cs that acted as an internal standard prior to analysis.
- To the residues from the second stage, a solution of 10 ml MeOH:H_2O 75:25 was added and the samples placed in an ultrasonic bath. for 40 min, then centrifuged at 3000 rpm for 15 min and the supernatant transferred to round-bottomed flasks. The procedure was repeated twice more and washings were combined in the flasks (total of 30 ml). The samples were taken to dryness by rotary evaporation at 40°C. Sample residues, following rotary evaporation, were re-dissolved in Milli-Q water and transferred quantitatively into volumetric flasks. Samples and standards were spiked with 100 μg l^{-1} Cs that acted as an internal standard prior to analysis.

9. Potassium hydroxide (KOH), 25% v/v, in MeOH solution.

Samples of approximately 0.25 g were accurately weighed into polyethylene centrifuge tubes to which 35 ml 0.1 M NH_4HCO_3 (pH 8) was added. They were placed in a shaking water bath at 37^{O}C for a minimum of 4 hrs, then centrifuged with the supernatant being decanted to waste. To the residues, 3 ml KOH 25% in MeOH was added and sonicated at 70^{O}C for 4 hours. The solutions were neutralized with 3 ml HCl (50% v/v), transferred quantitatively to volumetric flasks and made up to volume with Milli-Q water. Samples and standards were spiked with 100 μg l^{-1} Cs that acted as an internal standard prior to analysis.

10. Tetra-methylammonium hydroxide (TMAH), 25 % v/v, in MeOH

Samples of approximately 0.25 g were accurately weighed into polyethylene centrifuge tubes to which 35 ml 0.1 M NH_4HCO_3 (pH 8) was added. They were placed in a shaking water bath at 37^{O}C for a minimum of 4 hrs, then centrifuged with the supernatant being decanted to waste. To the residues, 3 ml TMAH 25% in MeOH was added and sonicated at 70^{O}C for 4 hours. The solutions were neutralized with HCl (50% v/v, 3 ml), transferred quantitatively to volumetric flasks and made up to volume with Milli-Q water. Samples and standards were spiked with 100 μg l^{-1} Cs that acted as an internal standard prior to analysis.

11. Multi-step extraction procedure using HCl and protease enzymolysis techniques.

The following extraction technique comprised two stages of extraction and analysis.

- Samples of approximately 0.25 g were accurately weighed into polyethylene centrifuge tubes to which 35 ml 0.01 M HCl (pH 2) was added. They were placed in a shaking water bath at $37^{O}C$ for a minimum of 4 hrs. The solutions were neutralized by the addition of 0.1M NaOH (approximately 3 ml).
- To this solution, protease was added and the samples placed in a shaking water bath as before for a minimum of 4 hrs. The samples were centrifuged and the supernatant transferred quantitatively to volumetric flasks. Samples and standards were spiked with 100 $\mu g\ l^{-1}$ Cs that acted as an internal standard for the analysis.

Mass balance calculations

The nitric acid microwave digestion technique, previously described, was used for the determination of any remaining selenium in residues following the various extraction procedures for the purposes of mass balance calculations.

4.3 Results and discussion

The results obtained from the extraction procedures detailed, are shown in Table 4-3. All results are given in mg kg^{-1} as the element. Extraction efficiencies were calculated based upon the 'total' selenium HNO_3 microwave digest values obtained and are shown in Table 4-4, together with mass balance data. The yeast sample obtained from Pharma Nord was not subjected to all extraction procedures as the selenium content was adequately retrieved by enzymolysis using either protease XIV or trypsin, following which, it was successfully analyzed by HPLC-ICP-MS for species determination.

The use of enzymolysis techniques for the extraction of selenium from biological samples without species conversion is frequently reported in the literature. A method developed by Gilon et *al.* [133] using protease with > 90% extraction efficiencies of the total selenium has been widely used by workers [179, 180]. Protease, a proteolytic enzyme, is capable of breaking peptide bonds of any protein present in a sample. This allows measurement of selenoamino acids but with the subsequent loss of information concerning, if present, any original selenium-containing proteins [181].

The results obtained in this study when using protease gave 90% extraction efficiencies for the selenized yeast samples obtained from Pharma Nord. However, the results obtained for yeast A and the biogurt were 19% and 15%, respectively, compared with the 'total' selenium values determined by the HNO_3

digest (nominal values specified by the manufacturers for yeast A are 1000 mg kg^{-1} and for Biogurt® 2000 mg kg^{-1}). The remaining selenium was found to be present in the residue. In view of this, enzymes were tested whose site of activity on the sample matrix may provide an alternative digestion process and hence, an improved extraction of the selenium species.

Table 4-3 Total selenium determination by all extraction techniques. Results given in mg kg^{-1} as the element, Se, and RSDs in parentheses.

Extraction technique		*PharmaNord yeast*	*Yeast A*	*Biogurt®*
HNO_3 microwave -	T[1]	1282 ± 24 (1.02)	984 ± 12 (0.6)	1980 ± 95 (2.5)
Protease –	T	1157 ± 73 (3.5)	190 ± 5.1 (1.6)	301 ± 27 (4.6)
	R[2]	123 ± 6	837 ± 41 (2.7)	1640 ± 111 (3.5)
Cellulase –	T	771 ± 35 (2.5)	123 ± 14 (5.9)	743 ± 46 (3.3)
	R	466 ± 25	632 ± 14 (1.2)	1055 ± 111 (3.2)
Pancreatin -	T	NP[3]	117 ± 4.5 (2.1)	411 ± 11 (1.5)
Trypsin -	T	1305 ± 92 (3.7)	109 ± 6.7 (3.4)	352 ± 42 (6.1)
MeOH:H_2O -	T	390 ± 19 (2.6)	141 ± 0.85 (0.3)	411 ± 30 (3.8)
	R	782 ± 49	616 ± 22 (1.9)	1666 ± 87 (2.9)
MeOH:H_2O -	T	NP	140 ± 16 (6.1)	527 ± 25 (2.5)
Freeze-dried -	R		513 ± 29 (3.01)	1380 ± 1.9 (0.07)
HCl -	T	NP	74.9 ± 5.5 (4.1)	1405 ± 79 (3.1)
	R		305 ± 5.1 (0.9)	42.6 ± 1.1 (1.5)
Stage i) NH_4HCO_3 -	T	180 ± 3.5 (1.1)	70.5 ± 6.1 (4.5)	1222 ± 102 (4.3)
Stage ii) protease -	T	NP	174 ± 16 (4.6)	44.8 ± 2.7 (3.5)
Stage iii) MeOH:H_2O -	T	NP	3.08 ± 0.39 (7.3)	53.6 ± 7.1 (6.6)
	R		278 ± 12 (2.4)	219 ± 22 (5.1)
KOH -	T	NP	714 ± 60 (4.7)	237 ± 22 (5.1)
TMAH -	T	NP	789 ± 74 (5.4)	292 ± 29 (5.6)
HCl + protease -	T	1191 ± 108 (4.7)	74.9 ± 2.6 (3.5)	934 ± 80 (4.6)
	R	87 ± 6	491 ± 29 (3.1)	155 ± 27 (9.4)

[1] T – totals in solution
[2] R – totals in residues, by HNO_3 microwave digestion, from individual techniques
[3] NP – not performed

Table 4-4 Extraction efficiencies and mass balance data for samples under investigation. Results are given in mg kg^{-1} as the element, Se.

	Extraction efficiencies, %			*Mass balance, %***		
	Pharma Nord	*Yeast A*	*Biogurt®*	*Pharma Nord*	*Yeast A*	*Biogurt®*
Protease	90 ± 4.6	19 ± 0.73	15 ± 1.9	99 ± 5	104 ± 6	98 ± 12
Cellulase	60 ± 1.6	12 ± 1.5	38 ± 3.9	96 ± 5	77 ± 4	91 ± 10
Pancreatin	NP	12 ± 0.6	21 ± 1.4		NP	NP
Trypsin	102 ± 8.9	11 ± 0.8	18 ± 2.8	102 ± 8.9	NP	NP
MeOH:H_2O	30 ± 2.0	14 ± 0.25	21 ± 2.4	91 ± 6	77 ± 3	105 ± 11
MeOH:H_2O Freeze-dried	NP	14 ± 1.8	27 ± 2.4		66 ± 6	96 ± 6
HCl	NP	7.2 ± 1.4	71 ± 7.1		39 ± 1	73 ± 8
Stage i) NH_4HCO_3	14 ± 2.5	7.3 ± 0.70	62 ± 7.7		53 ± 4	77 ± 11
Stage ii) protease	NP	18 ± 1.8	2.1 ± 0.24			
Stage iii) MeOH:H_2O	NP	0.3 ± 0.04	2.7 ± 0.51			
KOH	NP	73 ± 7.3	12 ± 1.6		NP	NP
TMAH	NP	80 ± 8.9	11 ± 2.1		NP	NP
HCl + protease	93 ± 10	7.6 ± 0.4	47 ± 6.0	99 ± 6	58 ± 4	55 ± 7

*NP = not performed

** Mass balance = (enzyme totals + residue totals) / totals by HNO_3 digests * 100

A cellulase extraction was performed which proved to be less successful for the Pharma Nord yeast, yielding only 60% of the selenium; cellulase gave poor extraction efficiencies (12%) for yeast A, as had the protease, which gave an extraction efficiency of 19%. Although cellulase targets cellulose cell walls which are found only in plant tissue, cellulase was considered due to the ambiguity surrounding the classification of yeasts. It was thought that cellulase may release any selenium species trapped in the cell walls of the samples as results obtained following the protease extraction suggested that most of the selenium remained in the insoluble residue.

The use of cellulase was not expected to be effective for the Biogurt® as this material was bacterial in nature. Despite this, the extraction of selenium from the biogurt using this enzyme gave a 38% efficiency. Although this is considerably low and would not provide a representative picture of the selenium species in the Biogurt®, it is more than twice the extraction efficiency obtained when using protease. The reason for this is unclear as although bacteria possess cell walls in addition to cell membranes they are analogous rather than homologous to that of plant cells. Bacterial cell walls largely contain peptidoglycan, polymers of modified sugars cross-linked by polypeptides, as opposed to plant cell walls which are constructed of cellulose.

Enzymolysis using trypsin proved to be effective for the extraction of selenium in the Pharma Nord yeast yielding 100% of the selenium present. However, the extraction efficiencies for yeast A and Biogurt® were found to be 11 and 18%,

respectively. Similar results were obtained when employing pancreatin for the two materials. The PharmaNord yeast was not tested using pancreatin as it contains a mixture of enzymes including protease and it was, therefore, considered that the extraction efficiency would prove to be satisfactory.

Mass balance calculations demonstrated that the remainder of the selenium from the cellulase and protease extraction procedures was to be found in the solid residue. Determination of the selenium content in the residues following pancreatin and trypsin digestion was not carried out as similar patterns of extraction efficiencies were seen with these enzymes leading to the supposition that the non-extractable selenium remained in the residue.

In response to the poor extraction efficiencies obtained for yeast A and Biogurt® using enzymes, methods were considered that might shatter the cell walls of the material to release the cell contents which might, therefore, enhance the extraction process. Commencing with the Pharma Nord yeast and yeast A samples, which were supplied in powder form, the samples were soaked in a sucrose solution to assess the activity of the yeasts. The Pharma Nord yeast responded by producing a gas, presumably CO_2, and gave off a characteristic yeast aroma. When viewed by light microscopy, budding of the cells was visualized indicating a live yeast sample. For yeast A, in similar circumstances, no activity was noted. Following on from this, the samples were soaked in water to attempt re-hydration prior to freezing with liquid N_2 (boiling point @ -196^{o}C) in order to assist in fracturing the cell walls. The two yeast samples were viewed

by electron microscopy before and after freezing in liquid N_2 to assess any apparent changes in the integrity of the cell walls. It was discovered that yeast A cells were already fractured prior to freezing and that the Pharma Nord yeast cells remained intact throughout. It was subsequently revealed that the cell walls of yeast A had been destroyed during the manufacturing process by the addition of papain, an enzyme capable of breaking down cell walls, at the broth stage. Figures 4-1 and 4-2 show the scanning electron micrographs obtained for the two yeast samples following freezing.

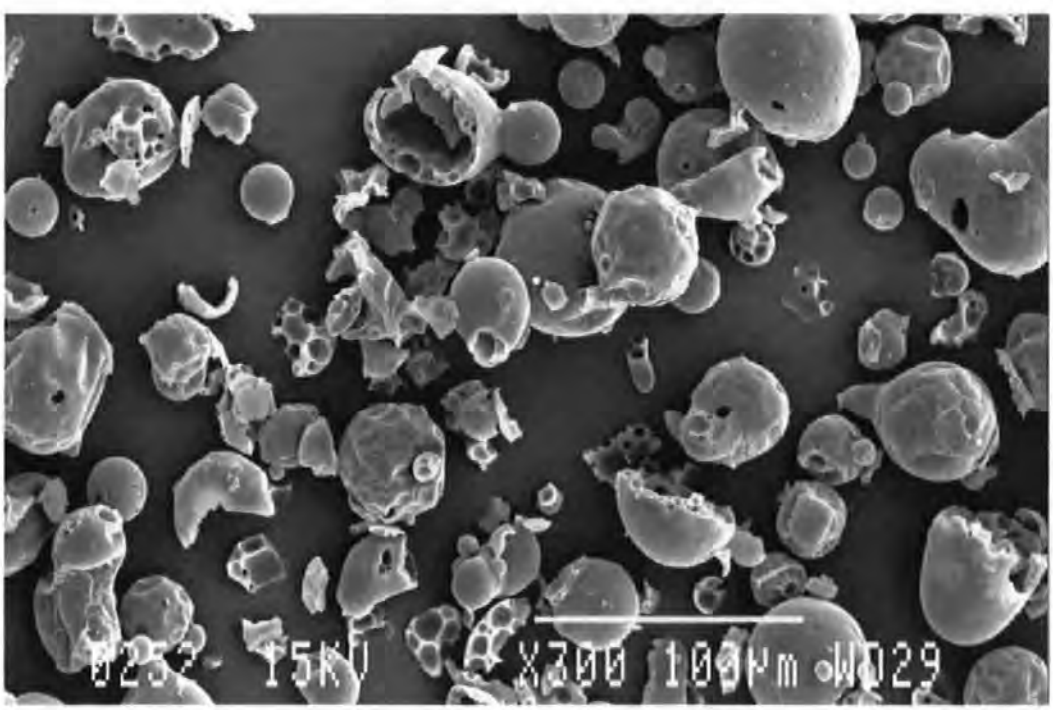


Figure 4-1 Yeast A viewed by scanning electron microscopy (see section 4.2.2.) following liquid nitrogen freezing.

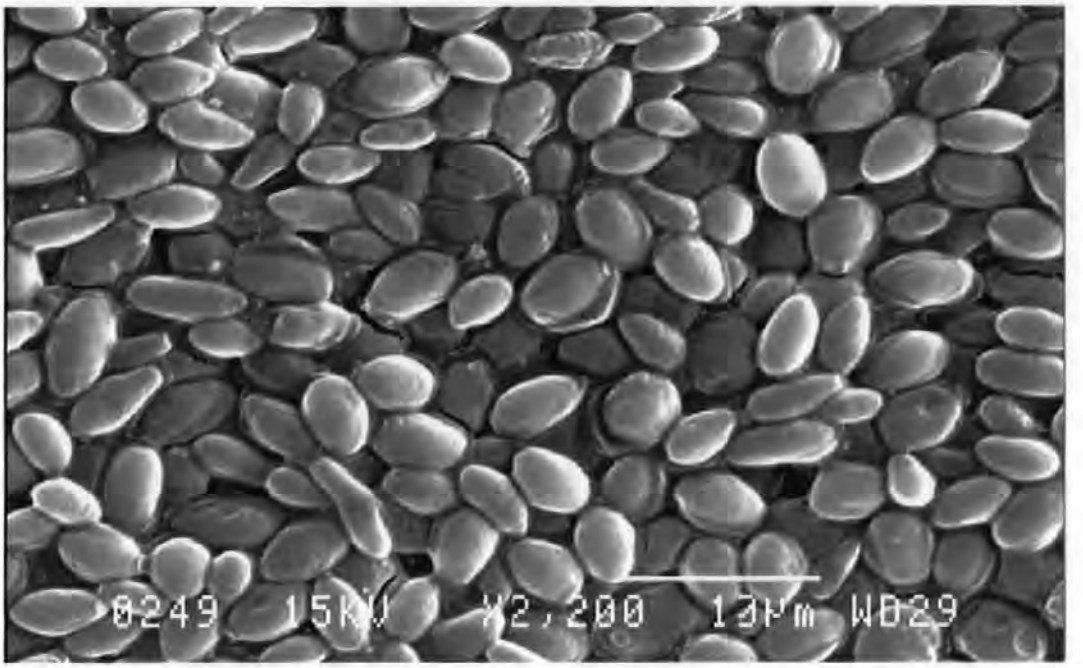


Figure 4-2 Pharma Nord yeast viewed by scanning electron microscopy (see section 4.2.2.) following liquid nitrogen freezing.

Electron microscopy of the Biogurt® sample gave no indication of the structure of the cells before or after freezing by liquid N_2. In light of these results no further work was carried out with liquid N_2.

MeOH:H_2O extraction of selenium compounds from a yeast matrix has been reported by Pedersen and Larsen [182]. The research carried out suggests that this technique is of limited use with extraction efficiencies of 15 – 20% being reported. However, due to the poor extractions obtained from the enzymolysis experiments for yeast A and biogurt a MeOH:H_2O extraction (procedures 5 and 6) was employed to see if the samples were more amenable to this than enzyme extraction. The solutions were prepared with the material as supplied from the manufacturers and following freeze-drying at –60^{O}C under vacuum (a slower process than freezing by liquid N_2 whereby the crystal structure of the ice may

pierce the cell walls precipitating the release of cell contents). A 30% extraction of the total selenium content was obtained for the Pharma Nord yeast which was an improvement on the previously reported research [182]. The general distribution of selenium in yeast samples has been studied [174] with approximately 10% being water-soluble and the remaining selenium being present in a bound form. Of this fraction it is thought that 50% of the selenium is probably bound to proteins or other large organic molecules. The improved extraction in this case, may arise from the selenium being compartmentalized in amounts that vary according to the sample type. Unfortunately, the extraction of selenium from yeast A and Biogurt® using MeOH:H_2O was 14% and 21%, respectively, supporting the research findings of Pedersen and Larsen [182]. The experimental results obtained with the addition of freeze-drying of the material gave a slightly better extraction, at 27%, for the Biogurt®. However, no improvement was seen for yeast A.

Analysis of the sample residues following MeOH:H_2O by HNO_3 microwave digestion demonstrated the presence of the remaining selenium for Pharma Nord yeast and the Biogurt® but not all of the selenium in yeast A was accounted for. A review of the mass balance for yeast A in most of the extraction techniques demonstrated similar findings. Occasionally this phenomenon was seen for Biogurt® as well, most particularly where HCl extraction has been used, including the sequential extraction by HCl and protease. One reason for poor mass balance results may be due to loss of analyte to walls of the containers. This conjecture does not account for the fact that yeast A is, in general, more susceptible to losses than the Biogurt®. Factors involved that could account for

the differences between the two samples may include matrix interferences in the plasma. The use of internal standards and matrix-matched standards were used, as far as was practicable, to reduce these effects.

A review of the results obtained for yeast A and Biogurt® samples in extraction procedures 7 (HCl, pH 2 digestion) and 8 (multi-step extraction using: 1, NH_4HCO_3; 2, protease; and 3, MeOH:H_2O) demonstrated a 71% extraction efficiency for the biogurt sample where the solution pH was strongly acidic (procedure 7, pH 2). Where NH_4HCO_3 has been used, prior to the introduction of an enzyme (procedure 8, stage i) the extraction efficiency was 62%. Research carried out by Emteborg *et al,* [183] demonstrated that extraction of selenium species from a sample of white clover improved with the addition of 0.28 M HCl (to a MeOH:H_2O 50:50 extractant) from 28.5% to 36.7%, and even more so with the use of 4% NH_3 in place of the HCl, to obtain a 47.6% extraction efficiency. It is thought that making samples alkaline liberates selenoamino acids from possible protein-binding sites [184]. In this instance, it appeared that both acidic and alkaline conditions improved the liberation of selenium species from the Biogurt® matrix. Unfortunately, this phenomenon was not seen for yeast A. Where HCl or NH_4HCO_3 have been used alone the extraction efficiency decreased to single figures. The introduction of protease in the second step of procedure 8 brought the extraction efficiency for yeast A to a similar level (19%) as that reported in procedure 1.

The extraction techniques discussed so far did not completely solubilize the yeast A or Biogurt® matrix. Procedure 8 was devised to utilize this effect in providing information on the distribution differently-bound selenium in the samples. However, with only 53% of yeast A and 77% of the Biogurt® yeast being recovered, this mass balance discrepancy lead to a larger uncertainty in the data obtained following this multi-step procedure.

Following the first stage of extraction using 0.1 M NH_4HCO_3, approximately 60% of the selenium was extracted from the Biogurt®. The results for yeast A yielded only 7% of the available selenium. The second stage, using protease, gave similar results for yeast A (18%, compared with 19%) as was seen previously using this enzyme. The Biogurt® results showed a much lower extraction (2%, compared with 15%), probably as a consequence of the relatively high extraction from stage one (62%). The MeOH:H_2O extraction, being the third stage, gave poor results for both samples.

In the case of Biogurt® for this sequential extraction procedure, most of the selenium was seen following the first stage of extraction using NH_4HCO_3 at pH 8 (62%). This was one of the highest extraction efficiencies of selenium seen for Biogurt® in any of the procedures considered. This suggested that the extractable selenium was predominantly water-soluble. Addition of enzymes appeared to have a suppressive effect where aqueous solutions were used reducing the overall extraction efficiency of the selenium.

The yeast A sample yielded the most selenium following the protease extraction (stage two) which was in keeping with the results obtained for the initial protease extraction technique (procedure 1). The Pharma Nord yeast subjected to the same sequential extraction procedure gave an extraction of 14% of the total selenium after the first stage (NH_4HCO_3, 0.1M, pH 8) indicating that this sample also required the use of the protease enzyme for release of the available selenium, where efficient extractions had previously been recorded following procedure 1.

The two-step procedure using 0.01 M HCl followed by a protease digest was chosen as this closely mimicked gastrointestinal conditions in humans. The results showed a highly efficient extraction for the Pharma Nord yeast at 93%. However, the extractions for yeast A were 7.6% and 47% for the Biogurt®. Mass balance calculations show a large discrepancy giving rise to uncertainty in the results obtained.

Further extraction methods utilized KOH (25%) in MeOH and TMAH (25%) in MeOH which gave complete solubulization of yeast A and the Biogurt® sample. Results for extraction efficiencies were 73 and 80%, respectively, for the yeast sample and 12 and 11% for the Biogurt®. The poor extraction obtained for the Biogurt® using KOH and TMAH is unclear. Workers that have reported the use of TMAH for the extraction of selenium species from yeast noted that there was degradation of the organic selenium species initially present to that of inorganic selenium, most probably selenite [181]. In this experiment, anion-exchange chromatography demonstrated the presence of selenite and selenate in yeast A,

following extraction with TMAH and KOH, by the matching of retention times with that of standards using HPLC-ICP-MS. A chromatogram of four selenium standards is shown in Figure 4-3. A chromatogram of yeast A following extraction using TMAH is shown in Figure 4-4 demonstrating the presence of selenite and selenate. Chromatograms of yeast A following KOH extraction were similar to the one shown for TMAH extraction and are therefore not reproduced here. In view of the poor extraction efficiency of selenium in the Biogurt® sample and that species conversion was shown to occur in yeast A, no speciation determination was carried out for the Biogurt® sample. The high alkalinity of the TMAH and KOH appears to cleave the selenium from the organic molecule to give the bare inorganic ions.

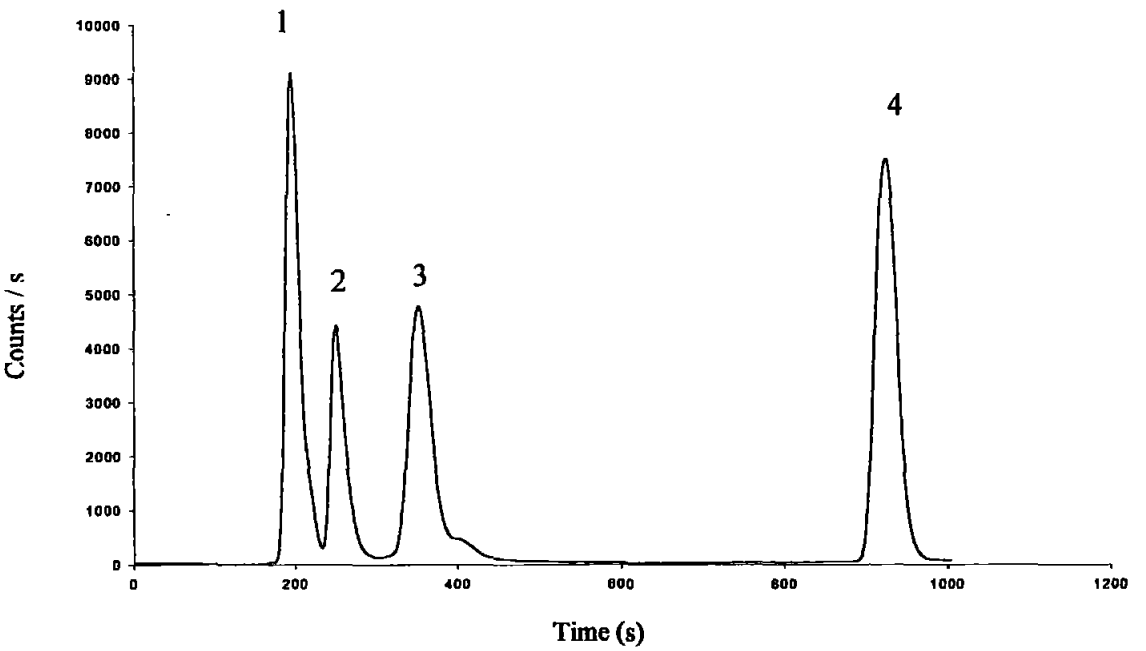


Figure 4-3 Chromatogram of 4 selenium standards, 100 μg l^{-1}, employing a Dionex AS 11 anion-exchange HPLC column using the conditions described in Table 4-2. Peaks: 1= Secys; 2 = SeMet; 3 = Se^{IV}; 4 = Se^{VI}.

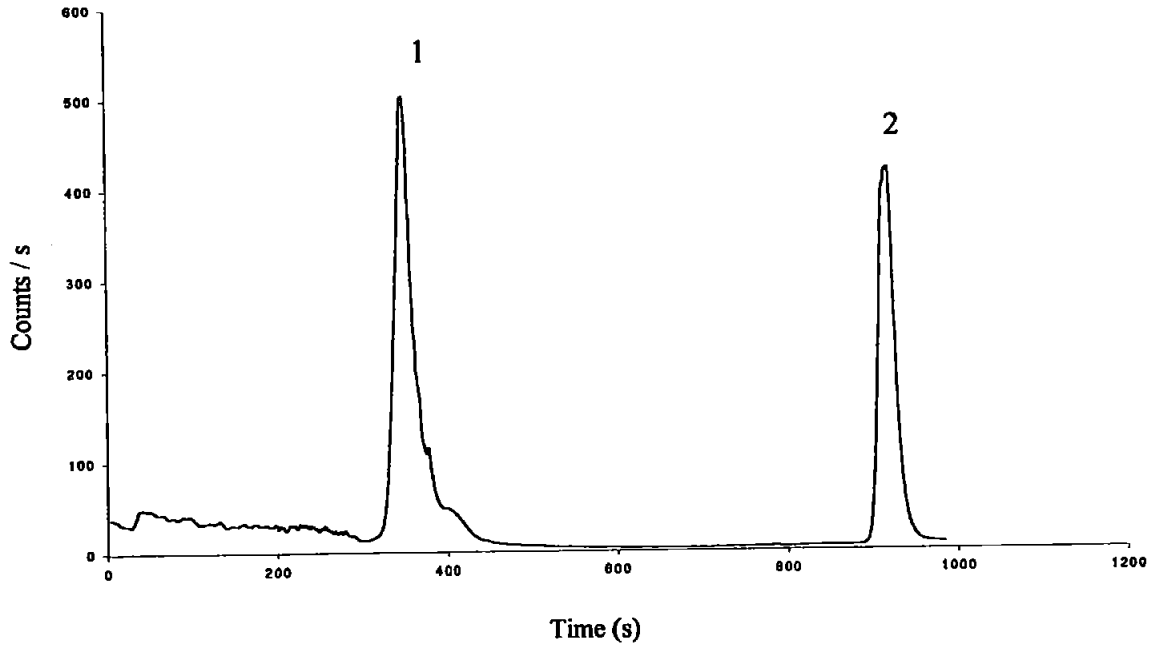


Figure 4-4 Chromatogram of yeast A following selenium extraction by TMAH employing a Dionex AS 11 anion-exchange HPLC column using the conditions described in Table 4-2. Peaks: 1 = Se^{IV}; 2 = Se^{VI}.

The two-step procedure using 0.01 M HCl followed by a protease digest (procedure 11), despite the limited extraction efficiencies (yeast A, 7.6% and Biogurt®, 47%), it was calculated that the extractant of yeast A should contain 76 mg kg^{-1} of selenium and for the Biogurt®, 940 mg kg^{-1} of selenium. Although the extractants might not provide an overall accurate reflection of the whole sample, investigations into the species present were embarked upon. The two extracts, together with the Pharma Nord yeast, were introduced onto a Dionex AS 11 anion-exchange column, using the chromatographic conditions described in Table 4-2, for the purposes of speciation determination. No inorganic selenium was detected in any sample and, therefore, further analysis was carried out using an isocratic elution programme employing 10 mM NH_4HCO_3 + 10% MeOH

adjusted to pH 5 with an eluent flow rate of 1.0 ml min^{-1}. A chromatogram of selenium standards is shown in Figure 4-5. Chromatograms of yeast A, Biogurt® and Pharma Nord yeast extracts are shown in Figures 4-6, 4-7 and 4-8, respectively. Both yeast A and the Biogurt® sample under investigation gave a peak with a retention time similar to SeCys whilst the Pharma Nord yeast gave a single peak matching the retention time of the SeMet standard.

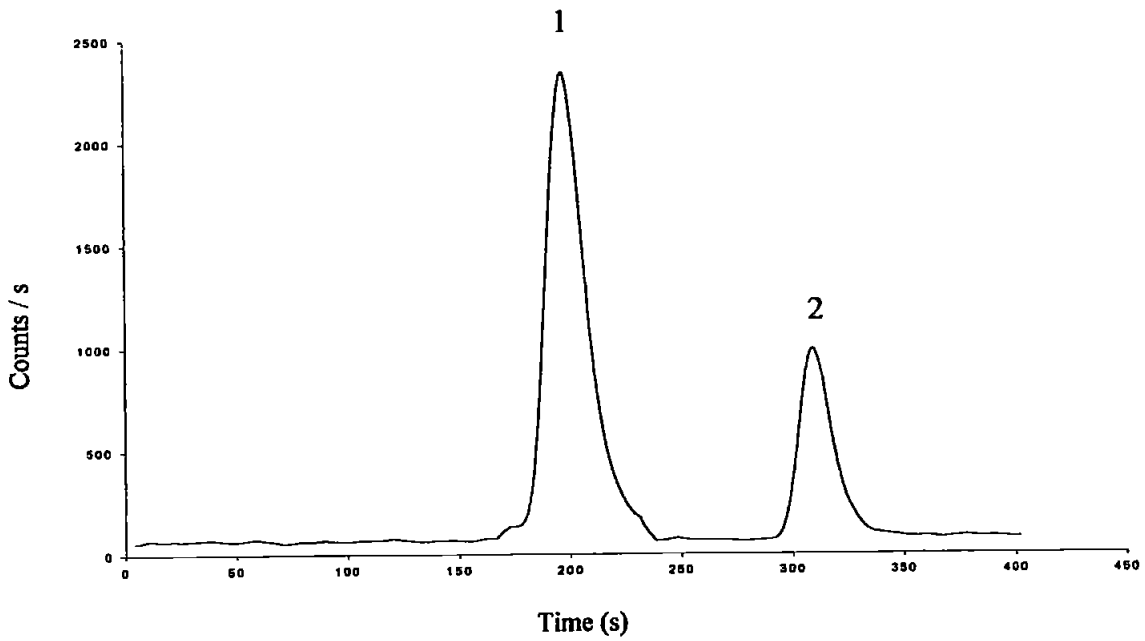


Figure 4-5 Chromatogram selenium standards, 50 μg l^{-1}, employing a Dionex AS 11 anion-exchange HPLC column using the conditions described in Table 4-2. Peaks: 1= SeCys; 2 = SeMet

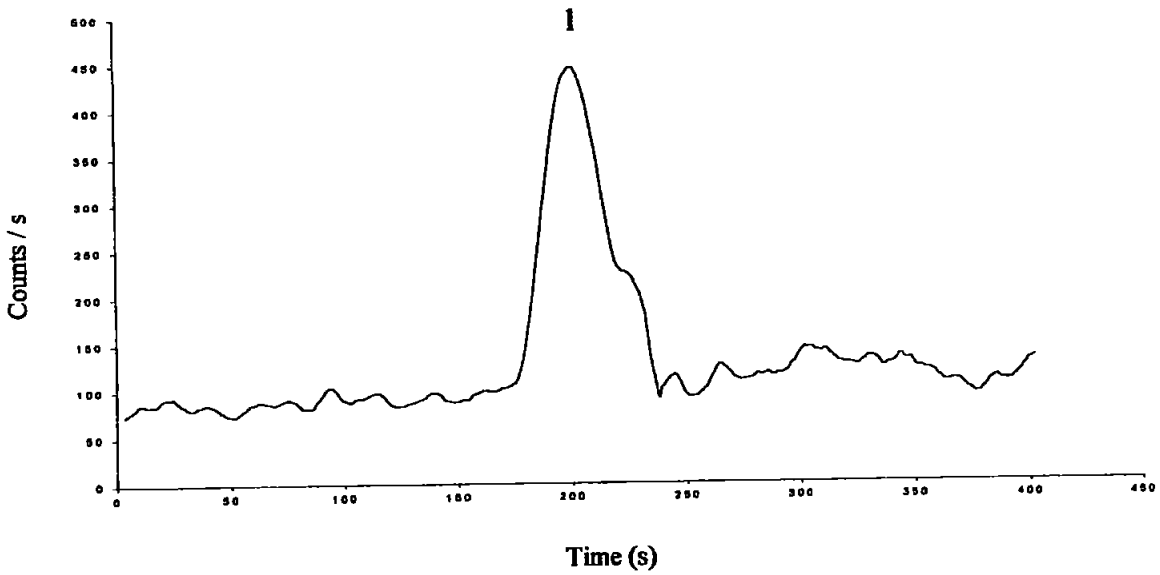


Figure 4-6 Chromatogram of yeast A following extraction by HCl and protease (procedure11) employing a Dionex AS 11 anion-exchange HPLC column using the conditions described in Table 4-2. Peak 1 ascribed to SeCys.

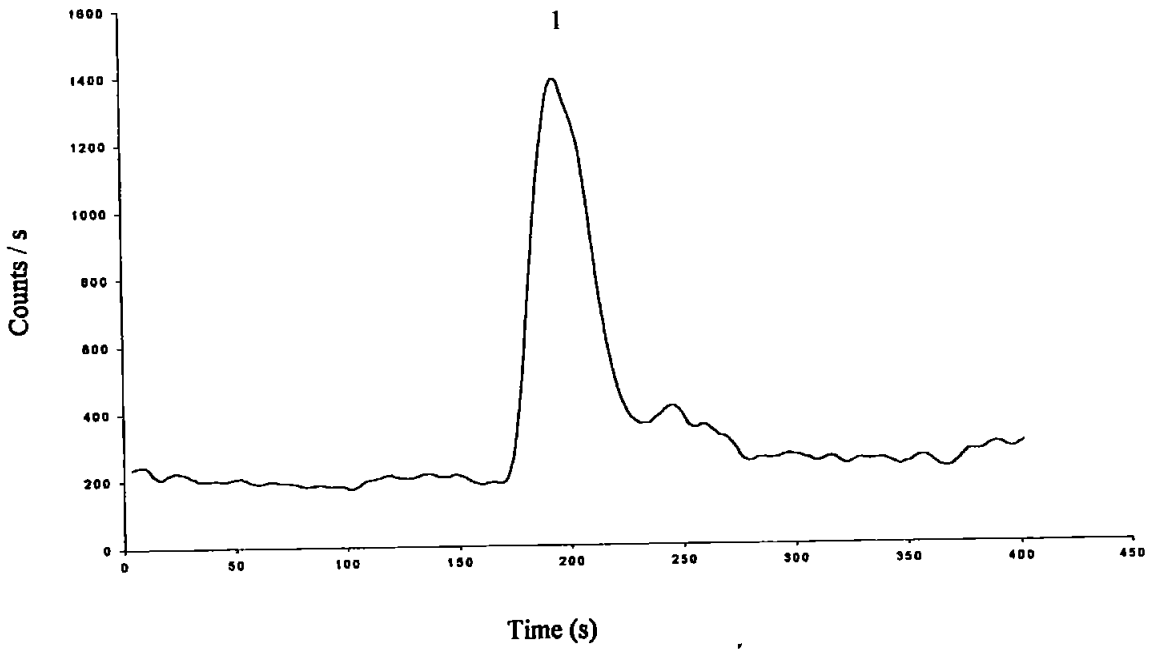


Figure 4-7 Chromatogram of biogurt following extraction by HCl and protease (procedure11) employing a Dionex AS 11 anion-exchange HPLC column using the conditions described in Table 4-2. Peak 1 ascribed to SeCys.

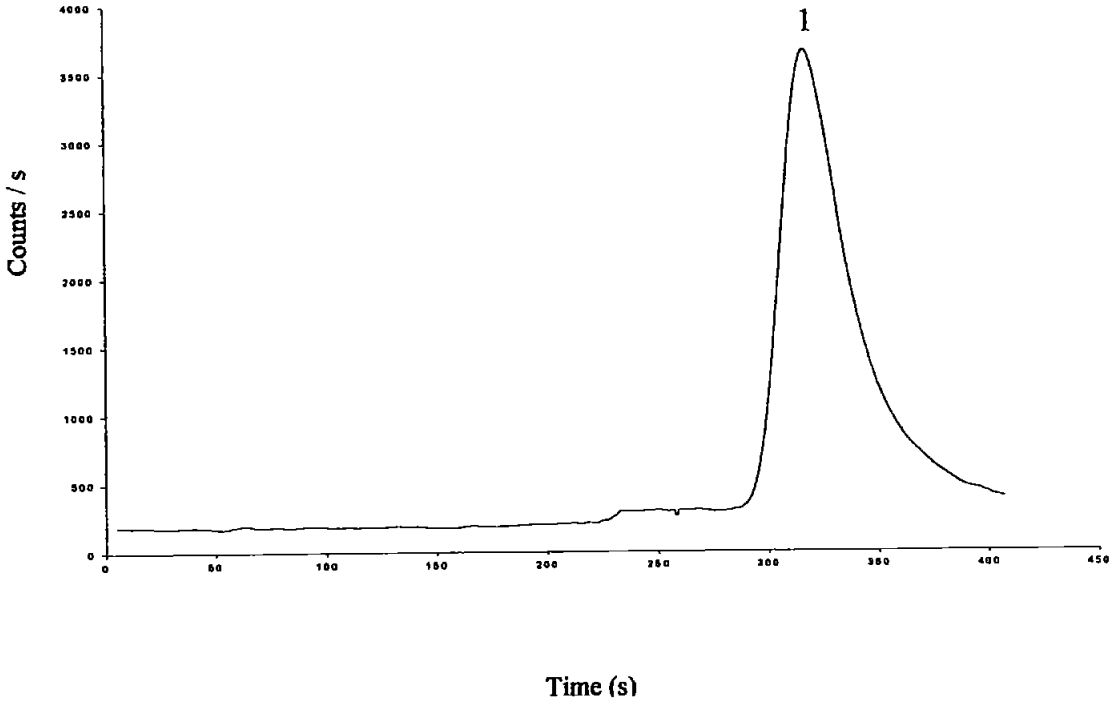


Figure 4-8 Chromatogram of Pharma Nord yeast following extraction by HCl and protease (procedure11) employing a Dionex AS 11 anion-exchange HPLC column using the conditions described in Table 4-2. Peak 1 ascribed to SeMet.

Research carried out by Bird *et al.* [174] demonstrated that where two methods of extraction had been compared, the first where yeast was added to water and shaken at 85 – 90^{O}C for 1 h and the second where protease had been used in a similar manner to that reported by Gilon *et al.* [133] and also used in this work, the speciation profile for the yeast differed. Both extraction methods demonstrated the presence of the same species (inorganic selenium, SeCys, SeMet and methylselenocysteine) but where protease had been used SeMet became the most dominant form. In the work presented here, the Pharma Nord yeast was found to contain SeMet as the major selenium-containing compound. The predominance of SeMet in the presence of protease suggests that SeMet is compartmentalized in

yeast within selenium-containing proteins of which 90% was extractable using protease.

These results suggested that the types of yeast (Pharma Nord yeast and yeast A) possessed some similarities in that they both required the use of protease to liberate selenium indicating the presence of selenium-containing proteins. However, the significant differences seen in the efficacy of the enzyme extraction suggested that the compartmentalization of the selenium in the two yeasts was substantially different from one another. Most of the selenium in yeast A (ranging from 37 – 85%) appeared to remain in the residue in a non-extractable form.

Quantification of the peaks gave the results that are shown in Table 4-5.

Table 4-5 Results for the speciation of selenium in the three samples under investigation. Results given in mg kg^{-1} as the element.

	Total HNO_3	*Total HCl and protease*	*SeCys*	*SeMet*
Pharma Nord yeast	1282 ± 24	1191 ± 108		1005 ± 71
Yeast A	984 ± 12	74.9 ± 2.6	1.59 ± 0.43	
Biogurt®	1980 ± 95	934 ± 80	5.25 ± 0.92	

The results obtained for the quantification of selenium by HPLC-ICP-MS demonstrated a poor mass balance for the yeast A and Biogurt® samples, with only 2% of the selenium in yeast A and less than 1% in the Biogurt® being accounted for. One way to check recovery of the selenium species injected onto the column was by evaluation of the peak areas obtained by flow-injection (FI)-ICP-MS compared with the area of the peaks obtained using anion-exchange HPLC-ICP-MS. Standards of SeCys and SeMet gave similar peak areas for both techniques as did the Pharma Nord yeast sample. However, the peaks obtained by FI-ICP-MS for yeast A and Biogurt® were significantly larger than those obtained using HPLC-ICP-MS. This suggested that the selenium species in these samples were strongly retained by the column. Lack of complete recovery of species due to on-column retention was also reported by Bird *et al.* [174] but only by 10 – 20% of the expected amount. Strongly acidic compounds may be retained on an anion-exchange column and, therefore, further investigations were carried out using a cation-exchange Partisil SCX 10 HPLC system with the conditions described in Section 4.2.4. A chromatogram of SeCys and SeMet standards together with the yeast A and Biogurt® samples is shown in Figure 4-9.

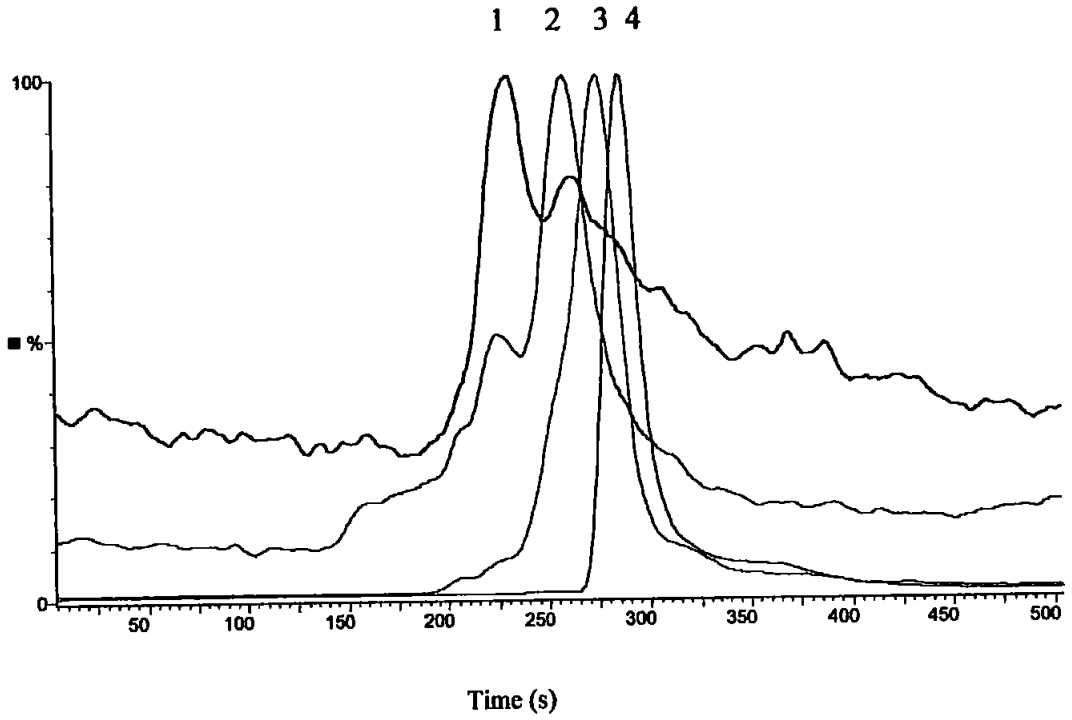


Figure 4-9 Chromatogram of the yeast A and Biogurt® samples and SeCys and SeMet standards by cation-exchange HPLC-ICP-MS using a Partisil SCX 10 column (250 x 4.6 mm) and a 20 mM pyridine eluent adjusted to pH3. Peaks: 1 = yeast A; 2 = Biogurt®; 3 = SeCys standard, 1 mg l^{-1}; 4 = SeMet standard, 1 mg l^{-1}.

The chromatogram shown in Figure 4-9 using cation-exchange conditions demonstrates the lack of resolution between the samples and standards. This can be explained by the zwitterionic character of the selenoamino acids and that under the pH conditions employed (pH 3) they will possess cationic groups and, therefore, have a limited affinity for the stationary phase. Although optimization has not been achieved here it can be seen that the yeast A and Biogurt® samples do not match the retention times of either of the standards used. Where anion-exchange chromatography was performed (Figures 4-5 to 4-8) the yeast A and Biogurt® samples appeared to elute with a similar retention time to that of

SeCys. However, the cation-exchange chromatography points towards the possibility that the samples may not contain SeCys or SeMet but other, chemically similar, amino acids. Comparison of peak areas by FI-ICP-MS and cation-exchange HPLC-ICP-MS also demonstrated the effect on-column of retention of sample analyte.

4.4 Conclusions

Research [131] has shown that yeast-based selenium food supplements can demonstrate a significant variety in the selenium species present with some brands showing generally low recoveries of any form of selenium, and may have matrix problems which make effective extraction difficult. Work carried out by Gilon, *et al.* [133] has demonstrated the efficacy of enzyme-based extractions using protease. In the work presented here, it was found that the selenium content, in the form of SeMet, was adequately extracted from the Pharma Nord yeast using protease which yielded 90% of the 'total' selenium content. However, the determination of 'total' selenium and selenium species in the yeast A and Biogurt® samples proved to be quite problematic. A variety of extraction methods were employed with limited success. Methods that avoided species conversion and with the highest extraction efficiencies were found to be: i) the use of protease for yeast A and: ii) the use of 0.01 M HCl for the Biogurt®. Speciation of these samples by anion and cation-exchange HPLC-ICP-MS was hampered, partly because of the limited extraction efficiencies of the samples and

by the retention of the analyte on-column and by the lack of standards available for matching of retention times.

Limitations may be imposed on the extraction techniques commonly available where identification of selenium species in these new, to be commercially available, bionutrients are studied. At present, the only selenium value obtainable is that of 'total' selenium using a HNO_3/H_2O_2 microwave digestion procedure. The retention of species information by 'softer' extraction processes appears to reduce the overall extraction efficiency with subsequent loss of information. The results obtained from this study seriously place in doubt the availability of the organo-selenium species in the yeast A and Biogurt® samples, for which they are supposedly designed to deliver.

Further work, with the aim of achieving a successful extraction for 'total' selenium and selenium species for yeast A, needs to continue with investigation into the use of β-gluconase, a specific yeast enzyme, although not available commercially, but which may promote a successful extraction. Consideration of the use of cell-permeabilizing surfactants to enhance cell wall degradation with procedures already used in this study may prove rewarding for extraction of selenium species in both the yeast A and Biogurt® samples.

Chapter five

5 Speciation of arsenic and selenium using high performance liquid chromatography with inductively coupled plasma mass spectrometry and electrospray mass spectrometry.

5.1 Introduction

Numerous instrumental methods have been developed for the separation, identification and determination of arsenic and selenium species. The most widely reported techniques have relied on high performance liquid chromatography together with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) [92]. The chemistries of arsenic and selenium readily allow for ion-exchange mechanisms to be employed. Control of the mobile phase by choice of competitive ion, its concentration and pH conditions can lead to the successful resolution by ion-exchange chromatography of a number of organic and inorganic arsenic and selenium species, individually [132, 185] and simultaneously [186]. Ion-pairing reversed phase chromatography is an alternative chromatographic mechanism that has often been employed, providing good separation of a number of arsenic and selenium species [187, 188]. However, species identification using these methods relies on the matching of retention times of analyte peaks with that of known standards. Due to the numerous forms and complexity of the analytes of interest and possible matrix interferences, co-elution of species may lead to erroneous results despite the use of an element specific detector. The availability of standards may also place limitations on the efficacy of the procedure. A case in

point is that of arsenoribofuranosides (arsenosugars), which have been known to co-elute with AsBet, DMA and MMA under cation and anion-exchange HPLC-ICP-MS conditions [189, 110, 116].

HPLC-ICP-MS has been used with success where materials have been widely studied and well-characterized. However, when analyzing materials that are new, or materials where total elemental concentrations are recorded but no information regarding the species is given and where standards are unavailable for peak identification, it would seem prudent to find instrumental techniques that can provide corroborative evidence to support that obtained by HPLC-ICP-MS. Research using molecular specific techniques such as electrospray ionization mass spectrometry (ESMS) has enhanced the capability of characterizing previously unidentified species [190]. The complementary use of element specific ICP-MS together with molecular specific ESMS following HPLC separation of the analytes of interest provides greater confidence in the assignment of species identified.

The electrospray process is a means of obtaining gas-phase ions from solution for the purpose of analysis by mass spectrometry. This is achieved by applying a strong electric field to a liquid passing through a capillary tip. An electric field is generated by applying a potential difference between the capillary tip and the counter electrode. ESMS can produce and detect positive and negative ions depending on the polarity of the electrodes. The choice will reflect on the experimental conditions used and in which mode the best results are obtained.

For data collection, ESMS analyzers can be used in the full-scan mode or selected ion monitoring (SIM) mode, depending on analytical requirements. The full scan mode will measure ions across a given mass range providing evidence of all molecular ions present and their abundance; selected ion monitoring looks at a limited number of ions dependent on the mass/masses selected by the operator. The full scan mode is particularly useful when attempting to identify previously unreported compounds. However, the mass spectrum obtained can be highly complex with difficulties arising where there is suppression of the analyte signal by concomitant matrix ions [191]. Using the SIM mode can reduce the effects of this problem. However, it can be extremely time-consuming unless there is some prior knowledge of the analytes of interest allowing the operator to select appropriate masses. The use of the tandem MS (mass spectrometry – mass spectrometry, MSMS) mode of ESMS provides essential information on molecular structure that can be used to characterize compounds. The MSMS function allows fragment patterns, by collision induced dissociation (CID), of parent molecular ions to be obtained. Fragmentation pathways are, in part, determined by the strength of the bonds which are to be broken and the stability of the product (daughter) ions. Fragment patterns are likely to be exclusive to a particular molecule and, therefore, explicitly distinguish between the analyte of interest and any other ion possessing the same molecular mass. Purification of the target analyte has also assisted in the identification of compounds by ESMS [113] by the reduction of matrix interferences.

The work presented here is based on the use of complementary HPLC-ICP-MS and HPLC-ESMS in the separation and identification of various arsenic species in extrants from a common brown seaweed, *Fucus spiralis* (IAEA-140, International Atomic Energy Agency, Belgium) and a kelp powder, *Ascophyllum nodosum,* (Queenswood Health Foods, Bridgewater, UK) available as a health supplement. A selenized yeast sample extrant (Pharma Nord, Vejle, Denmark) was subjected to the same techniques enabling identification of the selenium species present.

Marine algae were chosen for the study as they are known to contain arsenosugars [21] and they may form a significant part of the human diet, particularly in Eastern cultures such as the Japanese. It is also noted that marine algae constitute the basis of some Western vegetarian health supplements. Arsenosugars are considered to be essentially non-toxic forms of arsenic. However, research by Le *et al.* [192] suggested that arsenosugars could be converted by humans to the more toxic form of DMA, which is a suspected carcinogen. Research towards the identification and characterization of arsenosugars in biological samples is, therefore, vital to our further understanding of metabolic pathways and potential mechanisms of toxicities.

A selenized yeast sample was chosen for investigation due to the scientific interest propagated by the work of *Clark et al.* [47] where selenized yeast supplements were shown to reduce the incidence of some cancers by as much as 50%. The anti-carcinogenic effect of selenium is now known to be species

dependent and hence, the characterization and identification of the selenium species present in yeast has been at the forefront of research. The complementary use of HPLC with ICP-MS and ESMS can provide substantive evidence on the species present. The selenized yeast sample chosen (Pharma Nord) had previously been extensively investigated as part of a CRM feasibility study (Chapter 3). Although the predominant species identified, by all the external participating laboratories, was that of SeMet, this was mainly based upon their comparison with known available standards taken through an HPLC separation technique with element-specific detection. Hence, when a material is assessed for suitability as a CRM, corroborative evidence of the species present by ESMS can be, not only advantageous, but critical for future users.

A HPLC method for the speciation of arsenic and selenium compounds, compatible with both ICP-MS and ESMS detection was developed. This allowed direct comparisons to be made between the two techniques enhancing their complementary nature and abilities. As the most effective use of the electrospray process is achieved when ions are pre-formed in solution, an ion-exchange chromatographic system using a volatile mobile phase was the system of choice. Optimum HPLC conditions that were compatible with both ICP-MS and ESMS were achieved using an ammonium hydrogen carbonate solution with 10% methanol. Species identification using HPLC-ICP-MS was based upon the matching of peak retention times with that of known standards with further confirmation of species identity being obtained from ESMS data. Further characterization of species was obtained by the use of HPLC-ESMS in the

MSMS mode. Solid phase extraction (SPE) techniques were also considered in order to separate the bulk matrix from samples prior to analysis using ESMS and method development in this area was undertaken.

5.2 Experimental

5.2.1 Speciation of arsenic compounds using HPLC-ICP-MS and HPLC-ESMS

5.2.1.1 Instrumentation

ICP-MS measurements were performed using a VG PlasmaQuad 2+ (T.J.A. solutions, Winsford, Cheshire, UK), using conditions as described in Table 5-1. A Perkin-Elmer 410 high pressure pump (Perkin-Elmer, Norwalk, CT, USA) was used for control of the eluent flow rate. A Rheodyne 7125 injection valve (Rheodyne, Cocati, CA, USA) with a 20 µl sample loop was used for sample introduction by HPLC-ICP-MS and a 200 µl sample loop for HPLC-ESMS. Nitrogen, 4% v/v, for the reduction of $ArCl^+$ interferences on m/z 75, was added via a Signal series 850 gas blender (Signal, Camberley, Surrey) to the nebulizer gas flow.

Table 5-1 ICP-MS operating conditions for the determination of 'total' arsenic and species in sample extracts of marine algae using HPLC-ICP-MS, using m/z 75 for arsenic measurement.

ICP-MS	Plasma Quad 2+	
	V-groove nebulizer	
	Double-pass, water cooled Scott type spray chamber	
	Fassel torch – 1.5 mm bore injector	
Parameters	Argon plasma – 4% N_2 added for direct total As determination to the nebulizer gas flow	
	Nebulizer flow rate	0.81 l min^{-1}
	Coolant gas flow rate	13.1 l min^{-1}
	Auxiliary gas flow rate	0.8 l min^{-1}
	Forward power	1350 W
	Dwell time	500 ms

ESMS measurements were performed using a quadrupole ion trap (QIT) mass spectrometer with an electrospray ionization (ESI) interface (ThermoQuest Finnegan Mat LCQ, San Jose, CA, USA) using the conditions described in Table 5-2. The 'positive ion' mode was used throughout. Readings for pH were taken using a 3010 pH meter (Jenway, Ltd., Essex, UK). A portable membrane vacuum system (Vacuubrand GmbH & Co., Werthim, Germany) was used for solid phase extraction (SPE) work.

Table 5-2 Instrumental operating parameters for the identification of arsenic species present in marine algae using direct injection ESMS and HPLC-ESMS.

Instrument	*Operating conditions*	*Gas flow rates*
LCQ ESMS	Spray voltage – 4.5kV	
	Spray current – sample dependent	Direct injection
	Capillary temperature – 220°C	Sheath – 18 l min^{-1}
	Capillary voltage, 13.98 V	Auxiliary – 0 l min^{-1}
	Lens voltage, -14.98 V	HPLC
	Octapole 1 offset, -0.88 V	Sheath – 18 l min^{-1}
	Octapole 2 offset, -10.54	Auxiliary – 0.3 l min^{-1}

5.2.1.2 Chemicals and reagents

Chemicals were of analytical grade unless otherwise stated. All lab-ware was soaked in HNO_3 (10% v/v) for a minimum of 24 hours and rinsed thoroughly with MilliQ water (Millipore, Bedford, MA, USA) prior to use. Stock solutions of arsenobetaine (AsBet) (BCR - CRM 626, IRMM, Belgium), monomethylarsonic acid (MMA) (kindly donated by Dr. A. Moreda-Pineiro, University Santiago de Compostela, Spain), dimethylarsinic acid (DMA) and sodium arsenate (Sigma-Aldrich, Poole, Dorset, UK) at 1000 mg l^{-1} as the element were stored at 4°C in the dark. Standards were prepared daily from the stock solutions. Cellulase (Sigma-Aldrich) was used in the sample digestion procedure for the extraction of arsenic species. The mobile phase eluent was prepared using ammonium hydrogen carbonate (Sigma-Aldrich) and methanol (Fisher Chemicals).

5.2.1.3 HNO_3 digestion for total element determination

Microwave bombs (Savillex, Minetonka, Minnesota, USA) were pre-cleaned with 3 ml 69% v/v HNO_3 (Primer, Fisons, Loughborough, UK) in a Perfecto 800 W microwave oven (DeLonghi, Italy) on medium power for 2 mins. Samples of approximately 0.25 g were accurately weighed into the bombs and 4 ml HNO_3 (69%, v/v) together with 1 ml H_2O_2 (37%, v/v) were added. The bombs were loosely capped and left overnight to allow easily oxidised material to be destroyed. After predigestion, the bombs were swirled gently, sealed tightly and microwaved on medium power for 1 - 2 mins, or until the sample was a clear colour with no residue (indicating a completed digest). The samples were transferred quantitatively to 500 ml volumetric flasks and made up to volume with 2% HNO_3 giving an overall dilution of x2000. The samples and standards were spiked with indium to give a final concentration of 100 $\mu g\ l^{-1}$ Indium (In) which acted as an internal standard prior to analysis by ICP-MS using the conditions described in Table 5-1. The internal standard was used to correct for instrumental drift (sample viscosity effects, mass transport, etc.) over the analysis period

5.2.1.4 Enzymatic digestion procedures for extraction of arsenic species from marine algae

Samples of approximately 0.5 g were accurately weighed together with 0.05 g cellulase and approximately 40 ml CH_3COONH_4 buffer (0.1 M, adjusted to pH 5 using CH_3COOH). The suspensions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. Following enzymolysis digestion, the samples were centrifuged at 2500 rpm for 20 min, the supernatant transferred quantitatively to volumetric flasks and made up to volume with the CH_3COONH_4 buffer. The samples and standards were spiked with caesium to give a final concentration of 100 $\mu g\ l^{-1}$ Cs which acted as an internal standard prior to analysis by ICP-MS.

5.2.1.5 Chromatographic conditions for the determination of arsenic species using HPLC-ICP-MS and HPLC-ESMS

The chromatographic system consisted of a column (250 x 4.6 mm I.D.) packed with Hamilton PRP X100, a strong anion-exchange polymeric based resin of 10 μm diameter, with a guard column (50 x 4.6 mm I.D.) of the same resin. The mobile phase was 10 mM NH_4HCO_3 with 10% MeOH at pH 10 (adjusted with NH_3 solution). The eluent flow rate used throughout was 1 ml min^{-1}, with a 20 μl sample loop for HPLC-ICP-MS, and by using a post-column splitter, a flow rate of 150 $\mu l\ min^{-1}$ with a 200 μl sample loop was used for HPLC-ESMS.

5.2.1.6 Solid Phase Extraction (SPE) techniques

Method development using anion, cation and reversed phase SPE extraction cartridges was employed. Optimum conditions, in terms of analyte response determined using HPLC-ICP-MS, were found using 'Strata' anion exchange SPE cartridges (Phenomenex, Cheshire, UK). The cartridges were conditioned with MeOH (1 ml) followed by Milli-Q H_2O (5 ml). Sample extracts (1 ml) from the enzyme digestion procedure were introduced onto the SPE cartridge and the retained analytes were then eluted with NH_4HCO_3 (20 mM, pH 10, 1 ml). This solution was evaporated gently to dryness on a hot plate and re-dissolved in 200 µl of H_2O.

The *Fucus sp.* sample extracts, following anion SPE, were further purified by fraction collection subsequent to introduction onto the Hamilton PRP X100 anion-exchange column. The fractions were collected at times considered suitable for obtaining analytes from resolved chromatographic peaks as determined following HPLC-ICP-MS of the extract for the particular column eluent system used. The fraction collection program is shown in Table 5-3.

Table 5-3 Fraction collection programme for *Fucus sp.* samples using a Hamilton PRP X100 anion-exchange column with a mobile phase of 10 mM NH_4HCO_3 + 10% MeOH. Flow rate of 1 ml min^{-1} and a sample loop volume of 200 µl.

Fraction	*Collection time (s)*
1	120 – 180
2	200 – 240
3	240 – 300
4	330 - 420

Fractions were collected from the repeat injection of 10 samples onto a Hamilton PRP X100 column through a 200 µl loop using the conditions described in section 1.2.1.5. The collected fractions were gently evaporated to just dryness at approximately 50^{O}C on a hot plate and re-dissolved in 0.5 ml of MeOH with CH_3COOH (1%, v,v) for analysis by direct injection ESMS. For HPLC-ESMS analysis the collected fractions were again evaporated to dryness and re-dissolved in 0.5 ml of the mobile phase. For validation purposes, experiments were performed on standards, using the same conditions that had been applied to the samples, to ensure no species inter-conversion occurred during the SPE and on-column fractionation procedures that might have given rise to erroneous results.

5.3 Results and discussion

To commence the investigation of the marine algae an anion-exchange HPLC method was developed, based on the work of Madsen *et al.* [116] which was compatible with both ICP-MS and ESMS. In Madsen's work, a Hamilton PRP

X100 anion-exchange column was used for both HPLC-ICP-MS and HPLC-ESMS. The mobile phase used for HPLC-ICP-MS was a 20 mM $NH_4H_2PO_4$ solution adjusted to pH 5.6 with aqueous NH_3, whereas for HPLC-ESMS analysis the mobile phase was a 20 mM NH_4HCO_3 + 10% MeOH solution adjusted to pH 10.3 with aqueous NH_3. Volatile buffers are necessary in ESMS for the evaporation of the solvent leading to the presence of gas phase ions and hence, the change in mobile phases between ICP-MS and ESMS. The addition of MeOH plays a significant role in ESMS signal stability and sensitivity [191] which is attributed to the improved ion yield from charged MeOH droplets relative to charged water droplets. In this work, 10 mM NH_4HCO_3 + 10% MeOH adjusted to pH 10.2 with NH_3 was found to be suitable for both ICP-MS and ESMS detection of arsenic species. This both simplified and allowed direct comparison of chromatographic results from the coupled ICP-MS and ESMS system. The lower concentration of NH_4HCO_3 used assisted in reducing suppression of analyte signal due to concomitant ions in ESMS. Addition of organic solvents to the mobile phase are also known to reduce polyatomic interferences in ICP-MS [70].

Anion-exchange HPLC-ICP-MS analysis was initiated with the study of readily available arsenic standards. Figure 5-1 demonstrates the typical retention times for standards of AsBet, DMA, MMA and As^V.

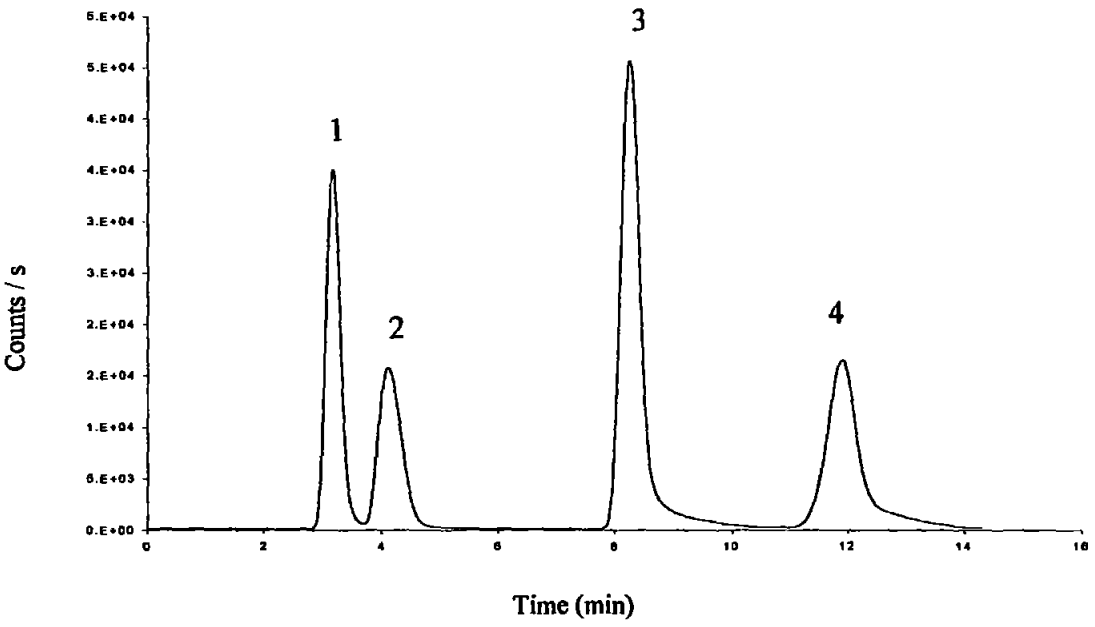


Figure 5-1 A typical chromatogram of arsenic standards, 250 μg l^{-1} each, using a Hamilton PRP X100 anion-exchange HPLC-ICP-MS (column dimensions of 250 x 4.6 mm I.D.). The mobile phase used was 10 mM NH_4HCO_3 with 10% MeOH at pH 10. Peaks: 1 = AsBet; 2 = DMA; 3 = MMA; 4 = As^V.

Analysis of arsenic standards was then performed using HPLC-ESMS in the positive ion mode. Electrospray can been used in the negative [193] or positive [191, 116] ion modes for the characterization of arsenosugars, other organic arsenic-containing compounds and inorganic arsenic. The work of Pergantis *et al.* [193] demonstrated an improved sensitivity in analyte signal in the negative ion mode. However, the work of Madsen *et al.* [116] successfully applied anion-exchange chromatography coupled with ESMS in the positive ion mode for the detection and characterization of some arsenosugars present in a marine algae. The positive mode is most frequently reported for the detection and identification of organic arsenic compounds but suffers in that it cannot detect inorganic arsenic.

A chromatogram, shown in Figure 5-2, demonstrates the retention time of AsBet, DMA and MMA standards obtained using this method. The chromatogram was obtained by overlaying the selected ion monitoring (SIM) spectra of m/z 179, 139 and 141 ($M+H^+$ ions of AsBet, DMA and MMA, respectively).

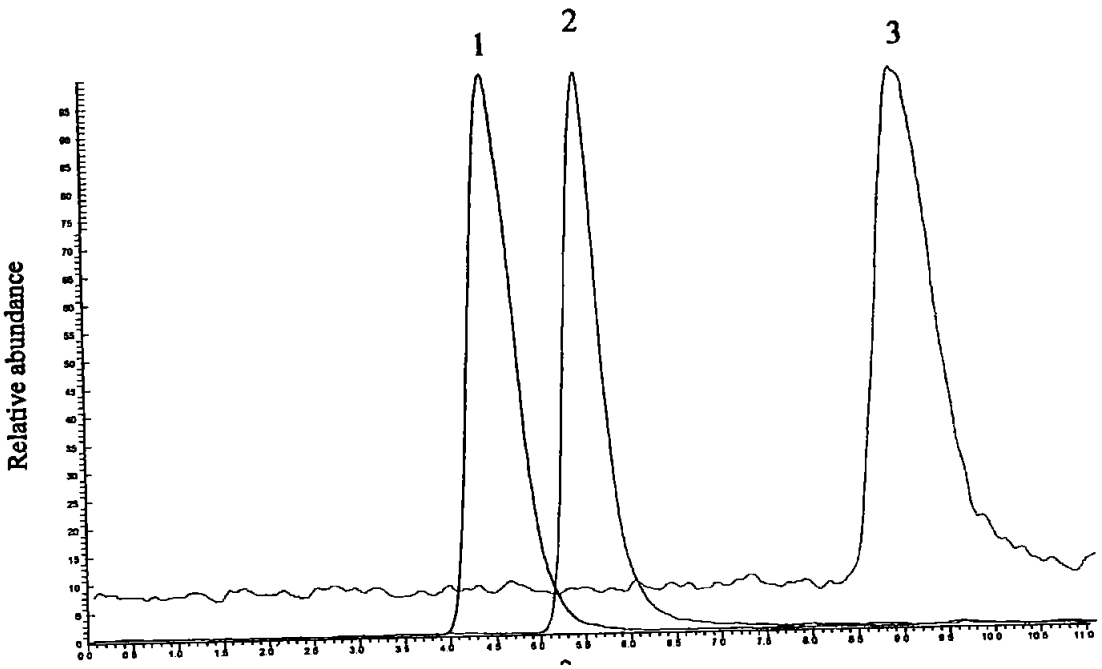


Figure 5-2 HPLC-ESMS of arsenic standards at 1000 μg l^{-1} with retention times in parentheses, given in mins: 1, AsBet (4.65); 2, DMA (5.68); 3, MMA (8.85). SIM mode with chromatograms overlaid of m/z 179, 139 and 141.

Although the same experimental conditions were used for both HPLC-ICP-MS and HPLC-ESMS, it can be seen that the retention times obtained for the standards by ESMS are significantly different from those obtained by ICP-MS. The retention times are shown in Table 5-4 and differ by approximately 1 min in

each case. The shift in elution times may be accounted for by the differences in the sample introduction train between the two techniques. This must be taken into consideration when making comparisons between the techniques for species identification.

Table 5-4 Comparison of retention times of arsenic standards using HPLC-ICP-MS and HPLC-ESMS under the same chromatographic conditions (as described in Section 5.2.1.5)

	Retention times HPLC-ICP-MS	*Retention times HPLC-ESMS*
AsBet	3.32	4.35
DMA	4.14	5.38
MMA	8.09	8.85

The probability that co-eluting species with the same molecular ions may be present in a sample cannot be overlooked and due to the significant shift in retention times of the arsenic species between the two techniques, further confirmation that the presence of ions at m/z 179, 139 and 141 corresponded with the expected $M+H^+$ molecular ions for AsBet, DMA and MMA was obtained by employing MSMS. The fragmentation patterns of MMA, DMA and AsBet obtained using MSMS are shown in Figures 5-3, 5-4 and 5-5, respectively.

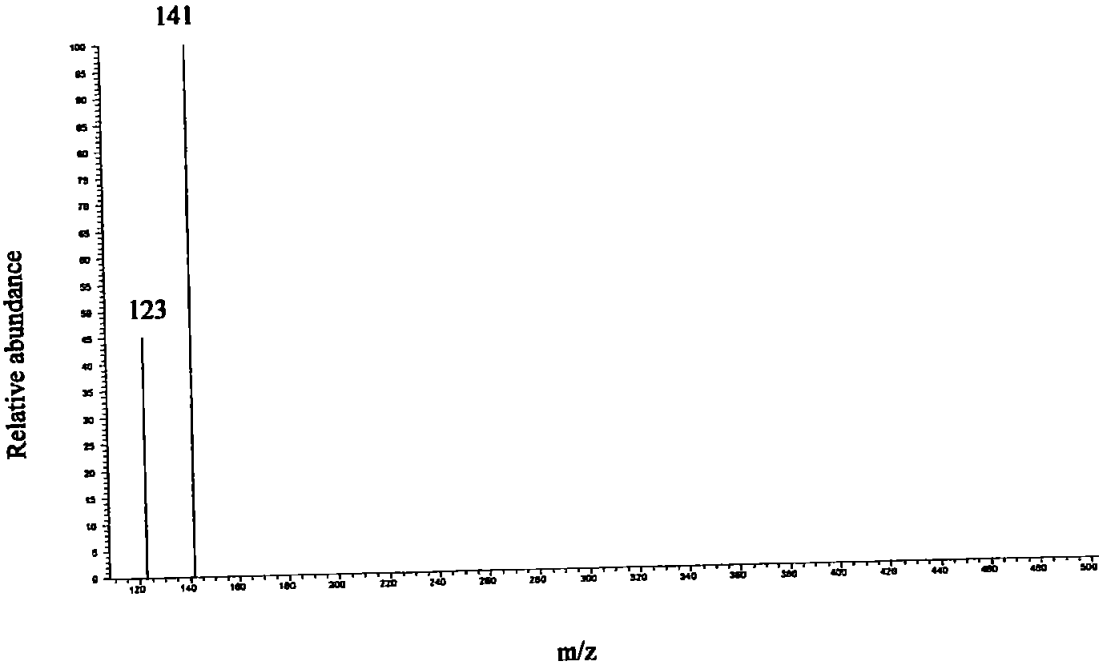


Figure 5-3 MSMS fragmentation pattern of MMA ($M+H^+$ ion 141) using the ESMS conditions shown in Table 5-7.

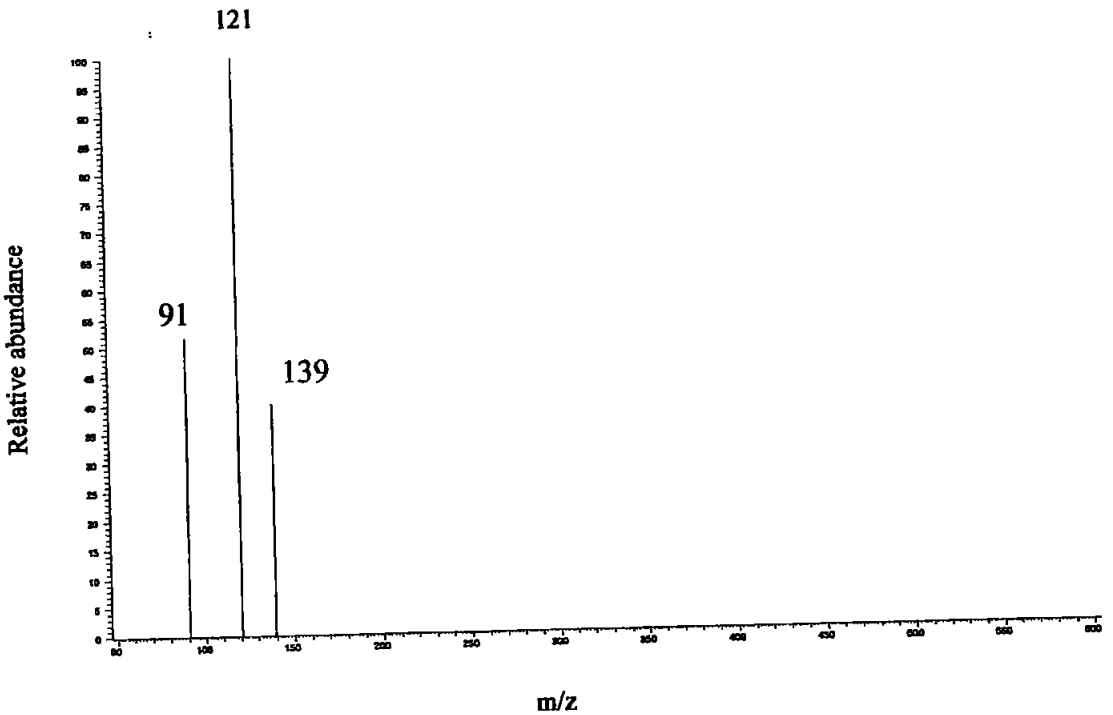


Figure 5-4 Fragmentation pattern of DMA ($M+H^+$ ion 139) using the ESMS conditions shown in Table 5-7.

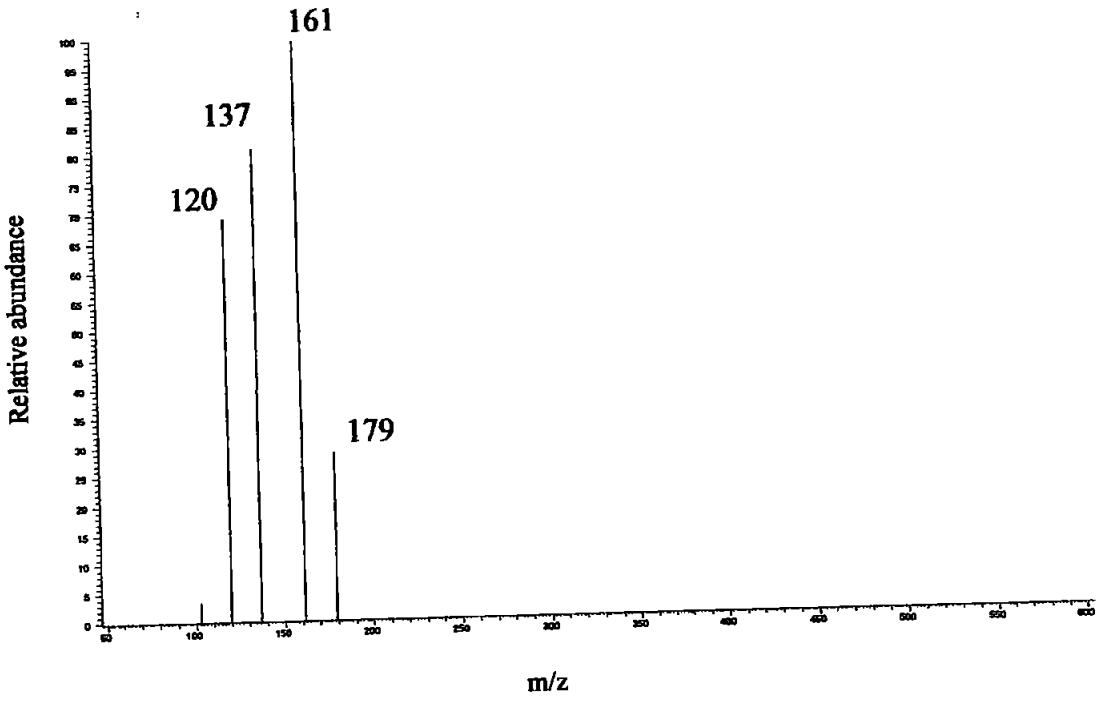


Figure 5-5 Fragmentation pattern of AsBet ($M+H^+$ ion 179) using the ESMS conditions shown in Table 5-7.

The structures of AsBet, DMA and MMA together with their identified fragmentation patterns, that correspond to the experimentally obtained results by MSMS [194], are shown in Figure 5-6.

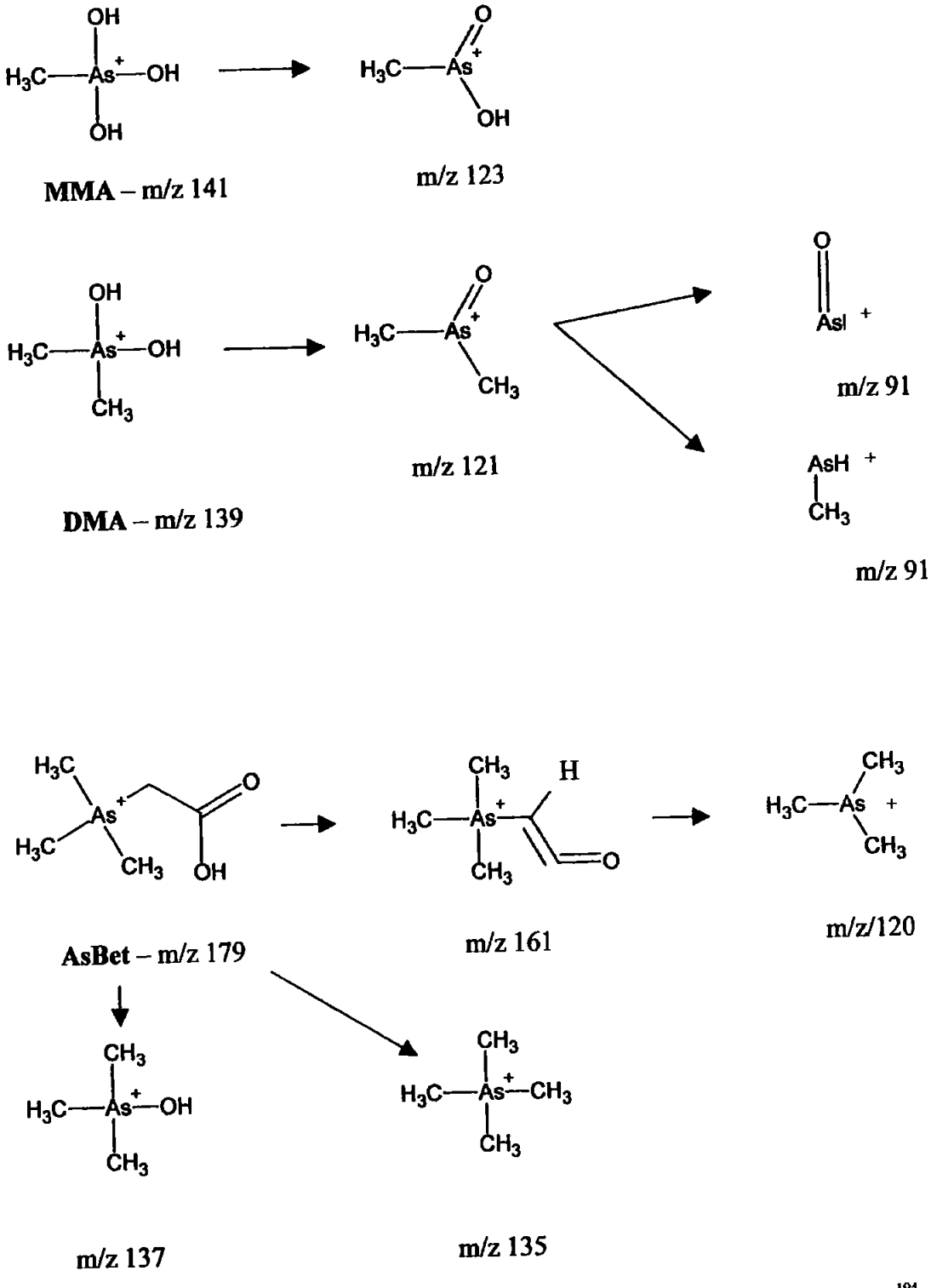


Figure 5-6 Structures of MMA, DMA and AsBet together with identified fragment pathways [194] using mass spectrometric analysis.

Reviewing the peaks obtained by HPLC-ICP-MS for standards of AsBet, DMA and MMA, which identify the presence of elemental arsenic, together with the molecular peaks of equivalent mass obtained by HPLC-ESMS and the fragmentation patterns seen by MSMS, there is overwhelming evidence for the species purity of the standards. The use of HPLC-ICP-MS and HPLC-ESMS as complementary techniques to one another in this way demonstrates their capability in providing information for species identification and verification in new materials or where further identification and characterization of species may be required. Knowledge of predicted fragmentation pathways for molecular ions obtained by using MSMS may prove vital where new materials are under investigation for identification and characterization of species, particularly where there is a lack of available standards for peak identification using HPLC-ICP-MS.

Having established optimum experimental conditions for HPLC-ICP-MS and HPLC-ESMS and the legitimacy of the procedure for species separation and identification, work with the marine algae samples was pursued. SPE techniques using on-column fraction collections were investigated for bulk matrix removal from samples prior to analysis using ESMS. Optimum conditions are given in section 5.2.1.6. Speciation retention and subsequent elution was verified using ICP-MS. However, the results following purification and fractionation techniques proved unsuccessful. One of the reasons for this was indicated by the loss of analytes of interest together with removal of the matrix leaving the analyte signal close to the limit of detection, as shown by ICP-MS response. Enzymatically digested sample extracts with no matrix removal or purification were, therefore,

introduced onto the HPLC column coupled with ESMS for analysis as described in section 5.2.1.5.

'Total' concentrations of arsenic present in the two marine samples, *Fucus spiralis* and *Ascophyllum nodosum*, under investigation were determined following HNO_3 and enzymolysis digestions using direct ICP-MS. The results are given in Table 5-5. The 'total' arsenic concentration from the enzyme digestions are given together with their extraction efficiencies, which were calculated as a ratio of the total concentrations obtained from the HNO_3 digests. The results are given with 95% confidence intervals and are in agreement with the given values.

Table 5-5 ' Total' As determined in HNO_3/H_2O_2 and enzymolysis extracts of *Fucus sp.* and *Ascophyllum nodosum* using ICP-MS. Results are given in mg kg^{-1} as the element

	Fucus sp.	*Ascophyllum nodosum*
HNO_3 acid digest	43.2 ± 1.0	47.6 ± 1.1
Enzyme digest	34.6 ± 2.8	36.7 ± 2.5
Enzyme extraction efficiency	80 ± 7 %	77 ± 6 %
Certified reference value	44.3 ± 2.1	44.0 ± 1.7[1]
LOD (3 x SD)	0.0028	NA[2]

[1] reference value only
[2] not available

Cellulase was used for the digestion of the algae giving an extraction efficiency of approximately 80%. This was considered to be an acceptable level in light of research using the more common MeOH or MeOH-H_2O extraction techniques that report efficiencies ranging from 20% [195] to > 85% [113]. The use of cellulase breaks down the cell wall releasing cellular contents with no inter-conversion of species.

Preliminary arsenic speciation studies on enzyme extracts of the algae were performed using HPLC-ICP-MS. Separation and detection of arsenic species by HPLC-ICP-MS, with ICP-MS being an element-specific detector, provided evidence of arsenic-containing compounds together with their respective elution times. The matching of retention times with that of known standards was employed for peak identification. It is known that matrix effects can alter retention times of standards and, therefore, spiking experiments with standards were carried out to confirm species by this technique. However, when analyzing marine algae, the likelihood of arsenosugars being present is exceptionally high for which there are no commercially available standards. This precluded the matching of retention times with standards for any unidentified peaks. The possibility of co-eluting species with that of standards could also not be excluded. Figure 5-7 (a repeat of Figure 5-1, provided for direct comparison) demonstrated the typical retention times for the available standards of AsBet, DMA, MMA and As^{V}. Figure 5-8 shows the chromatogram of *Fucus sp.* and Figure 5-9 that of *Ascophyllum nodosum* obtained using HPLC-ICP-MS. Comparison of the retention times with that of standards demonstrated that the first peak in Figure 5-8 eluted with a similar time to that of AsBet. However, it is known and well-established [196] that marine algae contain arsenosugars that elute with, or close to, the solvent front as does AsBet when using anion-exchange chromatography.

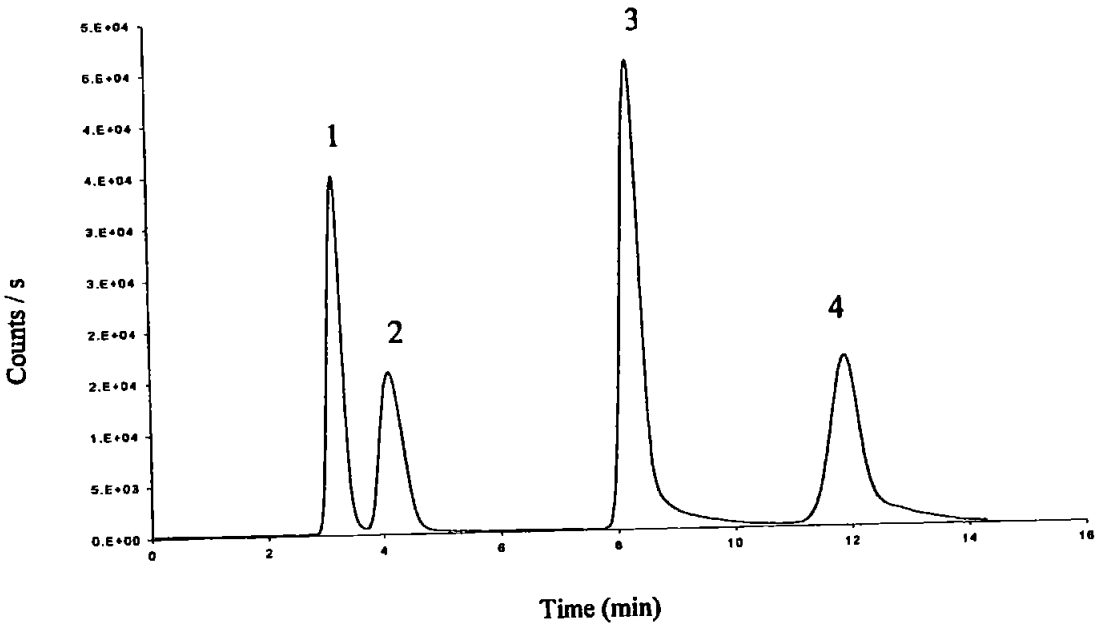


Figure 5-7 A typical chromatogram of arsenic standards, 250 μg l^{-1} each, using a Hamilton PRP X100 anion-exchange HPLC-ICP-MS (column dimensions of 250 x 4.6 mm I.D.). The mobile phase used was 10 mM NH_4HCO_3 with 10% MeOH at pH 10. Peaks: 1 = AsBet; 2 = DMA; 3 = MMA; 4 = As^V.

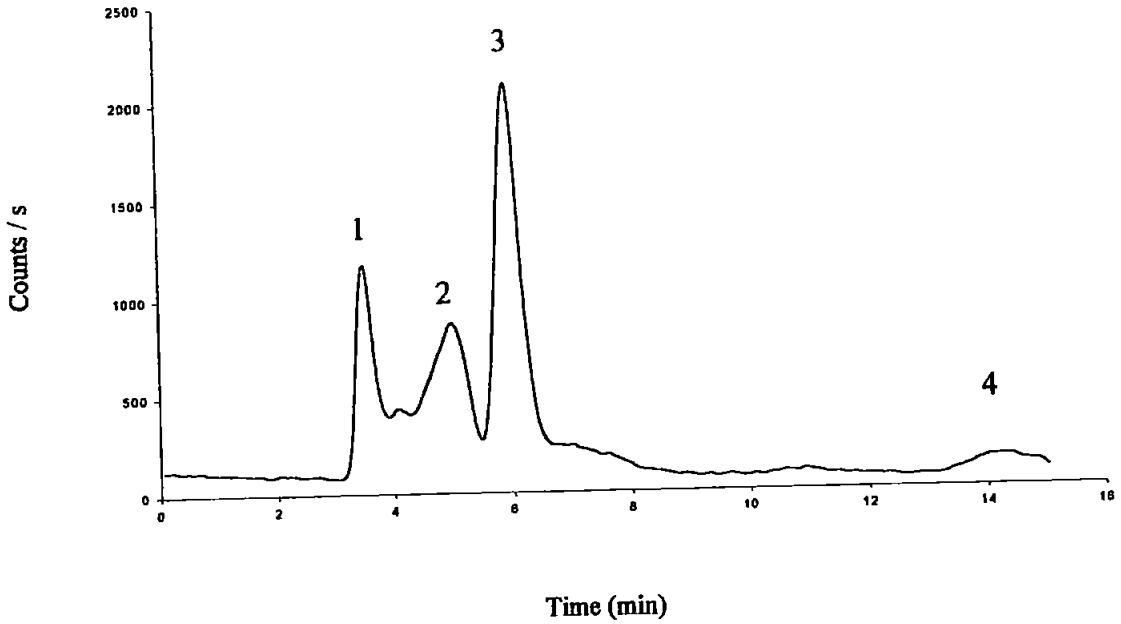


Figure 5-8 A chromatogram of the CRM IAEA-140 (seaweed), using a Hamilton PRP X100 anion-exchange HPLC-ICP-MS (column dimensions of 250 x 4.6 mm I.D.). The mobile phase used was 10 mM NH_4HCO_3 with 10% MeOH at pH 10. Peaks 1-4 nominally assigned prior to identification.

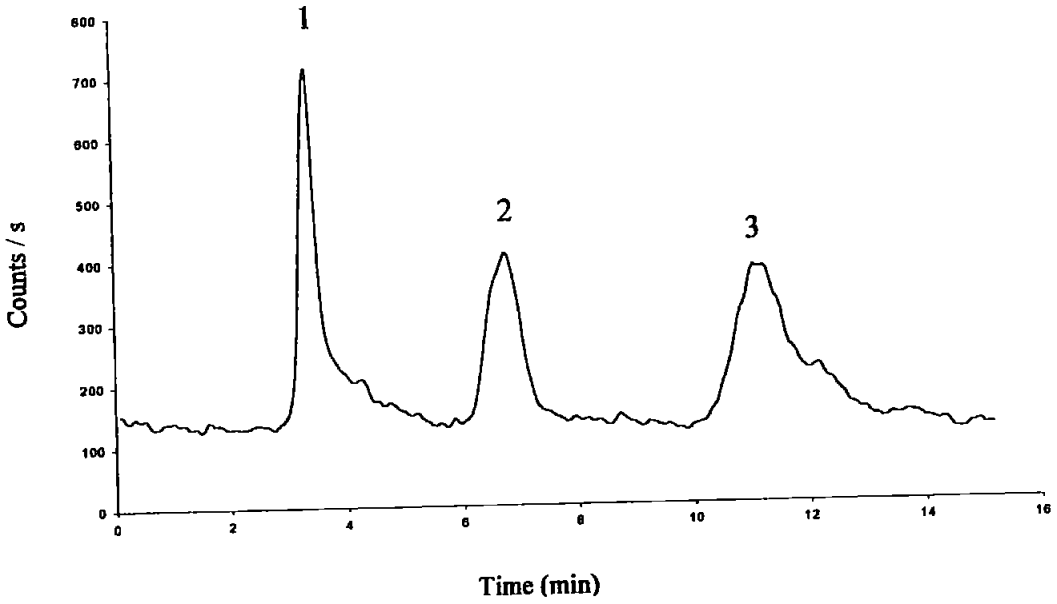


Figure 5-9 A chromatogram of *Ascophyllum nodosum*, using a Hamilton PRP X100 anion-exchange HPLC-ICP-MS (column dimensions of 250 x 4.6 mm I.D.). The mobile phase used was 10 mM NH_4HCO_3 with 10% MeOH at pH 10. Peaks 1-3 nominally assigned prior to identification.

A poorly resolved second peak may tentatively be ascribed to DMA and the third peak did not match any of the standards used and, therefore remained unidentified. The very small fourth peak may be attributed to inorganic arsenic. The chromatogram obtained for the *Ascophyllum nodosum* sample (Figure 5-9) showed the first peak eluting with a similar time to that of the AsBet standard. The second and third peaks did not closely match the elution times of any standard.

By using the same chromatographic system for HPLC-ESMS it was possible to examine peaks where arsenic-containing species were known to elute, as demonstrated by HPLC-ICP-MS, and obtain complementary molecular information leading to characterization of the compound. This was particularly

useful for arsenic-containing compounds that did not match retention times of that of known standards when using HPLC-ICP-MS, or where co-elution is known to exist (arsenosugars with AsBet).

The HPLC-ESMS chromatogram obtained from the *Ascophyllum nodosum* sample is shown in Figure 5-10. The electrospray process measures all molecular ions in a sample and, therefore, the chromatogram obtained did not give any well-defined peaks that might have corresponded to the peaks seen using HPLC-ICP-MS. There was evidently a broad, high background level of eluting molecular ions, probably attributable to the presence of the matrix.

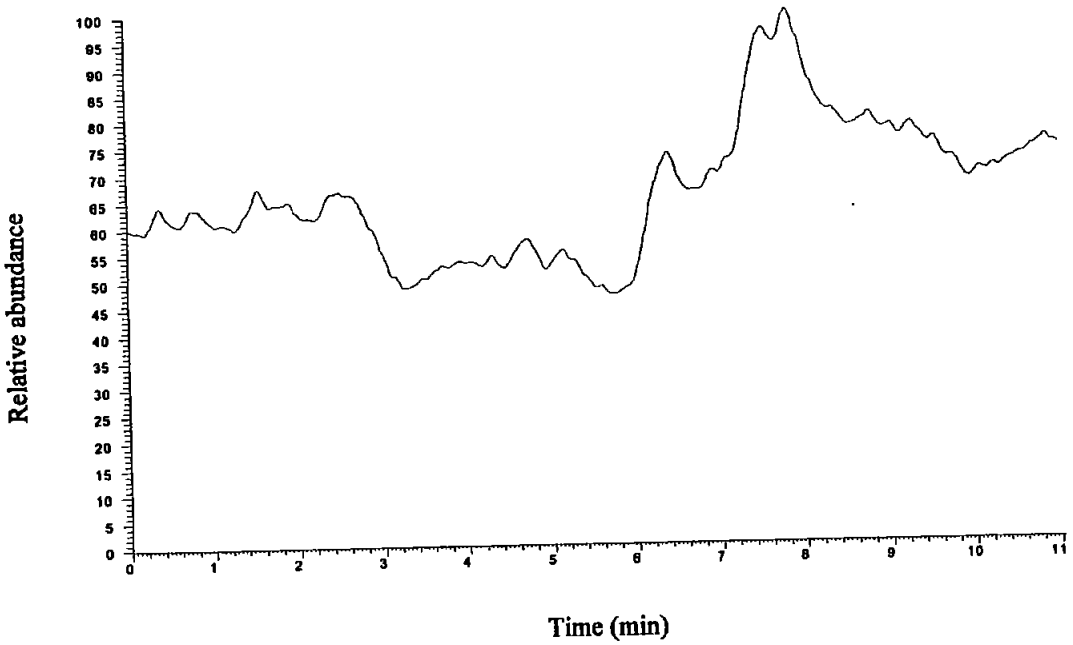


Figure 5-10 Chromatogram obtained of the *Ascophyllum nodosum* sample using ESMS with the conditions described in section 5.2.1.5 and Table 5-2.

However, observation of m/z 329 in the SIM mode, shown in Figure 5-11, provided evidence of a peak, having a similar elution time to that of AsBet (as seen in Figure 5-2 using HPLC-ESMS), which was attributed to the arsenosugar, previously identified by Edmonds and co-workers [18, 19], the structure for which is shown in Figure 5-12. This supported the findings by other workers that marine algae contain arsenosugars that may elute, under certain conditions, from a chromatographic column at a similar time to that of AsBet [196].

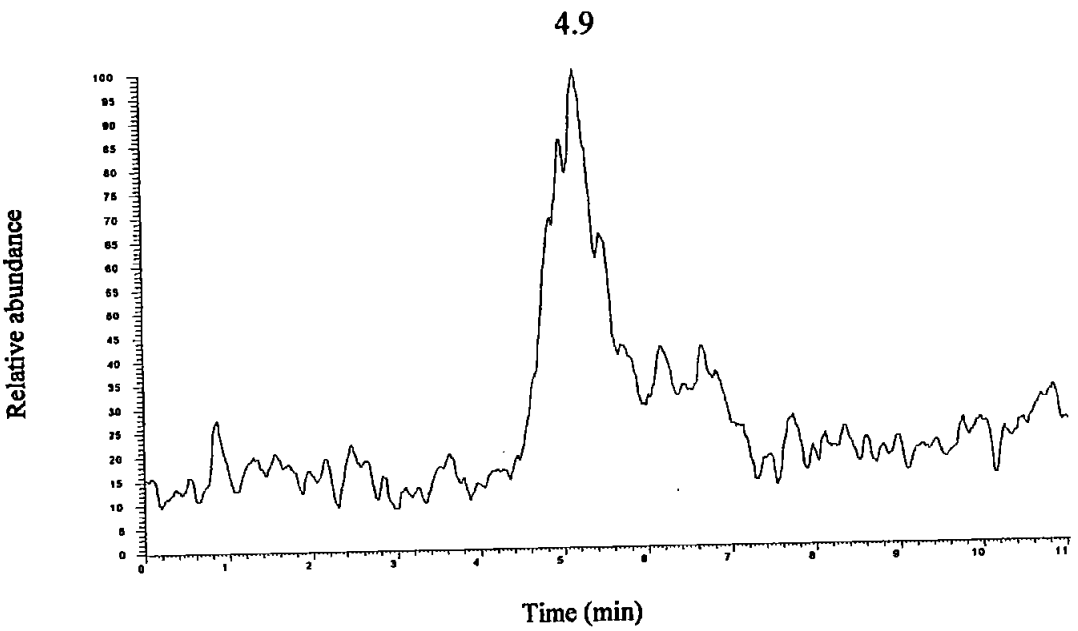
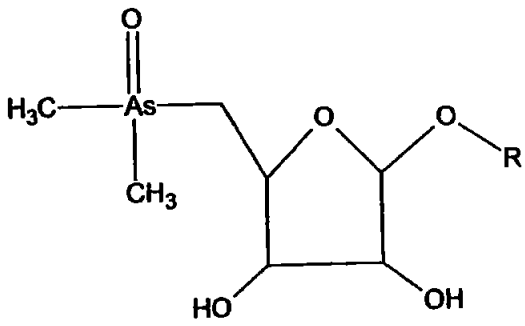


Figure 5-11 Chromatogram obtained of the *Ascophyllum nodosum* sample using ESMS in the SIM mode at m/z 329 with the conditions described in section 5.2.1.5 and Table 5-2. (peak apex time given – 4.9)



R = $CH_2CHOHCH_2OH$ (328)

Figure 5-12 Structure of the arsenosugar, commonly found in marine algae, having a $M+H^+$ 329 mass units [116].

A more detailed study of the *Fucus sp.* sample was undertaken for identification of arsenic species present within the sample matrix. A HPLC-ESMS chromatogram obtained from the *Fucus sp.* sample is shown in Figure 5-13. Three peaks are discernable above the baseline. Having taken into account the shift in retention times (see Table 5-4) between the techniques, the peaks may be considered representative of the first three peaks seen using HPLC-ICP-MS of the *Fucus sp.* sample (Figure 5-8).

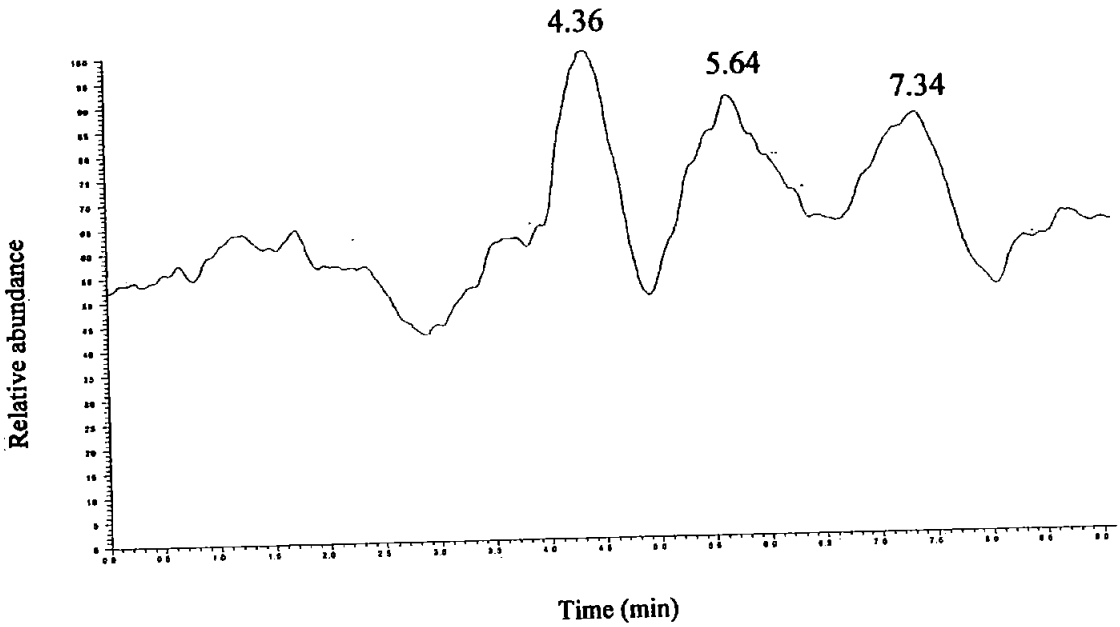


Figure 5-13 Chromatogram obtained of *Fucus sp.* using anion-exchange HPLC-ESMS with the conditions described in Section 5.2.1.5.

However, the complexity of chromatograms obtained by HPLC-ESMS is adequately demonstrated when viewing the mass spectrum obtained of protonated molecular ions by their mass/charge (*m/z*) ratio. A m/z spectrum corresponding to the second peak (retention time 5.64 min) is shown in Figure 5-14. A number of spectral peaks can be seen indicating the presence of a variety of molecular ions that have the same chromatographic elution times. Unlike ICP-MS, which is an element specific detector, all molecular ions amenable to the electrospray process are measured in the sample matrix, including the solvent. Although not the most dominant in the spectrum, the molecular ions at peaks *m/z* 241, 329 and 409 that correspond to known arsenic-containing compounds and can be clearly seen. The $M+H^+$ ion seen at m/z 241 has previously been identified by McSheehy *et al.* [110]

and the $M+H^+$ molecular ions at m/z 329 and 409 by Edmonds and co-workers [18, 19]. Using the SIM mode, the chromatogram shown in Figure 5-15, confirms that the masses 241, 329 and 409 co-elute.

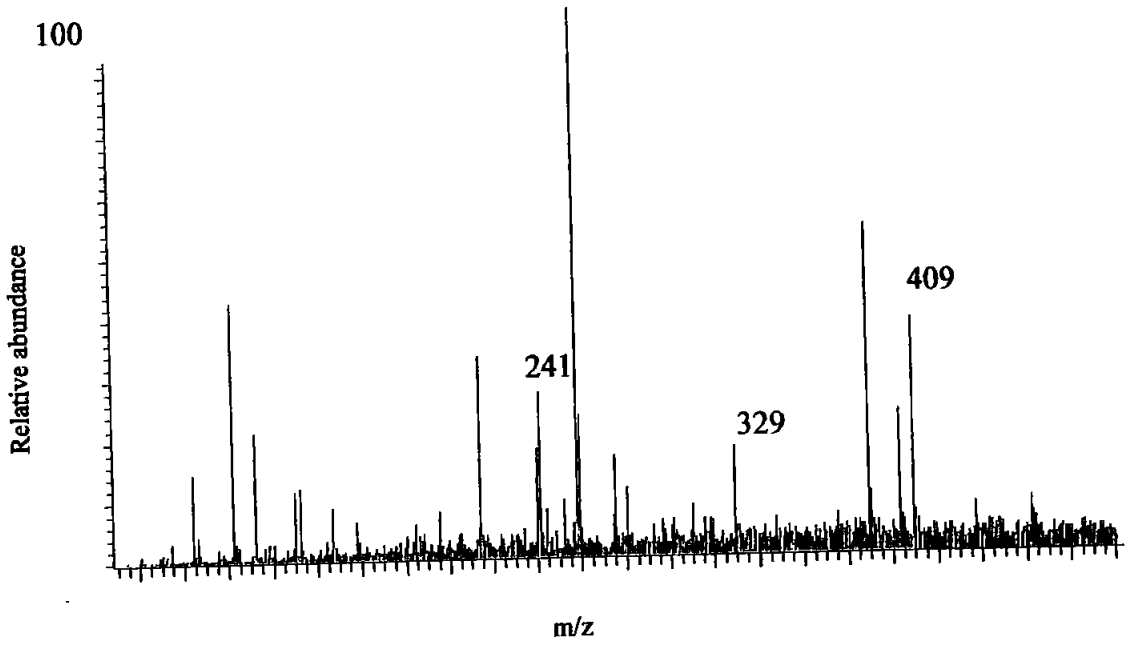


Figure 5-14 ESMS spectrum of middle peak (R_T 5.64 min) of the *Fucus sp.* chromatogram using anion-exchange HPLC-ESMS with the conditions described in Section 5.2.1.5.

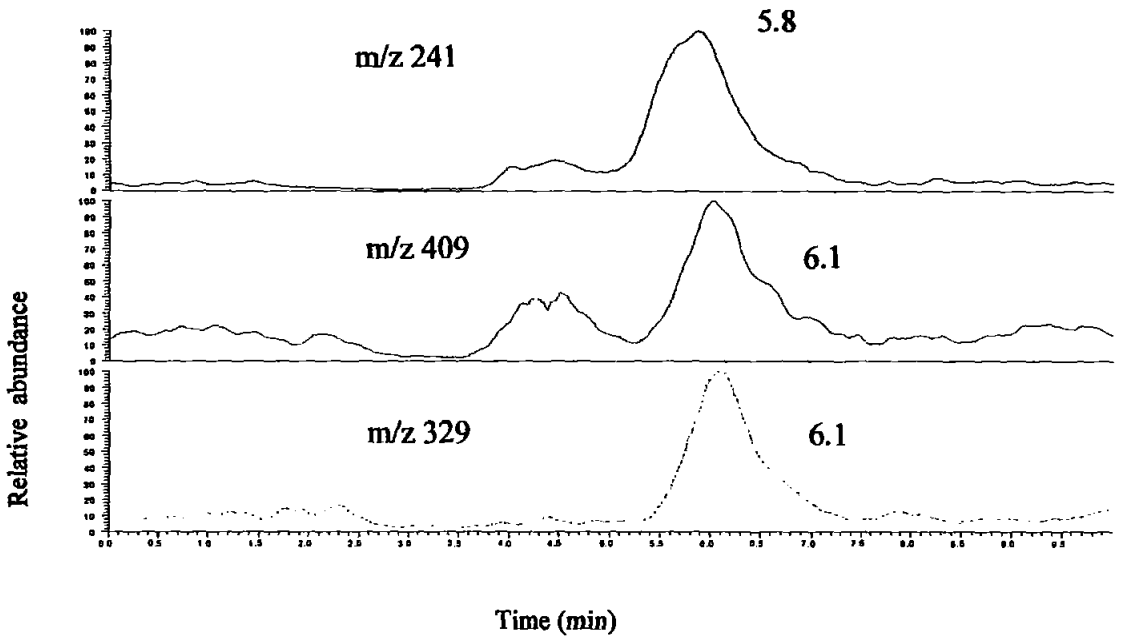


Figure 5-15 SIM chromatograms of the 3 arsenic species identified in middle peak of the *Fucus sp.* sample demonstrating retention times using anion-exchange HPLC-ESMS with the conditions described in Section 5.2.1.5.

The middle peak seen in Figure 5-13 has a retention range of 5.1 – 6.3 min with a peak maximum of 5.64 mins (Table 5-6). The molecular ions of 241, 329 and 409, although certainly present within the time range, show peak apices in the SIM mode with slightly longer elution times (shown in Figure 5-15) than the peak maximum. This suggests that they are only some of the eluting species contributing to the middle peak.

Table 5-6 Peak times for *Fucus sp.* sample using HPLC-ESMS. Retention range and peak points are obtained in the full ESMS mode. Time is given in minutes.

	Peak 1	*Peak 2*	*Peak 3*
Retention range (time)	4.0 – 4.8	5.1 – 6.3	6.8 – 7.8
Peak maximum (time)	4.36	5.64	7.34

The third peak, with a retention range of 6.8 – 7.8 min and a peak maximum at a time of 7.34 min, in the chromatogram of the *Fucus sp.* using HPLC-ESMS (Figure 5-13), was shown to contain a $M+H^+$ molecular ion at mass 483, which corresponds with an arsenosugar also previously identified by Edmonds and co-workers [18, 19]. A chromatogram, using the SIM mode, of this peak is shown in Figure 5-16. This compound may account for the unidentified third peak obtained by HPLC-ICP-MS of the *Fucus sp.* sample (Figure 5-13) having taken into account the shift in retention times of species between the techniques of HPLC-ICP-MS and HPLC-ESMS.

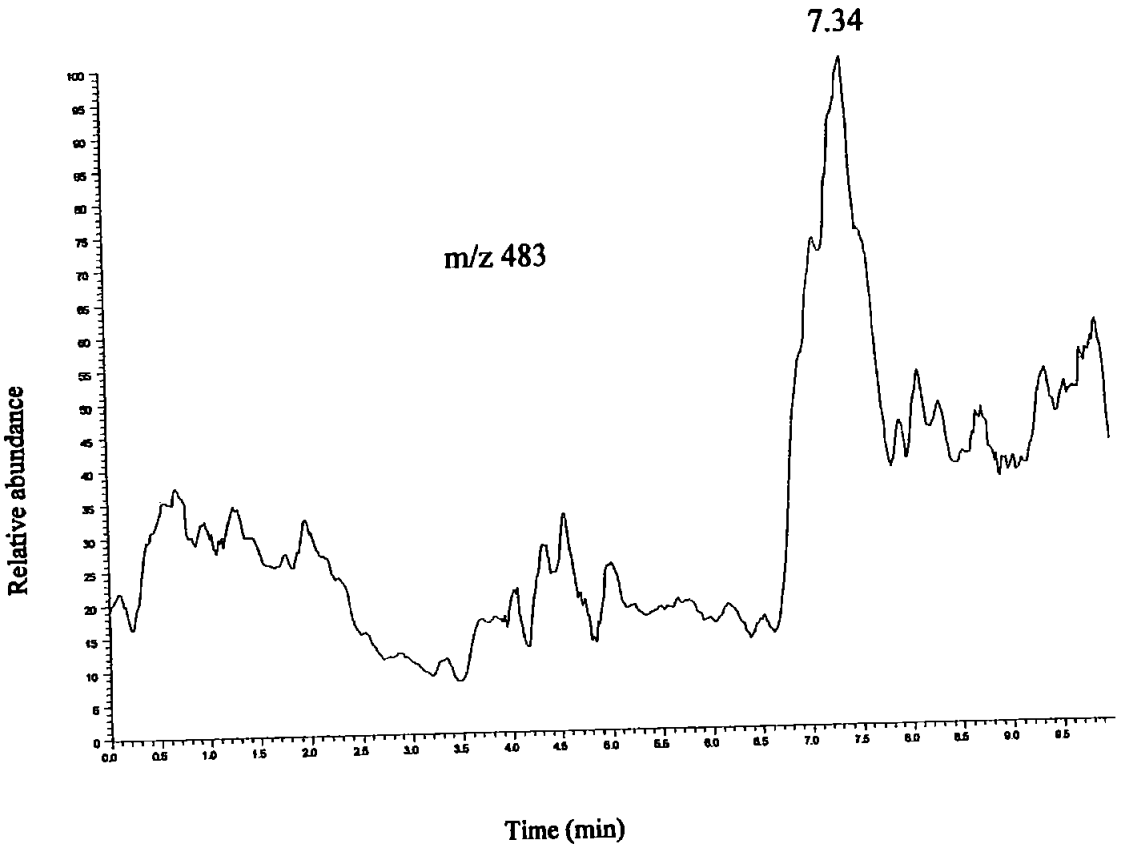
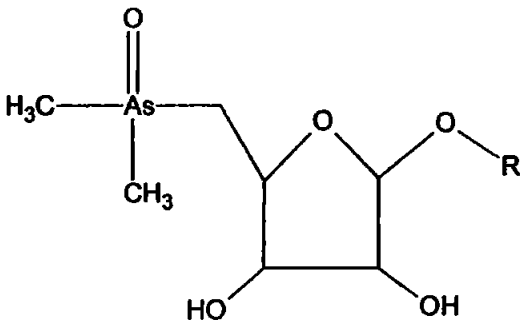


Figure 5-16 SIM of m/z 483 in the *Fucus sp.* sample demonstrating retention time using anion-exchange HPLC-ESMS with the conditions described in Section 5.2.1.5.

Due to major matrix elution and broad, high level background in the solvent front region, no arsenic-compounds were identified in the first peak obtained by HPLC-ESMS. However, using the SIM mode to look at mass 179, no peak was seen corresponding to this elution time or to any other time in the chromatogram. This suggests that peak 1 seen in the *Fucus sp.* sample by HPLC-ICP-MS (Figure 5-8) contains an arsenic-containing compound other than AsBet that co-elutes with this standard.

In all the sample chromatograms shown using HPLC-ESMS, the $M+H^+$ ion signals for the proposed masses of arsenic-containing compounds do not appear solely at their characteristic retention times but elsewhere in the chromatogram. These signals may be produced by matrix compounds with the same $M+H^+$ m/z value. Moreover, these ions may account entirely for the peak obtained where the suspected arsenic-containing compounds are thought to elute. In the case of real samples, the probability that an extract contains a matrix component of the same molecular mass is too great to ignore. The use of MSMS, where fragment patterns of the parent molecule can be studied, may provide more conclusive evidence of the actual molecular structure. By applying MSMS techniques it is possible to analyze product ions resulting from the collision induced dissociation (CID) of the parent ion. However, interpretation of the mass spectra is not always easy to achieve, particularly when confronted by previously unidentified compounds. *A priori* knowledge on the expected nature of the compound and an understanding of the expected molecular structures present can provide invaluable assistance. With this in mind, the structures of previously identified arsenic-containing compounds [18, 19, 110] having the same mass as those identified in this study are shown in Figure 5-17. The structure of the compound at mass 241 is shown in Figure 5-18.



R = $CH_2CHOHCH_2OH$ (328) : a

R = $CH_2CHOHCH_2OSO_3H$ (408) : b

R = $CH_2CHOHCH_2OPO_2HOCH_2CHOHCH_2OH$ (482) : c

Figure 5-17 Structures of three arsenosugars commonly found in marine algae [116]

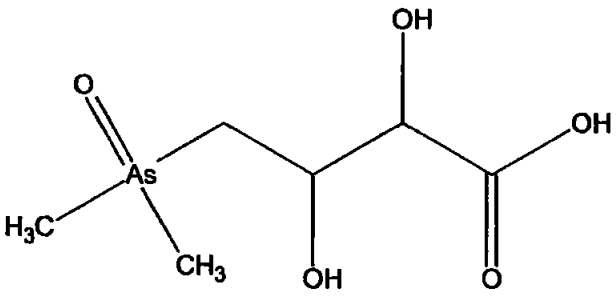


Figure 5-18 Structure of compound at m/z 241 [110], 3-[5`-deoxy-5`-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropylene glycol.

The structures of the three arsenosugars contain a common dimethylarsinoyl ribofuranosyl moiety at mass 237. Fragmentation patterns of the parent molecule

by MSMS should reveal the characteristic structures of these compounds. A common fragmentation pattern for arsenosugars is shown in Figure 5-19.

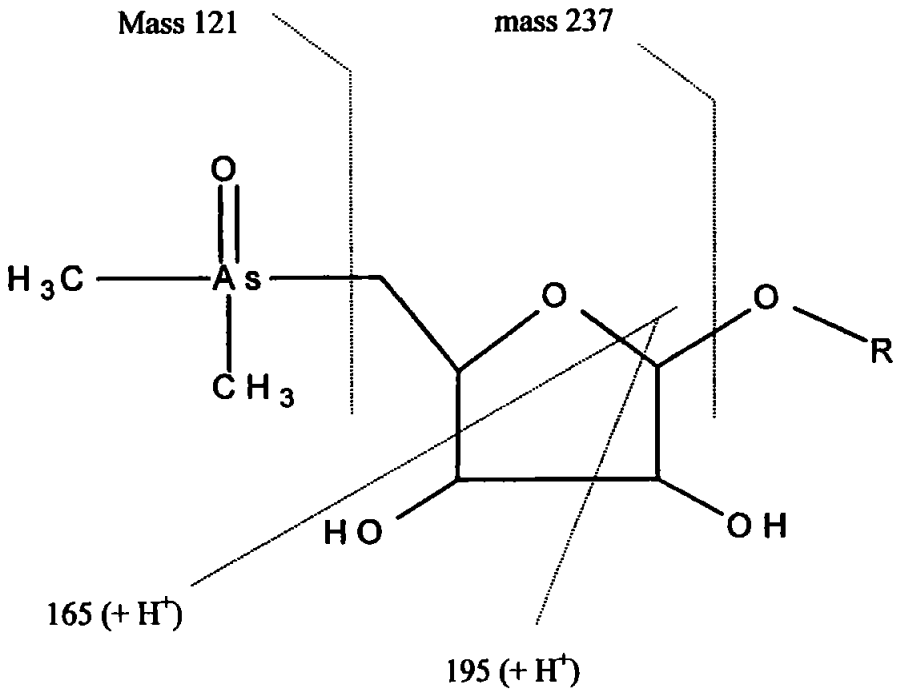


Figure 5-19 Characteristic fragmentation pattern of an 'arsenosugar' [57].

Tandem MS techniques, to fragment parent ions, were applied to the arsenic-containing molecular ions identified in the *Fucus sp.*sample. The fragmentation spectrum obtained for the molecular $M+H^+$ ion at m/z 329 is shown in Figure 5-20. The mass at 347 can be accounted for by a molecule of H_2O hydrating the original $M+H^+$ parent molecule. The characteristic arsenosugar fragments at masses 237 and 165 are visible. The masses at 285 and 213 may be attributable to

loss of CH_2 and OH groups as the molecule fragments. This molecule was, therefore, assigned as 3-[5`-deoxy-5`-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropylene glycol (Figure 5-14, a).

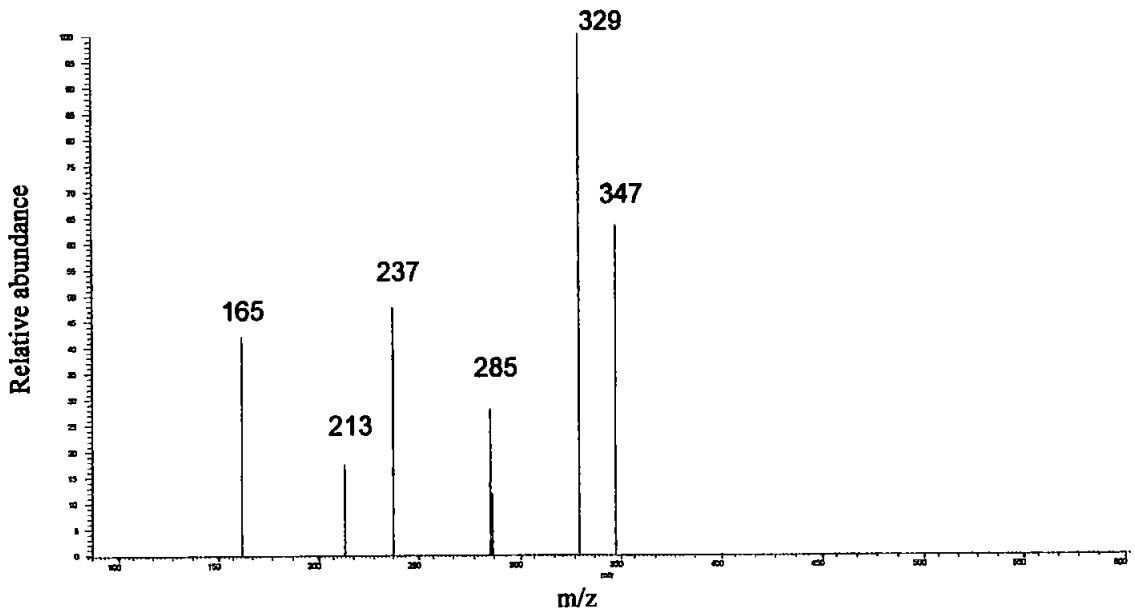


Figure 5-20 Tandem MS of m/z 329 in the *Fucus sp.* sample using anion-exchange HPLC-ESMS with the conditions described in Section 5.2.1.5.

The tandem ESMS spectrum of the $M+H^+$ ion at m/z 483 is shown in Figure 5-21. The m/z fragment at mass 237 is characteristic of an arsenosugar and dominates the spectrum. The parent ion at m/z 483 is also appreciably large. The lack of other ions suggests that the fragmenting voltage was not optimized in this case to provide the best structural evidence of the molecule. However, the information can be attributed to the presence of the arsenosugar, 3-[5`-deoxy-5`-

(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropyl-2,3-dihydroxypropyl phosphate (Figure 5-17, c).

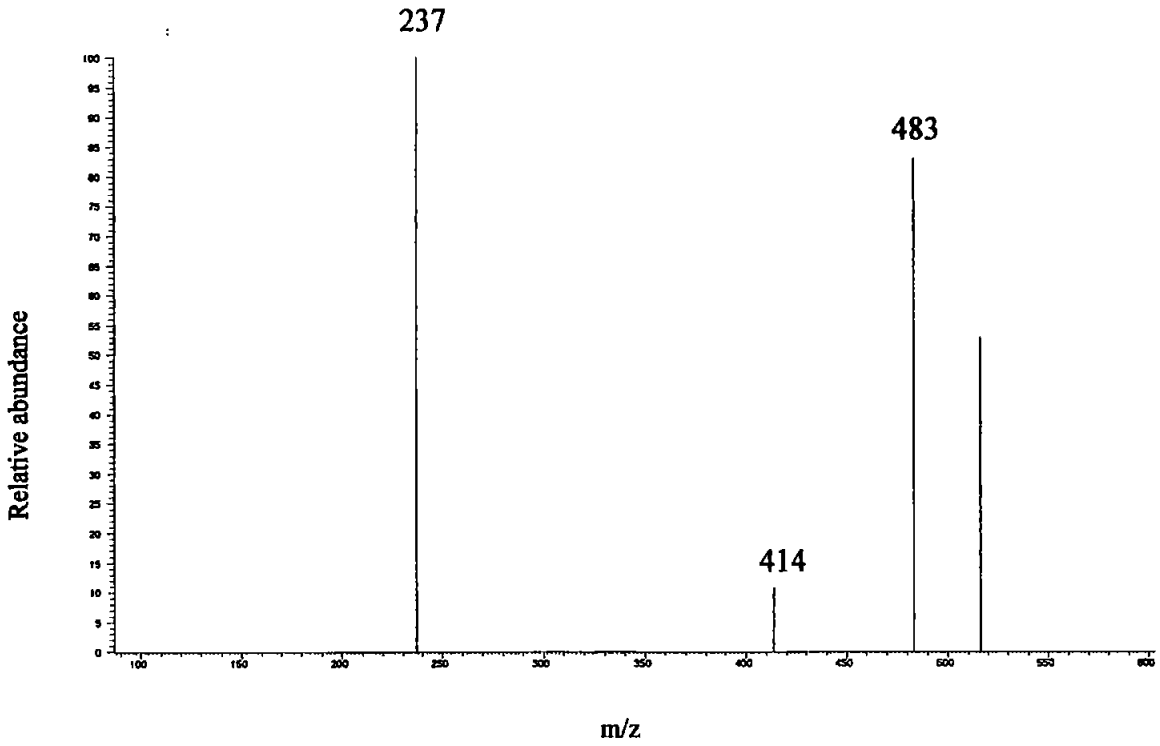


Figure 5-21 Tandem MS fragment pattern of compound at m/z 483 using anion-exchange HPLC-ESMS with the conditions described in Section 5.2.1.5.

The protonated molecular ion at m/z 409, that is frequently reported in the literature [113], has been assigned as an arsenosugar. The fragments at m/z 237 and 165, shown in Figure 5-22, support this assignment. The other peaks in the spectrum are more difficult to assign and may be from the loss of O, OH and C groups with rearrangements also occurring. The molecule at $M+H^+$ 409 was assigned as 3-[5`-deoxy-5`-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropyl hydrogen sulphate (Figure 5-17, b).

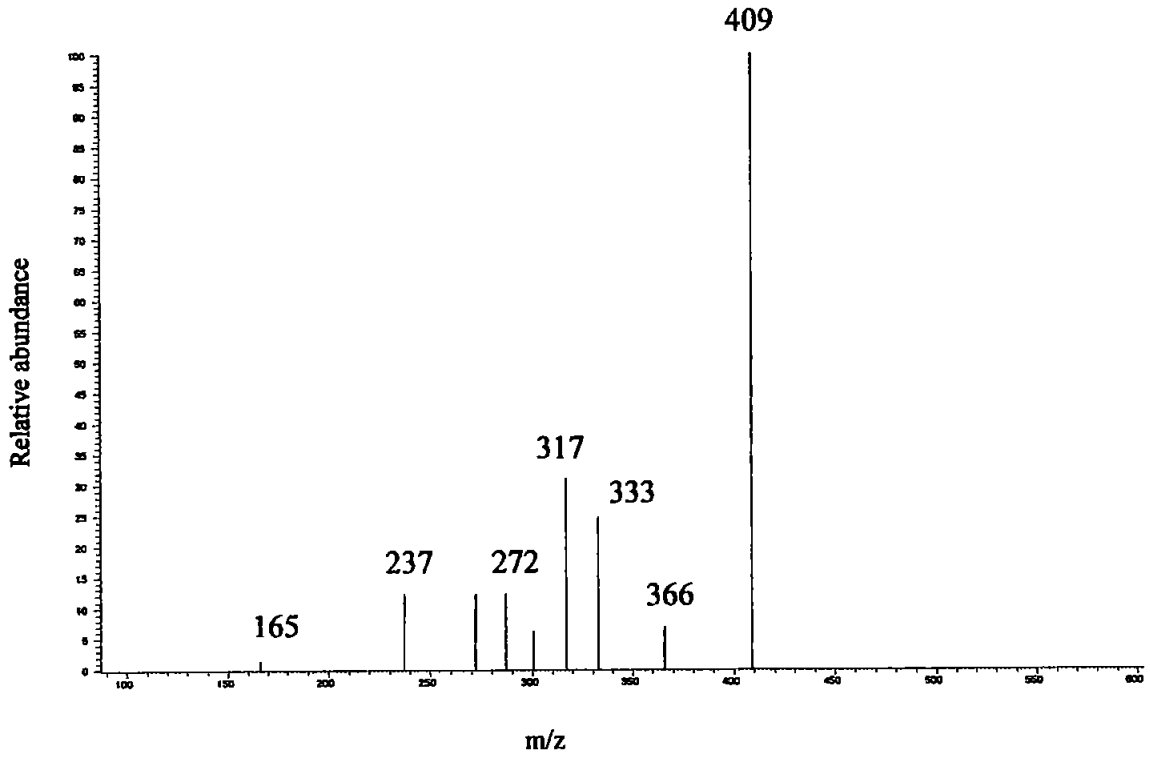


Figure 5-22 Tandem MSMS of compound at m/z 409 using anion-exchange HPLC-ESMS with the conditions described in Section 5.2.1.5.

The SIM at m/z 241, whose structure is shown in Figure 5-18, may be attributable to the arsenic compound 4-dimethlyarsinoyl-2,3-dihydroxybutanoic acid, recently reported by McSheehy, *et al* [110]. The fragments obtained in the MSMS spectrum (Fig 5-23) support this view as loss of 46 mass units at 195 represents a protonated carboxylic acid group and 121 represents the dimethylarsinoyl moiety. The loss of 30 mass units from 195 to 165 indicates the loss of a CHOH group present in the carbon chain of the molecule [110].

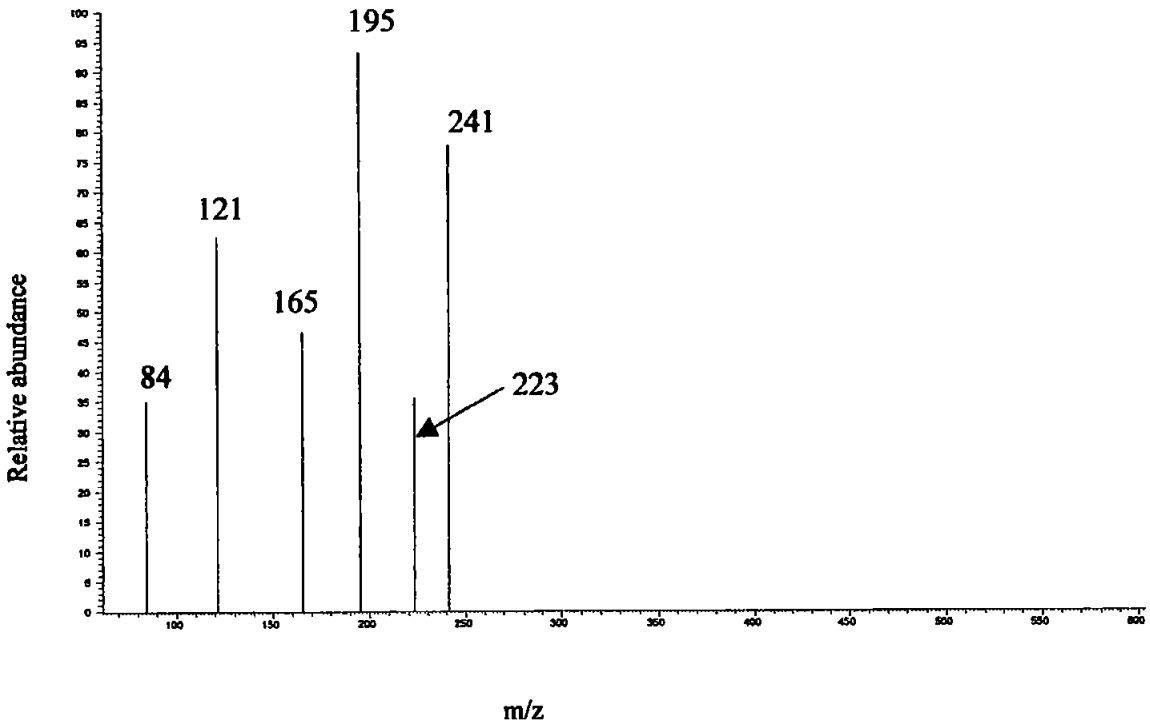


Figure 5-23 MS-MS of $M+H^+$ 241 using anion-exchange HPLC-ESMS with the conditions described in Section 5.2.1.5.

A short summary of the results obtained using HPLC-ICP-MS, which demonstrate the presence of arsenic-containing compounds in the *Fucus sp.* and *Ascophyllum nodosum* samples, and the results using HPLC-ESMS together with MSMS techniques providing molecular information suggest that the unresolved peak 2 (Figure 5-8) of the *Fucuc sp.* sample, using HPLC-ICP-MS, contains three compounds assigned as 4-dimethlyarsinoyl-2,3-dihydroxybutanoic acid ($M+H^+$, 241), 3-[5`-deoxy-5`-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropyl hydrogen sulphate ($M+H^+$, 409) and 3-[5`-deoxy-5`-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropylene glycol ($M+H^+$, 329). The third peak was assigned as 3-[5`-deoxy-5`-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-

hydroxypropyl-2,3-dihydroxypropyl phosphate ($M+H^+$, 483). No arsenic-containing compound was adequately identified for peak 1, although the absence of AsBet was confirmed. The investigations carried out on the *Ascophyllum nodosum* sample demonstrated the presence of an arsenosugar, m/z 329, having a similar retention to that of AsBet, which supports the findings of Gallagher *et al* [196], stating that marine algae contain arsenosugars that co-elute with AsBet.

5.3.1 Speciation of selenium compounds using HPLC-ICP-MS and HPLC-ESMS

5.3.1.1 Instrumentation

See section 5.2.1.1.

Table 5-7 Instrumental operating parameters for the identification of selenium species present in yeast using direct injection ESMS and HPLC-ESMS.

Instrument	*Operating conditions*	*Gas flow rates*
LCQ ESMS	Spray voltage – 4.5 kV	
	Spray current – sample dependent	Direct injection
	Capillary temperature – 220°C	Sheath – 18 l min^{-1}
	Capillary voltage, 9.95 V	Auxiliary – 0 l min^{-1}
	Lens voltage, -45.43 V	HPLC
	Octapole 1 offset, -5.71 V	Sheath – 18 l min^{-1}
	Octapole 2 offset, -6.62	Auxiliary – 0.3 l min^{-1}

5.3.1.2 Chemicals and reagents

Chemicals were of analytical grade unless otherwise stated. All lab-ware was soaked in HNO_3 (10% v/v) for a minimum of 24 hours and rinsed thoroughly with MilliQ water (Millipore, Bedford, MA, USA) prior to use. Stock solutions of selenomethionine (SeMet), selenocystine (SeCys), sodium selenate and sodium selenite (Sigma-Aldrich, Poole, Dorset, UK) at 1000 mg l^{-1} as the element were stored at $4^{O}C$ in the dark. Standards were prepared daily from the stock solutions. Protease (Sigma-Aldrich) was used for sample digestion procedures. Mobile phase eluents were prepared using ammonium hydrogen carbonate, ethanoic acid (Sigma-Aldrich) and methanol (Fisher Chemicals).

5.3.1.3 HNO_3 digestion for total element determination

Microwave bombs (Savillex, Minetonka, Minnesota, USA) were pre-cleaned with 3 ml 69% v/v HNO_3 (Primer, Fisons, Loughborough, UK) in a Perfecto 800 W microwave oven (DeLonghi, Italy) on medium power for 2 mins. Samples of approximately 0.25 g were accurately weighed into the bombs and 4 ml HNO_3 (69%, v/v) together with 1 ml H_2O_2 (37%, v/v) were added. The bombs were loosely capped and left overnight to allow easily oxidised material to be destroyed. After predigestion, the bombs were swirled gently, sealed tightly and microwaved on medium power for 1 - 2 mins, or until the sample was a clear colour with no residue (indicating a completed digest). The samples were transferred quantitatively to volumetric flasks and made up to volume with 2%

HNO_3 giving an overall dilution of x2000. The samples and standards were spiked with indium to give a final concentration of 100 μg l^{-1} Indium (In) which acted as an internal standard prior to analysis by ICP-MS using the conditions described in Table 5-1, section 5.2.1.1. The internal standard was used to correct for instrumental drift (sample viscosity effects, mass transport, etc.) over the analysis period.

5.3.1.4 Enzymatic digestion procedure for extraction of selenium species in yeast

Samples of approximately 0.25 g were accurately weighed together with 0.025 g protease and approximately 40 ml NH_4HCO_3 buffer (0.1 M, pH 8). The suspensions were homogenized in a 'Potter' homogenizer, transferred to polyethylene centrifuge tubes and placed in a shaking water bath at 37°C for a minimum of 4 hours. Following enzymolysis digestion, the samples were centrifuged at 2500 rpm for 20 min, the supernatant transferred quantitatively to volumetric flasks and made up to volume with the NH_4HCO_3 buffer. The samples and standards were spiked with caesium to give a final concentration of 100 μg l^{-1} Cs which acted as an internal standard prior to analysis by ICP-MS.

5.3.1.5 Chromatographic conditions for the determination of selenium species using HPLC-ICP-MS and HPLC-ESMS

The chromatographic system consisted of a Dionex AS 11 column (250 x 4.1 mm i.d.) packed with a strong anion-exchange polymeric based resin of 10 μm diameter with a guard column (50 x 4.1 mm i.d.) of the same material. The mobile phase was a step-gradient of 10 mM NH_4HCO_3 with 10% MeOH at pH 5 (adjusted with glacial CH_3COOH) and 50 mM NH_4HCO_3 with 10% MeOH at pH 5 (adjusted with glacial CH_3COOH). The elution program is given in Table 5-8. The eluent flow rate used throughout was 1 ml min^{-1}, with a 20 μl sample loop for HPLC-ICP-MS, and by using a post-column splitter, a flow rate of 150 μl min^{-1} with a 200 μl sample loop was used for HPLC-ESMS.

Table 5-8 Elution program for selenium speciation using HPLC-ICP-MS

	0 – 5 min	*5 – 9 min*	*9 – 15 min*
10 mM NH_4HCO_3 (adjusted to pH 5)	100 %	0 %	100 %
50 mM NH_4HCO_3 (adjusted to pH 5)	0 %	100 %	0 %

5.4 Results and discussion

The techniques used for the elucidation of arsenic-containing compounds in a marine algae were applied in a similar manner for the identification of selenium-containing compounds in the yeast sample. Protease was the enzyme of choice for extraction of species due to its efficiency, and also its successful use is frequently reported in the literature [197, 174]. However, one of the disadvantages encountered with the use of proteolytic enzymes is that information surrounding the original protein is lost and therefore other extraction techniques may be used to avoid this effect [111].

Total concentrations of selenium in the yeast sample extracts were determined following HNO_3 and enzymolysis digestions using direct ICP-MS. The results are given in Table 5-9. The totals from the enzyme digestions are given together with their extraction efficiencies, which were calculated as a ratio of the total concentrations obtained from the HNO_3 digests. The results are given with 95% confidence intervals and are in agreement with the reference values.

Table 5-9 Total selenium in HNO_3/H_2O_2 and enzyme digests of yeast using ICP-MS. Results are given in mg kg^{-1} as the element

	Total Se	*Extraction efficiencies*
HNO_3 acid digest	1282 ± 33	100 ± 5 %
Enzyme digest	1158 ± 99	90 ± 8 %
Reference value	1300	
LOD (3 x SD)	0.0041	

Speciation studies of selenium in yeast were performed by HPLC-ICP-MS prior to verification by HPLC-ESMS, using the conditions described in sections 5.2.1.1 and Table 5-7. Chromatographic conditions employed are described in Section 5.3.1.5 and Table 5-8. A typical chromatogram of selenium standards using HPLC-ICP-MS is shown in Figure 5-24 and that of the yeast sample in Figure 5-25.

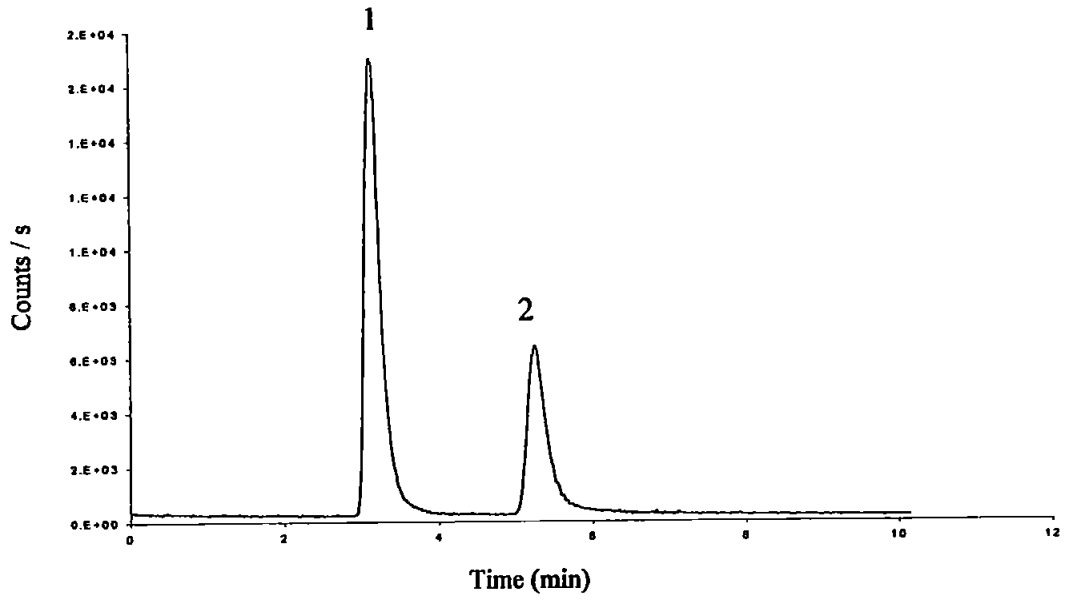


Figure 5-24 Typical chromatogram of Se standards by anion-exchange HPLC-ICP-MS as described in Section 5.3.1.5 and Table 5-8. Peak 1, SeCys at 1 mg l^{-1}; Peak 2, SeMet at 1 mg l^{-1} (all concentrations given in terms of the element)

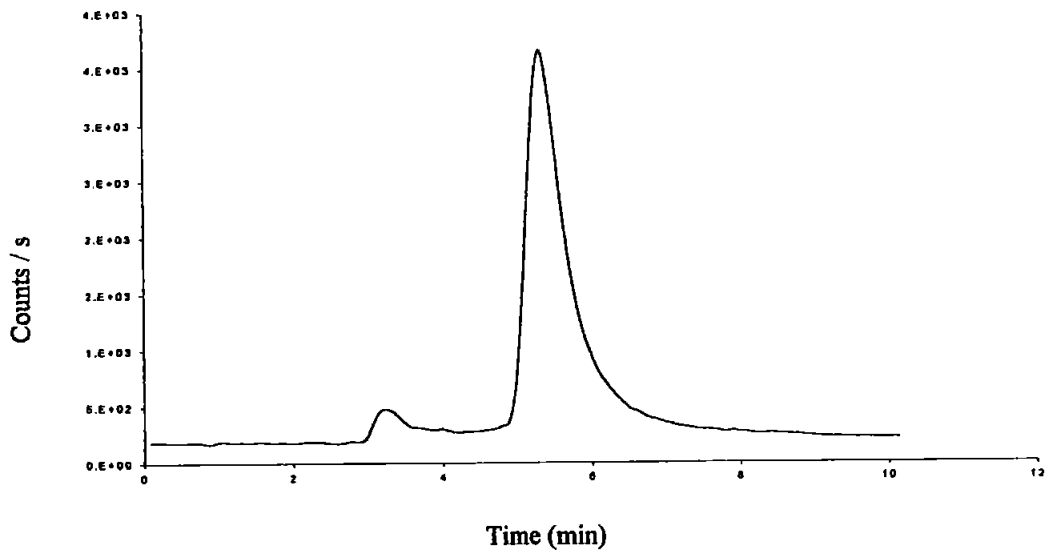


Figure 5-25 Chromatogram of yeast sample by anion-exchange HPLC-ICP-MS as described above.

The chromatogram of the yeast sample (Figure 5-25) suggested that the major peak seen corresponded to SeMet, having a similar elution time to that of the SeMet standard, and that a small peak of SeCys was also present. Analysis by HPLC-ESMS was employed for comparison of the peaks obtained in order to confirm their identity.

One of the advantages to working with selenium compounds is that selenium has 6 isotopes. The characteristic isotopic pattern can be used to assist in identifying selenium compounds present in a spectrum, without prior knowledge of the expected compound. The six isotopes together with the ratios of their natural abundance are shown in Table 5-10. Figure 5-26 demonstrates the presence of the isotopic pattern in SeMet based on its $M+H^+$ monoisotopic mass of 198.

Table 5-10 Selenium isotopes and their natural abundance

Se isotope	*Natural abundance, %*
74	0.9
76	9.1
77	7.5
78	23.5
80	50
82	9.0

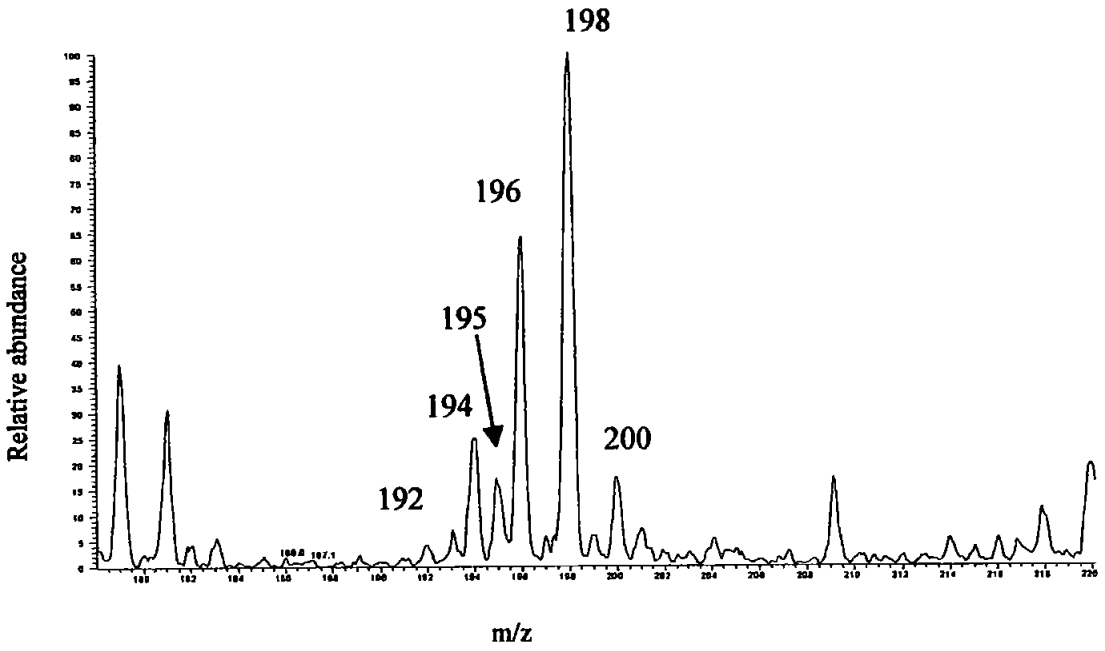


Figure 5-26 SeMet profile by ESMS, using the conditions described in table 5-7, demonstrating the characteristic isotopic pattern of a selenium-containing compound.

A standard of SeCys based on its $M+H^+$ monoisotopic mass of 337 is shown in Figure 5-27 which demonstrates the isotopic pattern that identifies a compound possessing a selenium-selenium bond in a m/z spectrum.

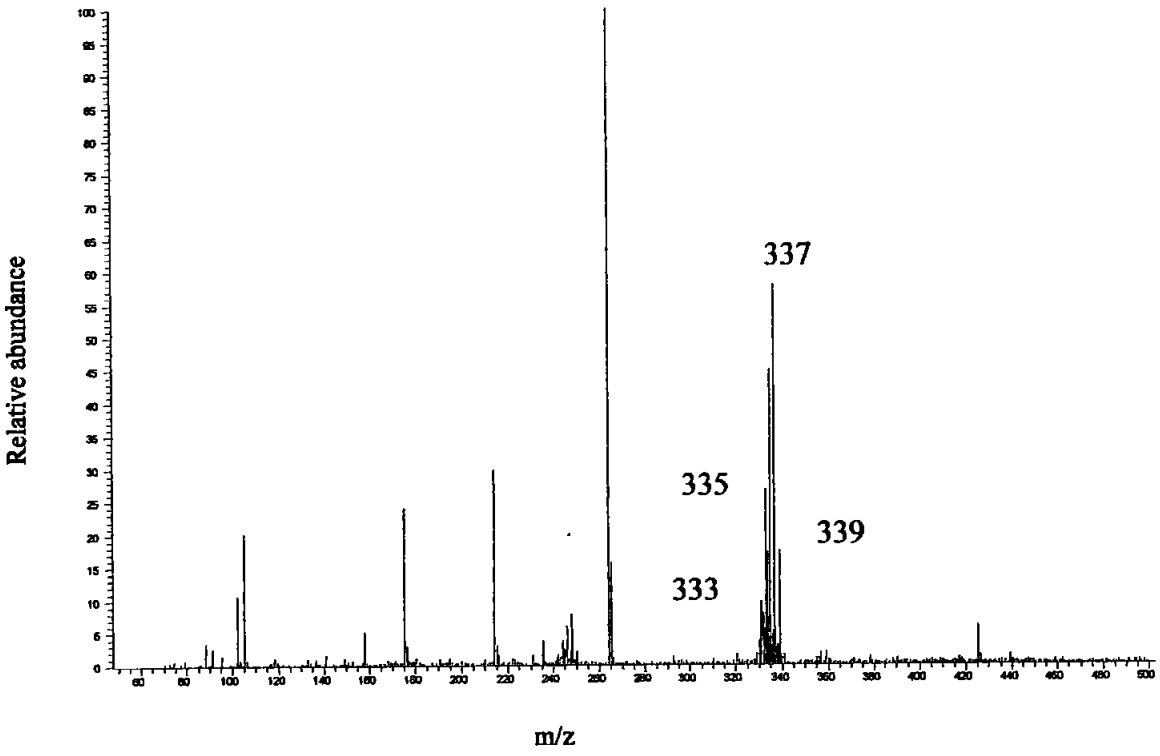


Figure 5-27 SeCys centroid profile in a m/z spectrum, using the conditions described in table 5-7, demonstrating the expected selenium-selenium bond isotopic pattern

When analyzing standards by HPLC-ESMS, a similar phenomenon was seen for the selenium species as was seen for the arsenic, i.e. that the elution times are slightly prolonged to those obtained by HPLC-ICP-MS, again probably due to the differences in the sample introduction train. Chromatograms by HPLC-ESMS for the selenium standards and yeast sample are shown in Figures 5-28 and 5-29, respectively. The chromatogram of the yeast sample (Figure 5-25) is limited in supplying information due to the high presence of matrix ions. However, if the SIM mode is used to pick out m/z 198 and 337, the SeMet and SeCys peaks can be seen. This chromatogram is shown in Figure 5-30. The altered elution times

are comparable to elution times for the SeCys and SeMet standards using HPLC-ESMS.

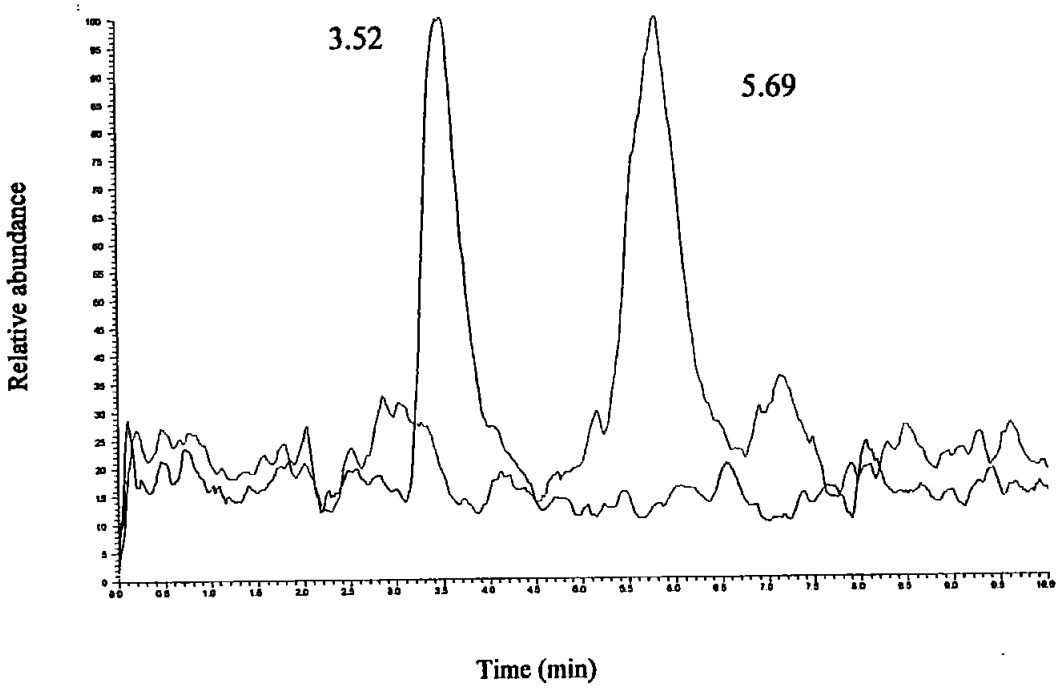


Figure 5-28 SeMet and SeCys standards in SIM mode using anion-exchange HPLC-ESMS with the conditions described in section 5.3.1.5. and Tables 5-7 and 5-8.

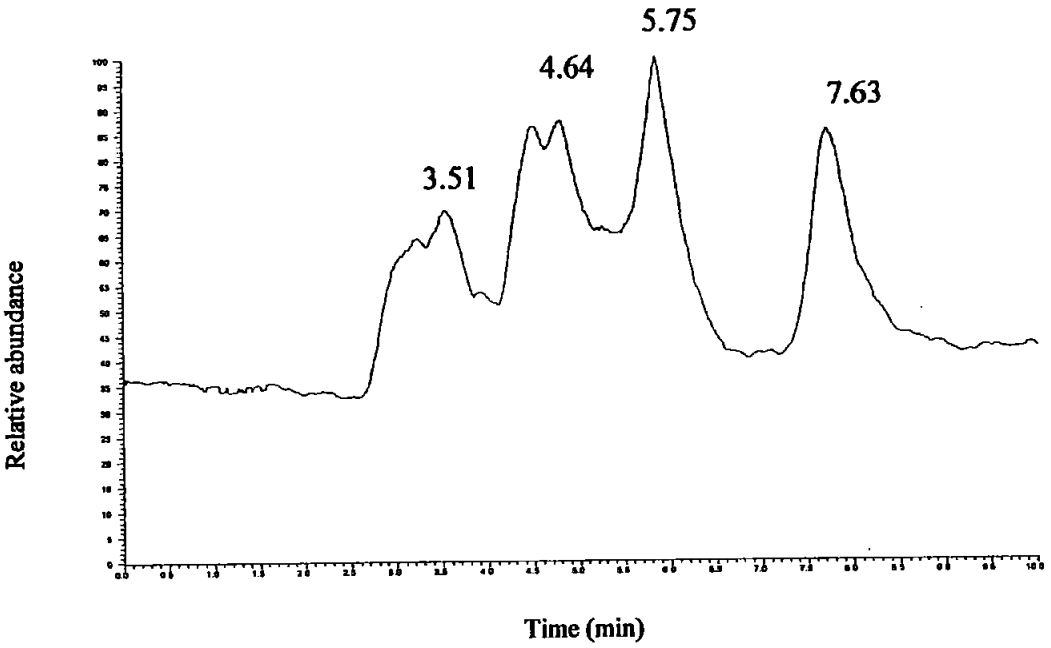


Figure 5-29 Chromatogram of yeast sample extract using anion-exchange HPLC-ESMS with the conditions described in section 5.3.1.5. and Tables 5-7 and 5-8.

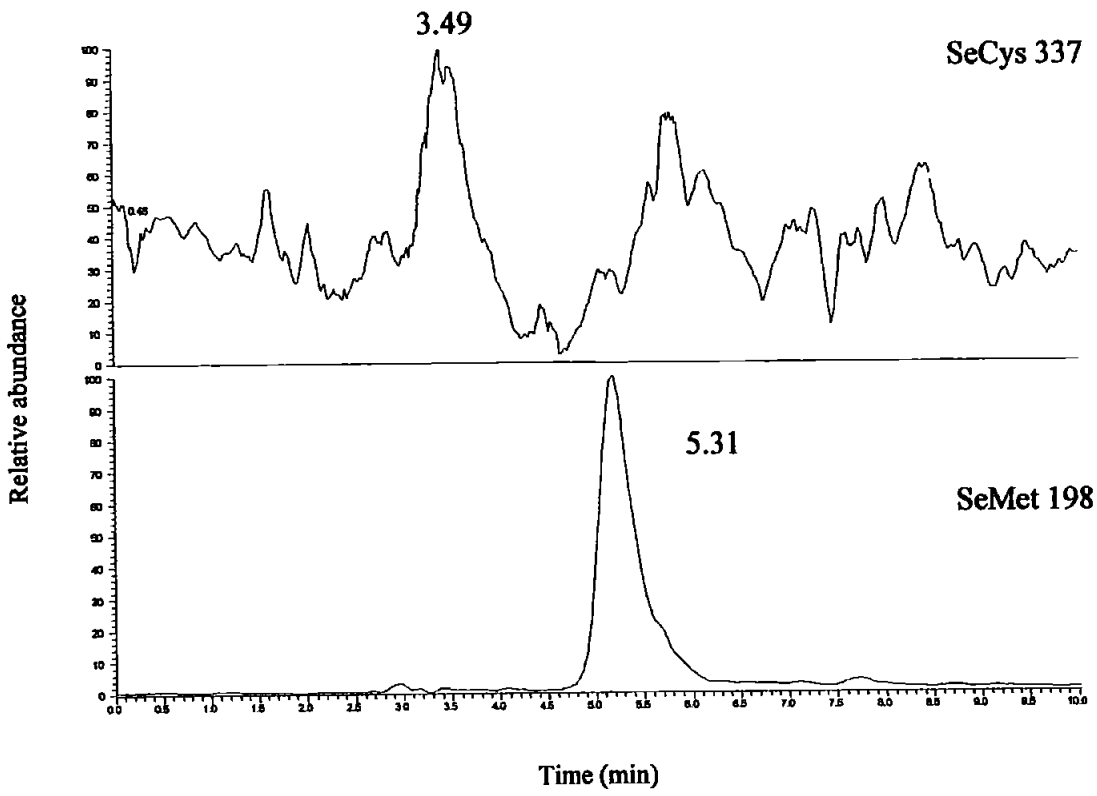
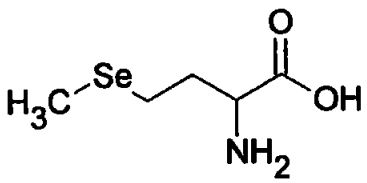
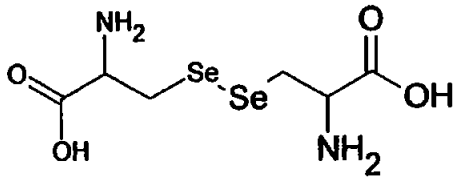


Figure 5-30 Yeast sample extract in SIM mode by HPLC-ESMS ESMS, using the conditions described in section 5.3.1.5. and Tables 5-7 and 5-8, demonstrating the response against retention times at mass 337 and 198.

The use of MSMS to observe the fragmentation patterns of the SeMet and SeCys standards was undertaken; the structures for which are shown in Figure 5-31. The fragmentation spectra by MSMS of these compounds are shown in Figures 5-32 and 5-33, respectively, and are consistent with their chemical structures.



Selenomethionine



Selenocystine

Figure 5-31 Structures of the two selenium compounds under investigation [36].

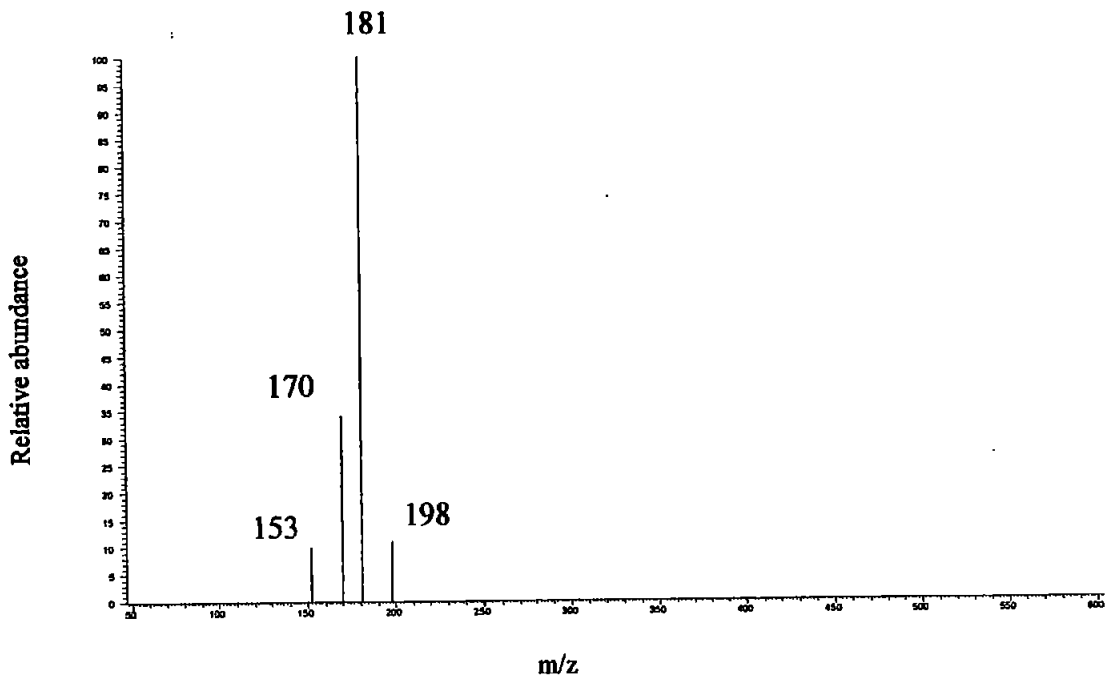


Figure 5-32 Tandem ESMS, using the conditions described in Table 5-7, of SeMet standard demonstrating the fragmentation pattern of the molecule.

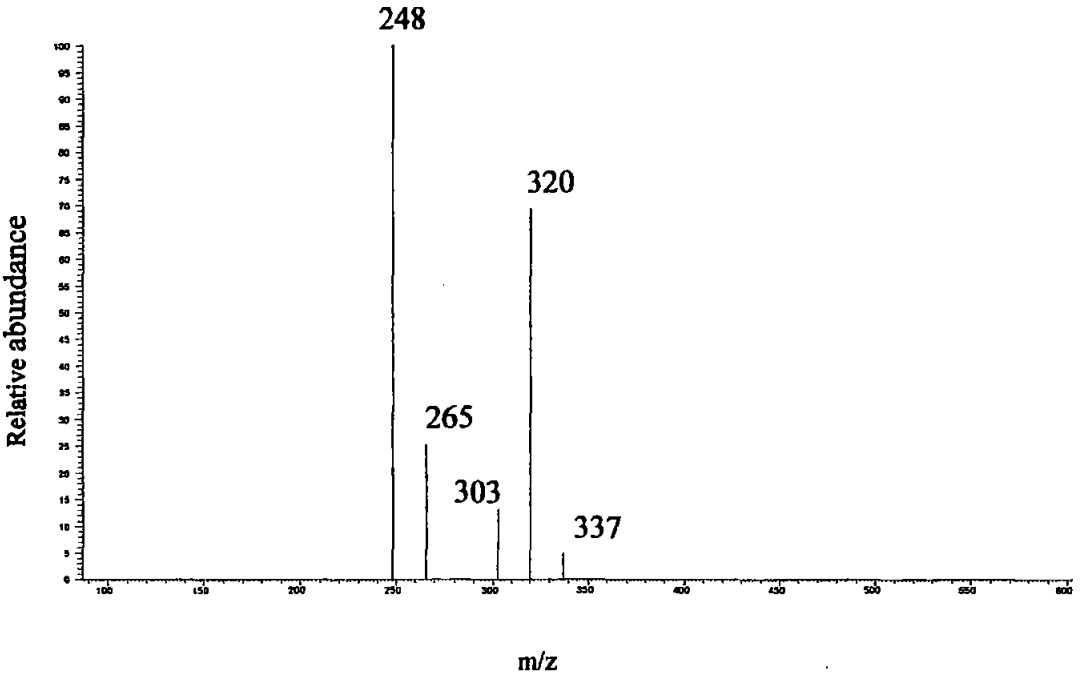


Figure 5-33 Tandem ESMS spectrum of a SeCys standard, using the conditions described in Table 5-7, demonstrating the fragmentation pattern of the molecule.

The fragmentation spectra are consistent with those found by other workers [65, 198] and represent the fragmentation of the amino acid functional groups from the molecule. The fragmentation pattern of the $M+H^+$ ion at m/z 198 in the yeast sample was found to be the same as that for the SeMet standard which confirmed that the major peak in the yeast sample was attributable to SeMet. No fragment pattern from the molecular ion at mass 337 in the yeast sample extract was obtained using MSMS. This was possibly due to the lower concentration of this analyte in the sample and that ESMS has a sensitivity of approximately x100 lower than ICP-MS.

The results using HPLC-ICP-MS and HPLC-ESMS, together with MSMS, demonstrate the presence of selenium-containing compounds in the yeast sample with the predominant species being that of SeMet.

5.5 Conclusions

The use of HPLC-ICP-MS in conjunction with HPLC-ESMS as complementary techniques to one another has proved beneficial in the identification and characterization of species that may otherwise remain unidentified, or in need of corroborative evidence, when using each technique in isolation. The chromatographic method developed requires minimal sample preparation time and is compatible with both methods of detection.

Sample analysis using HPLC-ICP-MS of arsenic species in the *Fucus sp.* CRM and the *Ascophyllum nodosum* demonstrated the presence of arsenic-containing compounds with a similar elution time to that of the AsBet standard used. Using HPLC-ESMS to provide complementary information, the co-eluting peak in the *Ascophyllum nodosum* sample was identified as that of the arsenosugar (328 mass units) 3-[5`-deoxy-5`-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropylene glycol. The co-eluting peak, having a similar elution time to that of AsBet, in the *Fucus sp.* was not identified. However, information was obtained confirming the absence of AsBet. Further studies of the *Fucus sp.* sample using HPLC-ESMS and MS-MS techniques gave a wealth of information regarding a number of arsenic-containing compounds present. Characterization of

the species demonstrated the presence of three previously identified arsenosugars [18, 19] supporting the research carried out by other workers in that arsenosugars form a major component of the arsenic found in marine algae [196].

Selenium speciation using HPLC-ICP-MS together with the information obtained using HPLC-ESMS in the selenized yeast sample, a prospective CRM, has presented information that would otherwise be unavailable using HPLC-ICP-MS alone. The major selenium-containing peak in the yeast sample matched the elution time of the SeMet standard used in HPLC-ICP-MS. However, the molecular information obtained using HPLC-ESMS provided an added certainty of the species present. This information is vital for materials that may be used as CRMs as the purpose of CRMs is to offer a route of validation for analytical procedures. Total elemental concentrations together with as much information as possible regarding species information will prove useful when analyzing materials of a similar nature. Where possible, materials being considered as CRMs for speciation analysis should also consider the use of ESMS to provide corroborative evidence of the 'actual' species present.

Chapter six

6 Conclusions and future work

6.1 Conclusions

Elemental speciation has become an essential component in the field of analytical science due to increased awareness of the varying nature of the species of the same element. Total elemental analysis is no longer adequate for the complete understanding of the biochemical impact of an element in its environment. Methodology designed to enable the separation, identification and quantification of elemental species has utilized instrumental techniques where elegant separation techniques have been hyphenated to sophisticated detectors according to requirements. The versatility obtained by the use of HPLC together with the low detection limit capabilities of ICP-MS, and their inherent compatibility, has played a significant role in the extensive number of speciation studies performed. However, for toxicological purposes, full speciation using HPLC may not always be necessary. Separation of relatively toxic from relatively non-toxic species may be sufficient for assessment and remediation.

Work was undertaken that focused on the separation of relatively toxic from relatively non-toxic species of arsenic and selenium found in samples of environmental importance, with particular emphasis placed upon the food chain. The aim was to develop a simple system that was efficient in separating, identifying and quantifying the species according to the broader screening characteristics of toxic versus less toxic whilst taking advantage of the their pK_a values in order to achieve this. A quick, cheap, low-pressure and portable method

allowing both selenium and arsenic species to be quantified, if present, in the same sample and at the same time using a suitable multi-element specific detector was developed. The arsenic species studied were inorganic As^{III} and As^{V}, which were broadly classified as relatively toxic, and AsBet and DMA, which were broadly classified as being relatively non-toxic. The selenium species investigated were inorganic Se^{IV} and Se^{VI}, which were broadly classified as relatively toxic, and SeMet, which were broadly classified as being relatively non-toxic.

A range of mini-columns of different dimensions were packed with two types of anion-exchange resin and a series of experiments based upon optimization of conditions to achieve the above aims were performed. The conditions studied were eluent competitive counter ion type and concentration, eluent flow-rate, column length, pH (to manipulate pK_a values) and sample injection volume. The preferred resolution was obtained using Hamilton PRP X100, 12-20 μm, resin. These column dimensions allowed suitable separation of species in the presence of competitive matrix ions from the sample digest without using unfavourably high back-pressures, thus allowing the use of a low-pressure pump to control the eluent flow-rate. A mobile phase of pH 10.2 created optimum conditions for major charge differences on the species resulting in adequate separation and resolution. Of the eluent competitive ion types studied; phosphate, phthalate and sulphate, for a range of concentrations, the most efficient for clearly separating both relatively toxic from relatively non-toxic arsenic and selenium species at the same time was found to be the sulphate counter ion at 10 mM concentration.

The optimum conditions of 10 mM K_2SO_4 aqueous mobile phase at pH 10.2 with an eluent flow-rate of 1.25 ml min^{-1} using a 100 x 3 mm column packed with Hamilton PRP X100, 12-20 μm, resin and a sample injection volume of 100 μl were clearly demonstrated using standards, digests of certified reference materials (DORM-2 and TORT-2) and digests of marine samples (plaice and oyster). Separation of the arsenic and selenium species into their relative toxicity classification was achieved in under 7 minutes. The conditions studied show linearity up to 500 μg l^{-1} for the arsenic and selenium species, based upon their inorganic and organic forms. Arsenic and selenium species were extracted from all samples using trypsin for the marine samples and protease for the Selenoprecise®. Extraction efficiencies were generally over 90% compared with the total arsenic and selenium values determined using a HNO_3/H_2O_2 digest. Mass balance calculations were performed for the CRMs TORT-2 and DORM-2, the oyster and plaice samples and the Selenoprecise®. The mass balance results compare more than favourably with the certified and reference values based upon organic and inorganic arsenic and selenium calibration.

The validity of this simple procedure for screening biota samples in terms of their arsenic and selenium toxicity was therefore demonstrated. Under the optimum conditions employed, limits of detection were determined to be in the range of 2-10 μg kg^{-1} for organic and inorganic arsenic and selenium species. The results for the plaice, oyster and Selenoprecise® samples show that over 90% of both the arsenic and selenium is present in the relatively non-toxic organic forms. This rapid (under 7 mins) screening technique allows a suitable estimate of the

implications for human health to be made for the samples with regard to arsenic and selenium without resorting to full speciation techniques.

To fully comprehend the biogeochemical cycling of elements in the environment it is imperative to identify and determine the species of these elements in conjunction with total element concentrations. To enable research in this vital field of science to progress, the preparation and certification of a variety of biologically and environmentally important materials for total element and species concentrations is required. The advantages of using the more complex HPLC methodology, where comprehensive separation and identification of species can be achieved using optimum chromatographic conditions, was demonstrated where samples of environmental importance were analysed as part of a feasibility study for the future production of CRMs.

The work carried out, as one of the collaborating partners, in a European CRM feasibility study was performed using ion-exchange HPLC-ICP-MS for the separation and determination of arsenic species in fish, rice, chicken and soil samples and selenium species in yeast and wheat samples. The role of the University of Plymouth was to carry out a preliminary study on a variety of fish types (plaice, monk, hake, haddock, cod, coley, pollack and whiting) to screen for one fish type that would be suitable for inclusion in the feasibility study as a candidate reference material. The plaice sample was chosen as it was decided that it met the criteria of merit based upon it having a relatively high level of arsenic, determined at 38.7 ± 1.9 mg kg^{-1} present in the form of AsBet, combined with the

best enzymatic extraction efficiency, determined at 98% using trypsin, seen for all fish types under investigation. The presence of AsBet, as the major species seen in the plaice sample extract, was confirmed using HG-AAS and anion and cation-exchange HPLC-ICP-MS. Following processing by IRMM, homogeneity and stability studies were performed on the plaice sample as part of the certification process. Results for the plaice sample indicated that sample units were homogenous, within and between unit, at an amount not less than 0.25 mg and that the material was stable within the temperature range of 4-40^{O}C and a time-scale of 0-7 months.

The inter-laboratory comparison study for determination of arsenic and selenium species in all candidate sample materials was performed with optimized conditions for each sample using ion-exchange HPLC-ICP-MS. Enzymatic extraction techniques for the speciation of the new candidate reference materials were used throughout, with the exception of the soil samples. Trypsin was used for extraction of arsenic species in the fish and chicken samples, cellulase for extraction of arsenic species in the rice sample and H_3PO_4 for the extraction of arsenic species in the soil sample. Protease was used for the extraction of selenium species in the yeast sample and trypsin for the extraction of selenium species in the wheat sample. Extraction efficiencies were determined to be in the range 92-100% demonstrating that the enzymes chosen were fit for purpose.

The results obtained demonstrated the presence of AsBet as the major species in the fish sample, inorganic arsenic as the major species in the rice sample, organic

simple methylated species of DMA and MMA were present in the chicken sample and the presence of inorganic arsenic species, in particular arsenate, dominated the soil samples. The yeast sample showed the presence of selenium as SeMet and the wheat sample contained both SeMet and SeCys, with SeMet being the dominant species. The presence of the various arsenic and selenium species in the samples appeared to conform to species found in similar samples by other workers. The use of CRMs for method validation lent credibility to the exercise. Quantification of samples were in close agreement with the certified or reference values given for the CRMs. The success of the feasibility study will be decided when all participating laboratories have submitted their results. It is hoped that the materials under investigation will be suitable to go forward, in the near future, for full certification.

The choice of selenium as a biologically important element, particularly in the human diet, was fully recognized following the clinical trial carried out by Clarke *et al.* [47] who demonstrated the efficacy of selenized yeast-based supplements in providing a degree of protection against carcinogens. Since this time, much research has been undertaken in order to identify the active form, or forms, of selenium that confer these anticarcinogenic properties. Research work, for the extraction of selenium species in two, new bio-natured nutrients, an alternative to the commercially available selenium supplements was undertaken. The sample types were a selenized yeast from a new process and a probiotic bacteria-based dried milk sample (Biogurt®). A variety of extraction methods were employed that included the use of enzymes, MeOH-based extractions and extraction with

KOH and TMAH. Fracturing of the cell walls of the sample materials was attempted by freezing in liquid nitrogen as an alternative method for releasing the cell contents. Examination of the samples, using scanning electron microscopy, following freezing showed no change in the cell structure and, therefore, did not enhance the extractability of selenium-containing compounds from the materials.

Methods that avoided species conversion with the highest extraction efficiencies were found to be the use of protease for the yeast sample (19%) and the use of 0.01 M HCl for the Biogurt® (71%). Information obtained from the speciation of these samples by anion and cation-exchange HPLC-ICP-MS was restricted due to the low extraction efficiencies obtained for the samples and that the chromatographic system was not fully optimized for resolution of the unknown compounds from the standards used. Validation of the methodology employed was obtained by the use of a well-studied yeast sample, available from Pharma Nord, where enzymatic extraction using both protease and trypsin gave extraction efficiencies of 90-100%. The selenium was found as SeMet at 1005 ± 71 mg kg^{-1}, as the element, which compared favourably with the results obtained by HNO_3/H_2O_2 digestion.

It is known that yeast-based selenium food supplements can demonstrate a significant variety in the selenium species present with some brands showing generally low recoveries of any form of selenium [131], and may have matrix problems, which make effective extraction difficult. The results from this study

seriously placed in doubt the availability of the organo-selenium species in the selenized yeast and Biogurt® samples, which they were designed to deliver.

The use of HPLC-ICP-MS as a sophisticated analytical technique is undeniable. However, a drawback associated with ICP-MS, an element-specific detector, is the loss of structural information. The introduction of ESMS, which retains molecular information, has enhanced the performance characteristics of HPLC-ICP-MS by its complementary use in the identification and characterization of previously unknown compounds.

The identification of compounds by HPLC-ICP-MS has relied upon the matching of retention times of analyte peaks to that of known standards. Ambiguities can arise due to problems associated with co-elution of compounds and where pure standards are not commercially available. The complementary use of HPLC-ESMS, where molecular information is retained, can overcome these problems and assist in the characterization of previously unidentified compounds. The use of HPLC-ESMS as complementary to that of HPLC-ICP-MS was used for part of the work towards this thesis in the identification of selenium-containing compounds in yeast and for arsenic-containing compounds in two marine algae. The yeast sample was chosen for the biological importance of the form of selenium present, in terms of bioavailability and toxicity with relation to human health. The marine algae samples were chosen as they are known to contain arsenosugars, for which there are no commercially available standards, and that arsenosugars may co-elute with known arsenic standards, i.e. AsBet and DMA, giving rise to potential errors in identification of species.

Development of an HPLC system that was compatible with ICP-MS and ESMS was undertaken so that comparisons between the two techniques could be made. Anion-exchange chromatography for the separation of arsenic and selenium compounds comprising an isocratic mobile phase of 10 mM NH_4HCO_3 + 10% MeOH, pH 10.2 for arsenic speciation and a gradient of 10-50 mM NH_4HCO_3 + 10% MeOH, pH 5.0 for selenium speciation was found to provide optimum conditions for use with both ICP-MS and ESMS detection. The volatile aqueous NH_4HCO_3 mobile phase and the presence of MeOH assisted in the formation of the gas phase ions. The presence of organic solvents is also advantageous for ICP-MS sample detection in the reduction of polyatomic interferences [70].

Analysis of the marine algae samples using HPLC-ICP-MS demonstrated the presence of arsenic-containing compounds in both samples. The first eluting peak in both samples was at a similar retention time to that of the standard, AsBet. Further work using HPLC-ESMS identified this peak, obtained from the *Ascophyllum nodosum* sample extract, as the arsenosugar 3-[5`-deoxy-5`-(dimethylarsinoyl)-β-ribofuranosyloxy]-2-hydroxypropylene glycol (mass units, 328). No arsenic-containing compound was identified for the peak in the *Fucus sp.* sample. However, the absence of AsBet was demonstrated using the SIM mode in ESMS at mass 179 ($M+H^+$ ion of AsBet) where no peaks were seen.

A full study of the *Fucus sp.* sample was undertaken. The presence of a number of previously identified arsenic-containing compounds [18, 19] when using HPLC-ESMS together with tandem MS techniques, to obtain fragment pathways of the

parent molecule which assisted in the characterization of the arsenic-containing compounds, were found. The peaks obtained using HPLC-ESMS corresponded to the peaks obtained using HPLC-ICP-MS (having taken into account the shift in elution times due to differences in the sample introduction train between the two techniques) where the presence of arsenic was confirmed due to the element-specificity of the ICP-MS detector.

A similar complementary use of HPLC-ICP-MS and HPLC-ESMS was undertaken when investigating the yeast sample, a potential certified reference material. The major species in the yeast sample was identified as SeMet. This was confirmed by the matching of elution times with that of standards when using HPLC-ICP-MS and from the fragmentation pathways seen using HPLC-ESMS in the tandem MS mode. The fact that element-specific and molecular-specific information was obtained using both techniques provided essential speciation information, of particular use where materials are to be used as CRMs.

The simplicity of the methodology was an asset when compared to other work. However, the limitations imposed upon the ESMS mode of action by matrix suppression were found to be unacceptable. The lack of seeing elemental ions in ESMS also contributed to limitations in species identification.

6.2 Future work

Future work, utilizing low-pressure mini-column liquid chromatography as an initial, rapid screening method, for assessing the toxicological impact of an element and its species may include the development of methodology for a wider range of environmentally important samples and to include a broader range of arsenic and selenium species. This may be achieved by further method development of the system already devised and by the inclusion of cation-exchange resins with optimization of chromatographic conditions. The use of resins for pre-concentration of samples to achieve lower limits of detection would be considerably useful for application in the study of waters and in the clinical application for the study of biological fluids.

In the work described in chapter two, the DMA under investigation was assumed to be less toxic than the inorganic forms of arsenic with methylation of inorganic forms being a detoxification pathway. However, research has come to light suggesting that MMA and DMA in the +III oxidation state are as cytotoxic as arsenite [199]. Further work towards the understanding of the stability of these compounds in the environment is needed together with the development in methodology capable of separating the +III from the +V oxidation state of the individual species.

The ability of analytical chemists to continue developments in methodology for the production of CRMs that are certified for species as well as 'total' elemental concentrations is particularly relevant when considering samples of biological

and environmental importance. The ability to separate and identify arsenosugars present in marine algae samples is vital for progress to be made in understanding the biochemical cycling of these compounds, the nature of their toxicities and relationship to human health. Due to the diversity of functional groups possessed by different arsenosugars and that some algae contain large amounts of lipid-bound arsenic, extraction procedures for obtaining the species may vary in their efficiencies. This makes the determination of species present in a sample difficult to achieve with low uncertainties. Method validation cannot be applied successfully due to the lack of CRMs certified for arsenosugars and the lack of commercially available standards. However, operationally defined methods of determination in candidate reference material may be a starting point for the inclusion of CRMs for arsenosugars to be made available commercially. Method development using HPLC-ICP-MS with the complementary technique of ESMS on a wide range of environmentally important samples will move forwards the possibility of full characterization and quantification of species present in a sample for use as certified reference materials. Work in the area of matrix removal, to minimize suppression of the analyte signal by concomitant ions, whilst keeping sample preparation to a minimum to make routine analysis possible, is required to facilitate and enhance the wealth of information that can be obtained.

Work is the area of species extraction from the sample matrix needs to focus upon methods that are efficient and do not cause species conversion to take place. In areas where selenoprotein information is required, the use of extraction

procedures that do not cleave aminoacid groups is fundamental to success. This requires the use of either water/methanol-based extractions or enzymatic extractions with non-proteolytic action. The possibility of devising sequential extraction procedures will enable the researcher to obtain information regarding the partition of selenium compounds within a sample matrix. Optimization of these extraction procedures is necessary for their use to become part of routine laboratory practice. The development of effective extraction procedures for the analysis of selenized yeast cannot be over-emphasized due to the necessity in the identification of the specific forms of selenium that may have a direct influence on anticarcinogenic activity within the human body.

Work in the separation, identification and determination of selenium compounds using chiral separations, isotope dilution techniques and ESMS instrumentation would further assist in understanding the absorption, metabolic fate and anticarcinogenic activity of selenium in foodstuffs or pharmaceutical preparations following consumption by the human population. In addition, the biochemistry of living organisms exhibits a strong enantioselectivity. Mammalian proteins are built exclusively of L-amino acids and research has suggested that the D-selenocystine is one third as toxic as L-selenocystine [200]. Consequently, research on the biological activity of selenomethionine and the analytical control of selenium-containing supplements require access to methodologies that are able to perform optical resolution and determination of D,L-selenomethionine enantiomers. This has been achieved by HPLC on cyclodextrin columns which requires a derivatization step [201] and more recently by the use of chiral crown

ether columns that do not require derivatization of the enantiomeric forms of the analyte [202].

It is crucial that analytical capabilities in the field of speciation continue to progress in order to appreciate, in greater detail, the effects of trace elements in the health of the general population. Coupled techniques, such as HPLC-ICP-MS and HPLC-ESMS, offer an attractive route towards this aim. Improved method development together with a wider choice of standards and CRMs for quality control and assurance will play an essential role in this.

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Courses, conferences and seminars attended

Courses.

Analytical Atomic Spectrometry Short Course – 4-8 Oct 99 University of Plymouth

AMS 592: Research Skills Oct-Dec 99 University of Plymouth

Conferences.

Young Researchers' Meeting R&D, UMIST, Manchester, UK, 16-17 April 2000.
Poster presentation: Advancement in Methodology for Speciation in Environmental Samples by ICP-MS

BNASS 10th Biennial Conference, Sheffield 17-20 July 2000.
Poster presentation: Advancement in Methodology for Speciation of Arsenic and Selenium using HPLC-ICP-MS.

4th Euroconference on Environmental Analytical Chemistry, Budapest, Hungary, 14-19 September 2000.
Poster presentation: Separation and Detection of Trace Metal Species in Environmental Samples by HPLC-ICP-MS.

Analytical Research Forum incorporating Research and Development Topics. University of East Anglia, Norwich, UK, 16-18 July, 2001.
Poster presentation: Advancement in Methodology for Simultaneous Separation of Arsenic and Selenium Species by LC-ICP-MS.

5th Euroconference on Environmental Analytical Chemistry. The Impact of (Bio-) Sensors and Bioanalytical Techniques in Environmental Analytical Chemistry. Blarney, Ireland, 8-12 September, 2001.
Poster presentation: Advancement in Methodology for Simultaneous Separation of Arsenic and Selenium Species by LC-ICP-MS.

BNASS 11th Biennial Conference, Loughborough University, 8-10 July 2002.
Oral presentation – Advancement in Methodology for the determination of Arsenic and Selenium species by HPLC-ICP-MS and ESMS.

Arsenic Speciation Workshop, Ghent, Belgium, 11-12 September, 2002.
Poster presentation: Advancement in Methodology for the Speciation of Arsenic and Selenium using HPLC-ICP-MS and ESMS.

2nd International Franco-Spanish Workshop on Bio-inorganic Analytical Chemistry, Pau, France, 26-28 September, 2002.
Oral presentation: Advancement in Methodology for the determination of Arsenic and Selenium species by HPLC-ICP-MS and ESMS

In-house seminar presentations.

June, 2000: Arsenic Speciation

May, 2001: Preparation and analysis of materials for certification

April, 2002: Advancement in Methodology for the determination of Arsenic and Selenium species by HPLC-ICP-MS and ESMS

Published work

1. Ebdon, L., Fitzpatrick, S. and Foulkes, M.E., *Chem. Anal. (Warsaw)*, 2002, **47**, 179-188.

2. Fitzpatrick, S., Ebdon, L. and Foulkes, M.E., *Intern. J. Environ. Anal. Chem.*, 2002, **82**, 835-841.