

Trace Analysis of Environmentally Important Species

by

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Declaration

I hereby certify that this material, which I now submit for the assessment on the programme of study leading to the award of Ph. D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

Signed: Catherine Dunne

Date: 14/10/94

Catherine Dunne

To my parents

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TRACE ANALYSIS OF ENVIRONMENTALLY IMPORTANT SPECIES

Catherine Dunne

Abstract

A flow injection hydride generation atomic absorption (AAS) method has been developed for the analysis of arsenic species. The technique has been optimised for the analysis of arsenite, arsenate, monomethylarsonate(MMA) and dimethylarsinate(DMA) with detection limits of 9, 35, 24 and 24 ppb respectively being achieved. The method described offers the advantage of the reproducible use of small volumes and the ability to achieve rapid sample throughput.

The optimised hydride generation AAS method was then investigated as a detector for HPLC. The resulting hyphenated technique allows the separation and detection of the individual arsenic species at ppm levels. As lower detection limits are required for the analysis of arsenic species in real samples an on-line preconcentration technique has been developed, resulting in improved detection limits and the removal of matrix interferences. Finally a matrix solid phase dispersion technique was developed for the extraction of arsenic species from fish which did not result in the loss of information on speciation.

A sensitive and reliable method was developed for the determination of aflatoxins B₁, B₂, G₁, and G₂, ochratoxin A and zearalenone in animal feed ingredients. A multi-toxin extraction and clean-up procedure was used, with dichloromethane:1 M hydrochloric acid (10:1) being used for the extraction and gel permeation chromatography being used for the clean-up. The liquid chromatographic method developed for the separation of the six mycotoxins involved gradient elution with reverse-phase C₁₈ column and fluorescence detection. Recoveries, repeatability and reproducibility have been determined on maize, palm and wheat. The detection limits varied depending on the type of feed.

Aims and objectives

The aim of this work was to develop and improve analytical methods for the determination of arsenic and arsenic species in the environment. A flow injection hydride generation atomic absorption method was developed for the determination of individual arsenic species or total arsenic. This method would improve detection limits of existing methods and provide an easy and rapid assay technique for arsenic analysis. A chromatographic separation technique was also investigated in order to provide information on the amount of each species present in a sample as this is vital where toxicity analysis are of interest. In order to provide speciation information and maintain the limits of detection obtained by the flow injection technique a preconcentration technique involving a column switching technique was also incorporated. This technique could also be used on-line. Finally a matrix solid phase dispersion technique was developed for the extraction of arsenic species from fish. To date there are many extraction techniques which try to extract the arsenic species from samples. Many of these techniques destroy the speciation information, or they involve lengthy and time consuming procedures. The matrix solid phase technique investigated here provides a partial solution to the problem, whereby three arsenic species were successfully extracted from fish samples without loss of speciation information.

Another project carried out was the development of a method for the determination of mycotoxins in feedstuffs. To date mycotoxins are usually determined singly with multiple clean-up steps. The method developed here allows for the determination of six mycotoxins with one clean-up procedure using gel permeation chromatography and a liquid chromatography procedure for the separation with fluorescence detection.

CHAPTER 1

Arsenic and analytical methods for the determination of arsenic.

1.1 INTRODUCTION

1.1.1 Properties of arsenic

Arsenic belongs to group VA of the periodic table. It has an atomic number of 33, an atomic weight of 74.91 and a melting point of 814 °C. Principle valences of arsenic are +3, +5 and -3. Metallic arsenic is stable in dry air, but when exposed to humid air the surface oxidises, giving a superficial golden bronze tarnish that turns black on further exposure. Elemental arsenic normally exists in the α -crystalline metallic form which is steel-grey in appearance and brittle in nature, and in the β -form, a dark-grey amorphous solid. The amorphous form is more stable to atmospheric oxidation. Upon heating in air both forms sublime and the vapour oxidises to arsenic trioxide, As_2O_3 . A persistent garlic-like odour is noted during oxidation. Arsenic vapour does not combine directly with hydrogen to form hydrides. Arsenic hydride (AsH_3 , arsine), a highly poisonous gas, forms if an intermetallic compound such as AlAs is hydrolysed or treated with hydrochloric acid. Heating to 250 °C decomposes arsine into its elements. Arsenic may be detected qualitatively as a yellow sulphide (As_2S_3) by precipitation from a strongly acid (HCl) solution. Trace quantities of arsenic may be detected by converting it to arsine. The arsine is decomposed by heating the gas in a small tube, an arsenic mirror is formed (Marsh test), or the arsine may be allowed to react with test paper impregnated with mercuric chloride (Gutzeit test)[1].

1.1.2 Occurrence of arsenic

The principle arsenic mineral is arsenopyrite ($FeAsS$, mispikel) other metal arsenide ores include iollingite ($FeAs_2$), nicolite ($NiAs$), cobalt glance ($CoAsS$),

gersdorffite (NiAsS), and smalite (CoAs_2). Naturally occurring arsenates and thioarsenates are common and most sulphide ores contain arsenic[2].

Arsenic is widely distributed about the earth and has a terrestrial abundance of $5 \times 10^{-4} \%$ of the earth's crust[2]. The quantity of arsenic associated with lead and copper ores may range from trace to 2 - 3 %, whereas the gold ores found in Sweden contain 7 - 11 % arsenic[1].

1.1.3 History

Ancient peoples, Greek, Romans, Arabic, Peruvian to name a few used arsenic and its compounds therapeutically and as poisons. Arsenic trioxide obtained during smelting copper is said to have been first prepared around 2000 BC. It was a favourite agent of medieval and renaissance poisoners. Arsenic in its element form is said to have been obtained first by Albertus Magus in 1250 AD. Writings of Paracelsus contain directions for its preparation[3].

Over 40 years ago Challenger[4] identified "Gosio gas" as trimethylarsine (Me_3As). Gosio gas is a volatile, toxic arsenic species produced by moulds growing on wallpaper with arsenic-containing pigments such as Sheelis green (copper arsenite) and Schweinfurt green (copper arsenite plus copper acetate). Gosio gas was responsible for a number of deaths, and the air in buildings in which it was being produced had a characteristic garlic-like odour. Lead arsenic dust from a painted ceiling was the source of the arsenic that caused health problems for Clare Bootle Luce when she was living in Rome as US Ambassador in 1954[5].

1.2 ARSENIC TOLERANCE/TOXICITY

1.2.1 Toxicity

The toxicity of arsenic ranges from very low to extremely high depending on the chemical state. In general inorganic forms are more toxic than organic forms, the trivalent arsenic being more toxic than the pentavalent form[6]. The toxicity of organic arsenics varies, monomethylarsonic acid (MMA) being more toxic than dimethylarsinic acid (DMA). Arsenobetaine is a major arsenic compound found in many sea foods and is non-toxic[7]. Metallic arsenic and arsenious sulphide have low toxicity whereas arsine (arsenic hydride), a gas, is extremely toxic[1]. Trivalent arsenicals react with sulphydryl groups in cells so as to inhibit sulphydryl containing enzyme systems essential to cellular metabolism. Arsine combines with haemoglobin and is oxidised to a hemolytic compound that does not appear to act by sulphydryl inhibition[3].

In practice the most dangerous arsenical preparations are dips, herbicides and defoliants in which the arsenicals are in a highly soluble trivalent form, usually trioxide or arsenite[8].

1.2.2 Symptoms

Acute arsenic poisoning symptoms are similar to those of "food poisoning" i.e. intense pain, projectile vomiting and diarrhoea. Symptoms of shock appear as fluid loss progresses. Hypoxic convulsions and coma may occur. Acute arsenic poisoning is fatal usually within 24 hours. Dimercaprol British anti-Lewisite (BAL) is an effective antidote, restoration of fluid and electrolyte balance is contributory[3].

Chronic arsenic poisoning mimics many diseases, weakness languor, anorexia, nausea, vomiting, diarrhoea and melanosis of the lower eyelids and clavicular areas[3]. Ulceration of the nasal septum is caused by airborne arsenic trioxide if proper precautions are not observed.

1.2.3 Permissible limits

The European Economic Communities has laid down quality standards in view of the importance for public health for human consumption of water. 50 µg L⁻¹ of arsenic is the maximum admissible concentration allowed for water supplied for consumption used in food production or in the manufacture, processing, preservation or marketing of products or substances intended for human consumption[9].

1.2.4 Handling precautions

For handling of arsenic trioxide special clothing and approved respirators should be worn and frequently changed to prevent dermatitis, particularly in the folds of the skin or moist areas. Careful personal hygiene must be observed, application of protective creams may prevent irritations. For processes treating arsenic fumes and dust, exhaust ventilation including filters should be provided[1].

1.2.5 Arsenic as a carcinogen

Arsenic is a known carcinogen. Blejer and Wagner[10] reviewed various epidemiological studies concerning cancer incidences in arsenic-exposed workers and found that workers inhaling inorganic arsenic compounds during copper

smelting, pesticide manufacture or in gold mine operations are at risk. Vintners and sheep-dip workers are also at risk. There are well documented investigations concerning the incidence of respiratory cancer among workers in copper refineries[11 - 13].

1.2.6 Natural arsenic content

Arsenic has no known vital function, it is ubiquitous in the biosphere. Most foods contain minute amounts averaging 0.02 ppm including meats, fish and poultry[14]. Typical values for arsenic in sea-water are 2 ppb, in fresh water a much wider variation can be encountered, commonly in the range 0.4 - 80 ppb[15]. Arsenic concentrations found in marine animals (dry weight basis) range from 0.31 ppm in salmon to a high of 340 ppm in the midgut gland of the carnivorous gastropod *Charonia sauliae*[5]. Dairy products contain much lower levels averaging 0.0033 ppm[14]. Normal blood arsenic levels vary between 3.5 and 7.5 µg per 100g[16]. Normal hair arsenic contents are usually found to be less than 1 µg per 100 g[3]. A wide range of arsenic levels in the heart, lungs, kidney and thigh muscle i.e. between 0.097 - 102 µg per 100 g tissue were reported by different analysts. However the region from which tissues were obtained and the method used for analysis may be a factor in the variability of the results[3].

1.3 USES OF ARSENIC COMPOUNDS

1.3.1 Medical

Arsenic compounds have been used as therapeutic agents since the fifth century BC, when the Greek physician Hippocrates recommended the use of arsenic

sulphide for the treatment of ulcerative abscesses. Similar arsenic preparations were prescribed for skin disorders, tuberculosis, asthma and leprosy around that time. During the Middle Ages, inorganic arsenic compounds were widely known and used extensively by physicians and professional poisoners[1]. More recently (1976) Fowler introduced a solution of 1 % arsenic trioxide which was employed in treating leukemia and psoriasis. In 1907 arsphenamine was discovered by Erilich and used for the treatment of syphilis. However since the introduction of penicillin the use of organic arsenicals in treating syphilis has declined[3].

Arsenicals have some applications in veterinary medicine. Various compounds were used extensively in treating chronic coughs, anaemia, blood diseases, petechial fever in horses etc.[3]. On the basis of present evidence the therapeutic use of inorganic arsenicals must be condemned.

1.3.2 Industrial

Arsenic and its compounds have had widespread industrial applications[17]. The major use of arsenic is in the agricultural field. Cacadylic acid, monosodium methyl arsonate (MSMA) and disodium methyl arsonate (DSMA) are used as herbicides. MSMA is used extensively in the cotton fields for the control of weeds. Sodium arsenite solutions have been used in cattle and sheep dips, for debarking trees and aquatic weed control. Arsenic acid is used in the formulation of wood preservatives. Arsenilic acid is used as a feed additive for poultry. Refined arsenic trioxide is used as a decolorizer and fining agent in the production of bottled glass. However arsenic for the glass industry has largely been replaced and is no longer a major market[17].

Arsenic is used in alloys mainly in combination with lead. Trace quantities of arsenic added to lead-antimony grid alloys used in lead acid batteries minimise

self-discharging characteristics of the batteries. Arsenic improves sphericity of lead ammunition and arsenic also has semiconductor applications. A major use is in the production of light emitting diodes where gallium arsenide is used[1]. Arsenic has come under a lot of scrutiny from an environmental and safety standpoint, so too have the compounds which compete with arsenic and overall to date it has been found that arsenic is more acceptable than the competing chemicals[17].

1.4 ARSENIC IN THE ENVIRONMENT

1.4.1 Arsenic compounds in the marine environment

The concentration of arsenic in marine and fresh water animals is considerably above the background concentration in the surrounding water. Typical values for sea-water are 2 ppb, in fresh water a much wider variation is found commonly in the range 0.4 - 80 ppb[15]. Cullen and Reimer[5] have reported arsenic concentrations in marine animals that ranged from 0.31 ppm in salmon to 340 ppm in the midgut gland of the carnivorous gastropod *Charonia sauliae*. The world record for arsenic accumulation is probably held by the polychaete worm *Tharyx marioni*[18]. The whole body concentration usually exceeds 200 ppm dry weight. Much of the arsenic concentration is in the pulps which comprise 4 % of the body weight. The concentration of arsenic in these organs is in the range 600 - 13000 ppm and the bulk of it appears to be in organic form. In 1977 arsenobetaine ($((\text{CH}_3)_3\text{As}^+\text{CH}_2\text{COO}^-)$) was isolated from rock lobster *Panulirus cygnus*[19]. Since its discovery it has been shown to be present and to be the most abundant arsenical in most marine animals. Arsenocholine ($((\text{CH}_3)_3\text{As}^+\text{CH}_2\text{CH}_2\text{OH})$) is another arsenical commonly found in marine life[20]. Trimethylarsine oxide ($((\text{CH}_3)_3\text{AsO})$) has been found as a minor component in a number of fish species. It is possible that this trimethylarsenic oxide is a

breakdown product of other arsenicals already present in the fish as its content is higher in frozen samples[5]. Inorganic arsenic appears to be converted to trimethylarsine oxide in the gut of fish[21].

The presence of organoarsenicals in marine organisms is commonly assumed to be due to the accumulation of compounds that have been synthesized from arsenate at low trophic levels[5]. Species at higher levels do not seem to be able to use arsenate for the production of such compounds as arsenobetaine. They accumulate organoarsenicals via the food chain and do not synthesize these compounds from inorganic arsenic.

1.4.2 Arsenic compounds in the terrestrial environment

As already mentioned arsenicals have found widespread use in the agricultural field eg. sodium arsenite for weed control and lead arsenate as a pesticide on fruit crops[1]. This has prompted a number of studies on the interaction of arsenic compounds on plants, man and other terrestrial animals. Another reason for the interest in terrestrial plants is that the arsenic content of plants could be used as a biogeochemical indicator. The typical arsenic content found in uncontaminated terrestrial plants is 0.2 ppm approximately, levels generally less than those encountered in the marine environment. Plants near mine wastes have been studied to assess arsenic accumulation. Samples of the little bluestem, *Andropogon scoparius*, taken from an arsenic mine exhibit a wide evolved tolerance of arsenic, most of it in the roots, which non-mine members of the species lack and have been found growing in soils containing 43,000 ppm arsenic (dry weight basis). Another indicator plant is the ox-eye daisy, *Crysanthemum leucanthemum*, which accumulates more arsenic in the leaves than the roots. Some species that contain high arsenic concentrations also contain high gold concentrations[5].

A number of studies have been made on the presence of inorganic arsenic and methylarsenic species in biological fluids[22, 23]. The total urinary excretion of arsenic per day ranges from 10 - 50 μg of arsenic from a normal diet[5]. The same determination on subjects who had eaten seafood prior to the analysis gave concentrations of inorganic arsenic and methyl arsenics unchanged while total arsenic concentration increased, thus indicating that "fish arsenic" is not metabolized by humans to any great extent[24].

1.4.3 Arsenic compounds in the atmosphere

Arsenic enters the atmosphere from natural and anthropogenic sources. Natural sources of arsenic include volcanic activity, wind erosion, sea spray, forest fires and low temperature volatilization. Anthropogenic sources of arsenic are mainly from smelting operations and fossil fuel combustion[25], the emissions consisting of arsenic trioxide [26]. Sea spray mainly contributes arsenate as this is the dominant species in sea water[27].

1.4.4 Arsenic compounds in aquatic systems

The concentration of arsenic in fresh water shows considerable variation with geological composition of the drainage area and the extent of anthropogenic input. Oceanic constituents tend to be less variable than their fresh water counterparts as surface water arsenic concentrations are subject to some seasonal variation due to biological uptake. Arsenic is transported both as dissolved species as well as that bound to suspended material and at the interface between fresh- and salt-water environments, this matter is deposited in estuarine and coastal sediments[5].

Marine waters principally contain arsenate followed by arsenite usually in much smaller quantities. The presence of arsenite is most evident in estuaries receiving arsenite rich river input introduced anthropogenically. Chemical and biological oxidation of arsenite, together with dilution effects reduce the impact of anthropogenic emissions far from shore. Microorganisms have the ability to reduce arsenate to arsenite and it has been found that there is a high incidence of arsenite in media of phytoplankton cultures[28]. In most river and marine waters only arsenate is found but this may be due to analytical limitations. MMA and DMA have been found but their presence is thought to be mainly as a consequence of phytoplankton activity.

1.4.5 Arsenic compounds in soils, sediments and fossil fuels

Concentrations of arsenic in soils are reflective of the parent rock material from which they were formed. African soils associated with gold deposits contain between 300 and 5000 ppm arsenic. The association of arsenic with valuable elements has led to its use as a geological marker. The use of inorganic and organic arsenicals as pesticides and herbicides has diminished in recent years. However, they are still used today and the fate of arsenicals has been studied more extensively because of their agricultural application. The largest accumulation of arsenic is in soils/sediments close to the source i.e. the parent rock, rather than from pesticide/herbicide applications or emissions from fossil fuel utilization. Arsenic is also present in coals and oil and is therefore introduced into the atmosphere as a consequence of fossil fuel utilisation. Arsenic content of sediments (ppm) are much higher than those of over lapping waters (ppb)[29]. The consistent appearance of arsenic in the manganese-iron oxide fractions of sediment extracts has led to the suggestions that coprecipitation of these oxides may be involved in the control of dissolved arsenic concentration

in the overlying water. Seydel[30] suggests that arsenic does not stay dissolved in water under oxidising conditions as long as there was iron present with which it could coprecipitate.

1.5 ANALYTICAL METHODS FOR THE DETERMINATION OF ARSENIC IN ENVIRONMENTAL SAMPLES

1.5.1 Early Work

Arsenic was one of the first elements for which quantitative analytical methods were developed, probably because of its use as a poison. In 1836 the Marsh-Berzelius test[3] was introduced. Arsine generated from an acidic solution is trapped on a mirrored surface. A black deposit is indicative of arsenic. The test is not specific for arsenic as other metals which form hydrides leave deposits on mirrored surfaces also.

Later the Reinsch test[3] was developed. A polished copper wire is placed in an acidified sample. The sample was heated for a half to one hour, the copper wire removed and if the foil remained bright arsenic was not present in more than trace quantities. A black or brown deposit indicated the presence of metal such as arsenic, mercury, antimony, silver, bismuth or lead.

The Gutzeit method[3] involves the production of arsine. The arsine produced reduces mercuric bromide impregnated onto a filter paper strip. A yellow to brownish stain produced is proportional to the quantity of arsenic present. Hydrogen sulphide also reduces mercuric bromide but this may be removed or trapped using lead acetate saturated cotton wool[3].

Titration methods for arsenic determination also exist. In a method using bromate, arsenic is distilled as arsenious chloride from an acid digest. The distillate is titrated with standard bromate solution using methyl orange as an indicator[31]. A titration of arsenic with standard hypochlorate solution has been described by Goldstone and Jacobs[32] whereas an iodimetric method was described by Cassel and Wichmann[33] where arsine evolved from an acidic solution is trapped in a mercuric chloride solution. The mercuric arsenide formed

is oxidised by excess mercuric chloride with the formation of mercurous chloride and arsenous acid. The arsenous acid is then oxidised with a weak iodine solution.

Early work focussed on the determination of total inorganic arsenic. But much attention has been switched to the development of analytical techniques which are capable of distinguishing between arsenic species and detecting them at μg levels. Analytical techniques for the separation of the arsenic species include liquid chromatography[34 - 39], gas chromatography[40 - 43], selective hydride generation[44 - 50] and voltammetry[51 - 53]. Methods for detecting arsenic include spectrophotometry[54, 55], flame and flameless atomic absorption spectrometry[56 - 60], atomic emission spectrometry[44, 61 - 63], voltammetry[51 - 53], neutron activation analysis[64], x-ray fluorescence[65, 66], atomic fluorescence spectrometry[67, 68] and mass spectrometry[47, 69, 70]. These detection techniques are more often coupled to the separation techniques.

1.5.2 Spectrophotometric methods

In general spectrophotometric methods are applicable to the speciation of inorganic arsenic but have been shown to be less sensitive than other methods. A silver diethyldithiocarbamate procedure was used by Howard et al.[54]. This method is susceptible to interferences from other trace metals and methylated arsenical species. Stauffer[55] determined arsenic spectrophotometrically using the molybdenum blue method in which arsenate forms a blue complex with molybdate which can be detected spectrophotometrically. Phosphate is a major interference in this method forming phosphomolybdate. The full development of the molybdate method requires 2 - 4 hours.

1.5.3 Neutron activation analysis

Neutron activation analysis is very sensitive and useful for the analysis of small samples[3]. Smith[64] analysed hair samples for the presence of arsenic. A sample after irradiation was digested with nitric and sulphuric acid and the arsenic was separated using a modified Gutzeit technique which produced arsine. The arsine was removed by trapping it in 1.6 % mercuric chloride and the activity of the liquid sample was then estimated using a geiger counter. This is not a popular method due to the unavailability of equipment.

1.5.4 Electrochemical methods

Electrochemical methods can be used for the direct speciation of inorganic arsenic but the indirect determination of the methylated arsenicals requires acid digestions[45]. Arnold and Johnson[71] reviewed the behaviour of arsenic in various media using polarography. In acidic solution the stepwise reduction of arsenate through arsenite to the elemental form and hence to arsine was possible. An initial oxidative attack is required to produce an aqueous solution of arsenate, usually sulphuric acid. Reducing agents employed include sulphur dioxide and potassium iodide. Electrochemical methods can distinguish between arsenite and arsenate since arsenate is not electroactive and has to be reduced by chemical means prior to the determination[51]. Voltammetric methods are of special interest whereby arsenite is first deposited onto a working electrode and then stripped. During the deposition step arsenic is effectively preconcentrated which makes this a very sensitive method. The voltammetric method is rapid and does not require expensive instrumentation. Bodwig et al.[51] determined arsenite and arsenate in natural waters by differential pulse anodic stripping voltammetry. Arsenic deposited onto a rotary gold electrode and followed by anodic stripping voltammetry allows fast and sensitive determination of arsenic. Detection limits

of $0.2 \mu\text{g L}^{-1}$ were achieved. Hua et al.[52] determined total arsenic in sea water by a flow constant current stripping analysis using gold fibre electrodes. The sample is acidified and arsenate reduced to arsenite with iodide before analysis. Detection limits of $0.15 \mu\text{g L}^{-1}$ were obtained. Jan and Smith[53] studied the behaviour of inorganic and organic arsenic using differential pulse voltammetry at a hanging mercury drop electrode and concluded that at certain conditions i.e. suitable pH and peak potential, it was possible to distinguish between individual organic arsenic compounds (i.e. phenylarsine oxide, triphenylarsine oxide and arsenazo) in mixtures with each other and in the presence of inorganic arsenic compounds.

1.5.5 Atomic absorption spectrometric methods

Detection limits for the analysis of arsenic using flame AAS are in the low ppm range which is too high for many analysis requirements. Consequently concentration techniques must be employed to make AAS practically applicable. Flameless AAS, mainly graphite furnace AAS, extends the detection of arsenic. Tsai and Bae[56] determined trace concentrations of arsenic in nickel based alloys by graphite furnace AAS. A detection limit of 0.3 ng g^{-1} was obtained. A pyrolytic graphite platform was used which helped reduce matrix interferences with stabilized temperatures. In flame AAS the air-acetylene flame normally used can absorb at 193.7 nm the most sensitive resonance line used for arsenic analysis[3]. A major improvement and reduction in sensitivity was brought about with the introduction of hydride generation used in conjunction with AAS. It was 1969 when Holak[57] used this procedure to improve arsenic determination in AAS. He generated arsine by reaction of zinc with hydrochloric acid and collected it in a liquid nitrogen trap which was then warmed and the arsine passed into an air-acetylene flame with a stream of nitrogen. Since then hydride

generation AAS has found widespread application. However the technique is not totally free from interferences but it does improve the sensitivity (ppb) and it is a simple fast procedure which may be used on-line.

Arsine generation is time and pH dependant and these parameters are not equivalent for inorganic and methylated forms of arsenic. Most early work was concerned with digestion procedures to convert all arsenic species to one species i.e. arsenite or arsenate before hydride generation AAS[58 - 60].

In recent years the main concern is with the determination of individual species rather than total arsenic mainly because of the differences in toxicity of the different species. This has brought about the use of coupling techniques. AAS is very popular as a detection step and is commonly coupled to high performance liquid chromatography (HPLC)[37 - 39]. Indirect couplings have been used with graphite furnace[72, 73] whereas direct couplings may be used with hydride generation AAS.

1.5.6 Atomic emission spectrometric methods

Both direct current plasma (DCP) and inductively coupled plasma (ICP) atomic emission spectrometry (AES) are used as detectors for the determination of arsenic. They are mainly coupled to liquid chromatography with ICP being the more popular emission technique used. The main advantage of plasma atomic emission is that the type of matrix problems experienced with flame sources are not common with plasma excitation sources i.e. a wider range of eluent composition can be employed without experiencing detector limitations, it is also a very sensitive technique. Urasa and Ferede[61] used DCP AES with ion chromatography for the determination of arsenite and arsenate in the presence of other anions. The ion chromatography eluent was aspirated directly into the DCP

allowing the determination of arsenite and arsenate simultaneously by measuring the AES. The chromatography separated out most interfering anions and others were not detected by DCP AES. Noilte[44] used continuous flow hydride generation ICP AES for multielement analysis including arsenic. A flow injection technique used with hydride generation ICP AES was used by Tioh et al.[62] to determine arsenic in glycerine. Rauret et al.[63] used an ion exchange HPLC with hydride generation ICP AES to determine arsenic species in aquatic media. Arsenite, arsenate MMA and DMA were determined. A gas/liquid separator was used to minimise the volume of solution reaching the plasma torch and to improve the separation of volatile hydrides. The use of the gas/liquid separator concentrates the hydride before detection and consequently detection limits are improved.

ICP and DCP as detectors are limited by the availability and cost of instrumentation.

1.5.7 Atomic fluorescence spectrometric methods

Although not a very popular technique it is more sensitive than AAS. Ebdon et al.[67] determined arsenic by continuous hydride generation AAS and atomic fluorescence spectrometry (AFS). AFS offers better sensitivity than AAS however there is some loss in precision because of problems encountered with a practical light source. Ebdon and Wilkinson[68] used a similar set-up to determine arsenic in coal. A perchloric acid digestion of the coal was used. Detection limits of 58 ng g^{-1} using AAS and 25 ng g^{-1} using AFS were obtained.

1.5.8 X-ray fluorescence spectrometric methods

X-ray fluorescence spectrometry (XRFS) is another technique which is not very popular for arsenic determination but however has been used. Campbell et al.[65] wet ashed plant and biological materials before determining arsenic by XRFS. The method was also applied to analyse arsenic in urine. Detection limits of 0.1 to 1 ppm were achieved however the technique is more suited to multielement analysis. Eltayeb and Grieken[66] determined arsenate and other metals using XRFs. Coprecipitation with aluminium hydroxide was used to extract and preconcentrate the elements from water. Detection limits were 0.2 - 0.8 $\mu\text{g L}^{-1}$ and recoveries for arsenate were 80 % approximately. The extraction procedure does however seem tedious.

1.5.9 Gas chromatographic methods

Gas chromatography (GC) is limited to the analysis of volatile materials. Derivatisation of non-volatile compounds is often used to facilitate analysis by GC. In the determination of arsenic by GC, hydride generation is often used to produce a volatile hydride. The arsenic hydrides produced may be separated by GC. Skogerboe and Bejmuk[40] determined arsenic by GC after hydride generation. Other elements were determined simultaneously ie. germanium and antimony. Arsenic, germanium and antimony were removed from aqueous samples via hydride generation and reliably determined by GC with thermal conductivity detection[40]. It was found that arsine could not be determined with a flame ionisation detector and it was thought that this may have been due to the formation of stable oxides in the flame. Hahn et al.[41] determined arsenic, selenium, germanium and tin simultaneously by hydride generation GC with AAS as the detector. Hollow cathode lamps for each element were moulded on a lamp turret. The turret kept the lamps in a continuous state of operation (10 mA)

and allowed them to be rapidly interchanged. The main advantages of this technique were in terms of cost and convenience. The sequential technique makes more efficient use of sample and reagents than a series of single element determinations. Clark et al.[42] used an on-column hydride generation method for the production of volatile hydrides of arsenic, tin and antimony for GC analysis. The top of the column was modified ("doped") with sodium tetraborohydride. Solutions of hydride forming metals and metalloids were converted to their hydrides on injection onto the column. An AAS detector was used. Siu et al.[43] used GC with electron capture detection and applied an arsenic derivatisation step. 2,3-dimercaptopropanol (BAL) was allowed to react with arsenite to form an arsenite-BAL derivative which was found to give good sensitivity when analysed using electron capture detection. Arsenate must be reduced to arsenite for derivatisation. The detection limits were 10 pg arsenic in absolute terms.

1.5.10 Selective hydride generation AAS

It is well known that the reduction of arsenic compounds with sodium tetraborohydride is pH dependant[44, 45]. This instigated interest in the exploitation of its dependency. Anderson et al.[45] investigated not only the use of pH but also reaction matrix, chelating agents and redox agents for the determination of arsenite, arsenate, MMA and DMA using continuous hydride generation and AAS for detection. Several combinations of media and reagents investigated showed selectivity towards the reduction of individual arsenic species. Hence to obtain quantitative information on the species present in one sample several runs with different media and reagents were required.

Howard and Arab-Zavar[46] used a cold trapping technique for selective determination of inorganic arsenic, MMA and DMA. The species were reduced

with sodium tetraborohydride and trapped at $-196\text{ }^{\circ}\text{C}$. The trap is allowed to warm to room temperature and the arsines volatilise in order of increasing boiling point and are swept to the atomisation cell. The cold trapping procedure effectively preconcentrates several arsenic species and allows sequential determination. It has found widespread application[47 - 49]. Later Anderson et al.[50] compared the selective reduction procedure to the well established cold trapping technique. It was found that the selective reduction procedure was more precise than the cold trapping procedure but the detection limits were not as good. However both of these selective methods are relatively slow.

1.5.11 Liquid chromatographic methods

Chromatography techniques, i.e. column chromatography, ion exchange and HPLC, have been widely applied to the determination of arsenic species[36 -41, 70]. These techniques are always used in combination with other detection techniques, i.e. AAS, AES. Liquid chromatography has been used for inorganic and organic species of arsenic and it is not limited to the analysis of volatile compounds. Grabinski[72] used column chromatography with flameless AAS to determine arsenite, arsenate, MMA and DMA. Anion and cation exchange resins were used for the separation of the individual species. These were slurry packed onto the one column. Detection limits for each individual arsenic species were 10 ppb. However, the elution pattern involved four changes of solvent composition and the column was also regenerated after each chromatogram with ammonium hydroxide and hydrochloric acid resulting in a very labour intensive method. Iverson et al.[34] also used column chromatography to separate arsenite, arsenate, MMA and DMA and used flameless AAS for detection. A cation column was used with gravity flow but to speed up analysis columns were operated under nitrogen. Detection limits of $2\text{ }\mu\text{g L}^{-1}$ of arsenic were achieved.

Fractions were collected from the column effluent and analysed by AAS. The procedure was not online. Ricci et al.[35] developed an automated method using ion chromatography with hydride generation flameless AAS for the speciation of arsenic compounds in air samples. Two buffers were used for elution. Detection limits of less than 10 ng ml^{-1} were obtained for each species. Pacey and Ford[36] also used ion exchange for arsenic speciation and used graphite furnace AAS for detection. Anion and cation exchange columns were used. The cation column was used to separate and determine DMA and the anion column for arsenate and MMA. Total arsenic was determined and therefore arsenite could be determined by difference. Three determinations were required to find the speciation information of four arsenic species which makes analysis time lengthy. Detection limits were 4 ng ml^{-1} or less depending on the species determined.

Tye et al.[38] used HPLC for the separation of arsenite, arsenate, MMA and DMA and successfully applied this technique to the analysis of water samples. Hydride generation AAS was used for the detection. Preconcentration of arsenate, MMA and DMA was carried out the species being loaded onto an anion exchange column with sulphuric acid $10^{-4} \%$ v/v and eluted with ammonium carbonate. Detection limits of 2 ng or less for each species were obtained when an injection volume of one ml was used. Chana and Smith[37] separated arsenite, arsenate, MMA and DMA by HPLC and detected the separated species by hydride generation flameless AAS. The method was applied to the analysis of arsenic species in urine at the $\mu\text{g ml}^{-1}$ levels. An anion exchange column was used. A C_{18} reverse phase guard column was used to remove most of the organic components from urine that would otherwise bind irreversibly to the packing material in the anion exchange column. A phosphate buffer was used for separation and elution of the arsenic species. This method was continuous and ideally suited for routine monitoring of liquid samples. Branch et al.[39] coupled HPLC to AAS for the determination of arsenic species. ICP mass spectrometry

could also have been used as the detector. Two systems were used, one using a phosphate buffer and the other using sulphate. Detection limits in the order of 5 - 10 ng ml⁻¹ were achieved. Arsenobetaine was also determined by this set-up. Gradient elution was used for optimum separation and the method was efficient, highly reproducible and gave rapid separation.

1.5.12 Mass spectrometric methods

Mass spectrometry (MS) has not found widespread use in the analysis of arsenic species yet probably because of its unavailability and high cost however it has been used with GC[47, 69] and HPLC[39, 70]. Branch et al.[39] used ICP MS with HPLC. Enhanced detection limits were achieved of the order 5 - 10 ng ml⁻¹ for each species. Cullen and Dodd[70] used graphite furnace AAS and MS with HPLC. MS offers more positive identification of coeluent peaks.

1.6 CONCLUSION

Early work was concerned with identifying the presence of arsenic. Many analytical techniques have been developed since then both for the separation of arsenic species and for its detection. Spectrophotometric methods are not very popular as they suffer from many interferences. Neutron activation analysis is a very sensitive technique but the unavailability of instrumentation has limited its use. Electrochemical techniques are most suitable for the determination of inorganic arsenic but these techniques are extremely sensitive (low ppb levels). Atomic absorption spectrometry must be one of the most popular methods because of the wide availability of the instrumentation associated with the

technique. The technique is almost always used with hydride generation which increases the sensitivity many fold (ppb). Hydride generation AAS is often coupled to another technique e.g. HPLC. Flameless AAS is more popular than flame as interferences associated with the flame are overcome. Atomic emission spectrometry is another well established technique, especially inductively coupled plasma AES, which is a very sensitive technique which allows a wide range of solvents to be used and hence limits problems associated with coupling techniques. Mass spectrometry is often used with ICP AES but due to its high cost it is not readily available in laboratories for its use in coupling techniques. Atomic fluorescence and x-ray fluorescence spectrometry are very sensitive techniques but are not very popular probably because of their limited use.

Gas chromatography has been used but it is limited to volatile components which means derivatisation has to be used if arsenic compounds are to be analysed. Hydride generation is again often used with this technique as it has the advantage in that several elements may be determined in one run. Selective hydride generation where speciation is carried out by exact control of the hydride requires a lot of operator manipulation which makes it more time consuming. Conditions need to be carefully controlled and with the availability of GC and HPLC little use is found for selective hydride generation today.

Each method provides advantages and disadvantages and must be considered with regard to the scope of the study and the availability of laboratory facilities. HPLC is potentially the most suitable technique for the separation of arsenic species especially when combined with hydride generation AAS or AES. This technique will be discussed in detail in the next chapter.

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CHAPTER 2

The analysis of arsenic species using hydride generation atomic absorption spectrometry.

2.1 INTRODUCTION

Hydride generation has been utilised for over 100 years in the Marsh reaction[1] and the Gutzeit test[1] in both quantitative and qualitative analysis respectively. But it was not until the late 1960's that it was used with atomic absorption spectrometry. Holak[2] in 1969 generated arsine by reaction of zinc with hydrochloric acid and eventually passed it onto an argon/hydrogen flame for measurement by AAS. Since then there have been many publications dealing with hydride generation with AAS detection[3 - 9]. Initial use of hydride generation AAS was with selenium and arsenic as there was problems associated with the determination of these metals by flame AAS[10]. Since then hydride generation has found widespread application in the determination of virtually all elements capable of forming volatile hydrides and these include antimony, arsenic, bismuth, germanium, lead, selenium, tellurium and tin[11]. The toxicity of very low levels of these elements necessitates trace level analysis. The low wavelength of their resonance lines coupled with low nebulisation efficiency of AAS techniques has led to the popularity of hydride generation since 100 % transport efficiency of the element into the atomisation cell is possible. This factor alone significantly increases the detection capabilities for analysis[5].

Hydride generation has become very popular because it is a relatively simple technique and the apparatus is of low cost. The method involves preconcentration of the analyte and separation from the matrix. This results in greater sensitivity and suppression of interferences during atomisation, however, some interferences still occur.

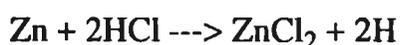
The hydride technique can be divided into three steps: hydride generation and release; hydride transport and hydride atomisation.

2.2 HYDRIDE GENERATION AND RELEASE

Hydride generation and release can be defined as the conversion of an analyte in the acidified sample to the corresponding hydride and its transfer to the gaseous phase. Many agents have been used for the conversion of analyte to hydride, including mixtures of zinc and hydrochloric acid[2], magnesium and titanium chloride reacted with hydrochloric acid and sulphuric acid[12], an aqueous slurry of aluminium reacted with hydrochloric acid[13] and sodium tetrahydroborate reacted with acid[14]. The reactions used in hydride generation can be classified into two categories, metal/acid reductions or sodium tetrahydroborate/acid reductions.

2.2.1 Metal/acid reductions

The most frequently used of the metal/acid reduction was zinc/hydrochloric acid as shown below.



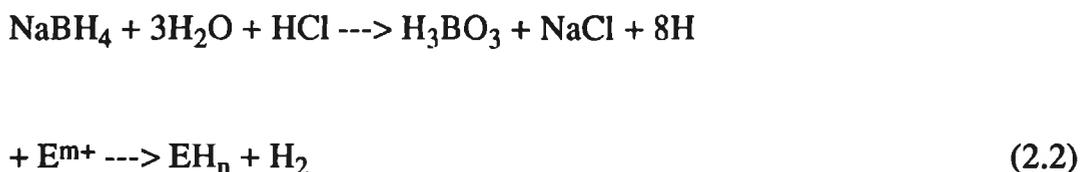
Where E is the analyte element and m may or may not equal n[11].

Flasks equipped with dosing fittings that allowed the introduction of granular zinc to an acid solution of analyte without opening the system to the atmosphere were most frequently used as reaction vessels[15]. A balloon system which functioned to collect reactant products with subsequent rapid expulsion to the

atom reservoir was introduced to sharpen the peak response[16]. However this system never found widespread use as the reaction products which carried substantial amounts of acid vapour quickly degraded the balloon surface. Another system introduced by Holak[2] used a condensation tube. A U-tube kept at liquid nitrogen temperature trapped the hydrides by condensation, they were subsequently vented to the atom reservoir by means of a carrier gas. The main disadvantage of the metal/acid reduction is that it is slow reaction, times of 20 minutes at least are required to ensure quantitative reaction. The inability to automate the system and the fact that it is only capable of producing hydrides for arsenic, selenium, antimony and bismuth have also hindered its use[12].

2.2.2 Sodium tetrahydroborate/acid reduction

The reaction of the sodium tetrahydroborate/acid reduction is shown below:



Where E is the analyte element and m may or may not equal n.

Since the introduction of sodium tetrahydroborate/acid reduction with AAS in 1972[14] it has virtually replaced metal/acid reduction. Initially sodium tetrahydroborate tablets were used. Similar reaction vessels and balloon systems were used as those in the metal/acid reduction but for automation purposes aqueous sodium tetrahydroborate was introduced. Continuous flow systems could now be used using peristaltic pumps. The sodium tetrahydroborate/acid

system offers several advantages over the metal/acid system, it lends itself to automation, produces hydrides faster i.e. 10 - 30 seconds, and it produces hydrides with a wider range of elements.

For acidification hydrochloric acid is most often used. Sulphuric and nitric acids have also been used[17 - 19]. The response from each acid, with an element or a species of an element, differs with increasing concentration of the acid. At higher concentrations of acid the response either reaches a plateau or decreases. The reasons are not completely understood but factors such as rapid reduction, decomposition at higher acid concentration, degassing and interferences due to sulphate and nitrate may influence the response[17].

Optimum conditions for hydride generation depend on type of analyte i.e. arsenic, selenium, tellurium etc, and the valency of the element i.e. arsenite or arsenate. Parameters such as the type, volume and concentration of acid and reducing agent, carrier gas flow rate, type of atomisation cell, its temperature and whether masking or releasing agents are used, all affect the signal obtained.

When generating the hydride of a particular analyte the valence state is important. Arsenic may be determined as arsenite or arsenate but as arsenate there is a slower rate of hydride formation and therefore gives a poorer response[17]. Selenium must be in the +4 oxidation state for conversion to the hydride[20].

The presence of masking and releasing agents is not always required but when interferences are present they are almost essential. Depending on the interferent and analyte present the masking agent varies. Anderson et al.[17] studied the affect of different chelating agents on metal interferences in different media in the determination of arsenic. It was found that in a citric acid-citrate matrix

ethylenediaminetetraacetic acid (EDTA) enhanced the interference effect of copper II whereas both thiosemicarbazide and thiourea were effective masks. In an acetic acid reaction matrix the combination of EDTA and thiourea or thiosemicarbazide were shown to prevent interferences from many metals. Bye[21] studied the effect of iron III as a releasing agent for nickel II interference and showed that it was very effective at certain concentrations. The amount of reducing agent also plays an important role in hydride generation. Yamamoto et al.[22] used low concentrations of sodium tetrahydroborate and found that some metal ions can be tolerated.

2.3 HYDRIDE TRANSFER

There are two basic modes of hydride transfer as described by Dedina[23], these are direct transfer and collection.

2.3.1 Direct transfer

In direct transfer the hydride released from the sample solution is directly transferred to the atomiser. There are a number of direct transfer methods available including continuous flow, flow injection and batch[19, 20, 22, 24 - 26].

2.3.1.1 Continuous flow transfer

The transfer of a metal hydride using a continuous flow method involves both the acidified sample solution and reducing solution flowing continuously at a constant rate to a gas/liquid separator to establish a steady state analytical signal. Continuous flow is a reliable, rapid and convenient technique well suited to the analysis of large numbers of water samples in a geochemical laboratory[17]. It does however use large quantities of sample.

2.3.1.2 Flow injection transfer

In flow injection hydride generation AAS acid and reducing solution flow continuously at a constant rate to the gas/liquid separator and a limited volume of sample is injected into the acid stream. The signal produced is thus transient. The flow injection method is very simple, rapid and precise. The flow injection method improves the sensitivity over batch or continuous flow methods. This is mainly due to the use of a transient signal because an excess amount of sample is not necessary to obtain a transient signal[24].

2.3.1.3 Batch transfer

In the batch mode a limited volume of sample is reduced i.e. the reductant is added to a known volume of sample in an acid solution. The batch system gives a transient signal and the absorbance maximum depends upon the analyte mass

and not its concentration[27]. The sensitivity can be increased by simply applying larger volumes of sample solution.

2.3.2 Collection transfer

In collection mode, the hydride is collected in a collection device until the evolution is complete and then it is transported to the atomiser. The collection technique may be divided into pressure and cold trap collection.

2.3.2.1 Pressure collection

In the pressure mode, collection is carried out in a closed vessel where the hydride released from the sample is collected under pressure, together with hydrogen resulting from the borohydride decomposition. Narasaki and Ikeda[28] describe a system using pressure mode collection in the determination of arsenic and selenium where the hydride is stored in a gas liquid separator up to an appropriate pressure and then swept automatically to an atomic absorption furnace. Detection limits of 0.3 ppb were obtained for arsenic determinations.

2.3.2.2 Cold trap collection

In the cold trap mode, a U-tube is immersed in liquid nitrogen, through which hydrogen passes freely and in which the hydride is collected. The hydride is

purged into the atomiser with the aid of a heating bath. Anderson et al.[29] used a cold trapping technique in the determination of arsenic in water samples. A glass bead U-trap suspended in liquid nitrogen was used. The procedure effectively preconcentrated the hydride which ultimately passes as a "plug" of analyte to the detector.

2.3.3 Conclusion

The batch method gives better sensitivity than the continuous system but the continuous system is more suitable for automation as precision is improved and interferences are reduced. Direct methods have the advantage of being simple and fast but the disadvantage of all excess hydrogen generated being swept into the atomiser. The collection methods have better sensitivity than direct transfer methods as all the metal hydride is analysed at once but suffers from the disadvantage that unstable hydrides cannot be analysed using this system. Overall continuous system has been found to be optimum.

2.4 HYDRIDE ATOMISATION

The atomisation of hydride takes place in the optical beam of an atomic absorption spectrometer. Originally an argon-hydrogen diffusion flame[30] was used to atomise the hydrides, because it was transparent at the wavelength of arsenic (193.7 nm), but now electrically[10, 31, 32] or flame heated[17, 20] quartz tubes are routinely used. Other systems include flame-in-tube atomisers[23, 33, 34] and graphite tube furnaces[35, 36].

2.4.1 Flame-in-tube atomisers

Flame in tube atomisers are most often externally unheated quartz tubes with a flame burning inside. Siemer[33] was the first to describe this type of atomiser. His design was a T-tube with a fuel rich hydrogen-oxygen flame burning near the T-tube junction. Nakashima[34] used a different arrangement consisting of a long absorption tube aligned in the optical path, with an argon (or nitrogen)-air-hydrogen flame.

A typical flame-in-tube atomizer is shown in figure 2.1. It consists of two parts, an intake part and a T-tube, both made of quartz, connected by a standard joint. The horizontal bar of the T-tube is aligned in the optical path. The hydride is transported from a generator by a flow of hydrogen which enters the left side of the intake section. Oxygen is introduced through a capillary into the right side but the oxygen flow is much smaller than the flow of hydrogen. A very small, almost invisible flame burns at the end of the capillary which is usually mounted 2 to 10 mm in front of the T-tube junction, so that the flame burns in the inlet arm of the T. The inlet arm diameter and dimensions of the bar are varied considerably to match actual demands. The inlet arm diameters may vary from 2.5 to 10 mm approximately. The bar of the T-tube may vary from 20 to 160 mm long and 3 to 15 mm in diameter. With respect to bar-tube dimensions, T-tubes, and consequently atomisers can be classified into two types: large tubes with lengths around 150 mm and diameters between 7 to 10 mm and small tubes with a length 60 to 90 mm and a diameter 3.2 to 3.8 mm[23].

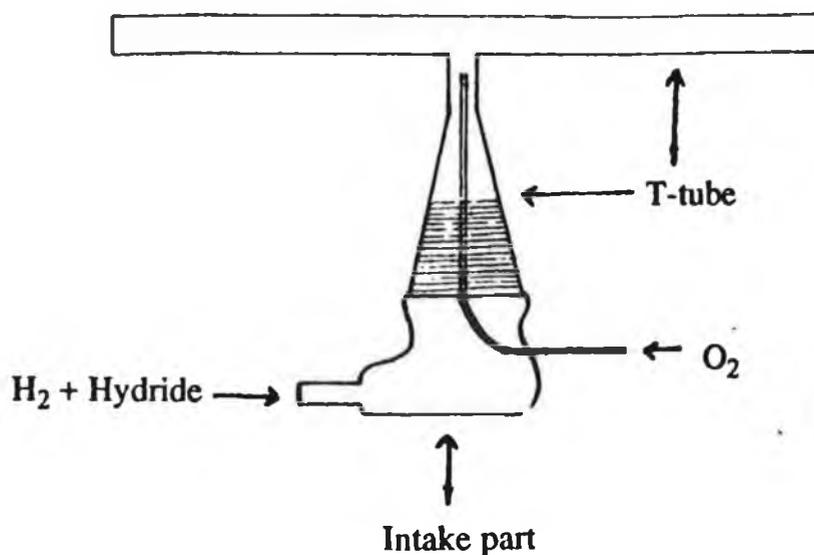


Fig. 2.1 Schematic diagram of flame-in-tube atomiser.

The temperature in the atomiser bar-tube depends on the hydrogen and oxygen flows. Dedina[23] reports that there is a marked dependence of the analytical response on oxygen inlet flow to the flame. With increasing flow a rapid rise of the signal is observed followed by a plateau. This indicates that increasing the oxygen flow into the flame beyond the optimum does not change the atomisation efficiency. The optimum oxygen flow rate is independent of hydrogen flow rate, it depends only on the inlet arm diameter (the larger the arm diameter the higher the oxygen flow required for maximum sensitivity).

Free radicals are generated in the reaction zone of the hydrogen - oxygen flame as follows:

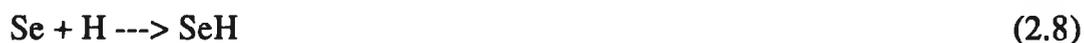


Only OH and H radicals are formed in the presence of excess hydrogen due to the very fast reaction a balanced state between these species is readily established in which, under given conditions, OH radicals are outnumbered by H radicals at least by a few orders of magnitude and therefore can be neglected. Thus it may be assumed that only H radicals are formed in quantities corresponding to the total amount of oxygen i.e. two radicals for each oxygen molecule.

Dedina[23] reports that the mechanism of atomisation proceeds most probably via interaction of hydride species with H radicals. For selenium two consecutive reactions take place:



Recombination reactions of the type:



are not significant because they are strongly exothermic and consequently their rate constants are relatively small. The probability of formation of free analyte atoms is therefore proportional to the number of collisions with H radicals. Therefore local atomisation efficiency increases with the growing number of free radicals in the inlet arm of the T-tube and it may be assumed that at the optimum oxygen flow the hydride is completely atomised.

Free analyte atoms formed are transported to the T-tube bar by a flow of hydrogen. The pattern of the distribution of free atoms is governed by the hydride supply to the atomiser and by the removal of free atoms from the bar-tube. The removal proceeds by forced convection, expelling free analyte atoms, and simultaneously by the decay of free atoms in the bar-tube. The lower the hydrogen purge flow the slower the forced convection and the higher the sensitivity. The lower the hydrogen flow the more time for the free atoms to decay, but there is a "slow flow limit" below which all analyte free atoms decay completely before reaching the end of the bar-tube. Thus the total number of free analyte atoms in the bar-tube and consequently the sensitivity are directly dependent on the hydrogen flow rate. The opposite case also exists a "fast flow limit", when the gas flow is so fast that no significant free atom decay takes place in the atomiser bar-tube.

Dedina[37] used a hydrogen-oxygen flame-in-quartz tube method to investigate the interference of volatile hydride forming elements in the analysis of selenium and found that the large T-tube is optimum for sensitivity but it is not suitable when other hydride-forming elements (arsenic, antimony, tin and bismuth) are present in the sample. The small T-tube with a better suppression of analyte decay interferences was found to be a better solution in most cases. The analyte decay interferences could be further reduced either by lowering the probability of free analyte atom contact with the bar surface (by increased hydrogen flow or by

using a shorter or narrower T-tube bar) or by heating the T-tube bar. The magnitude of the radical population interference decreases with a smaller inlet bore or with a higher oxygen flow. A decrease in tube diameter was found to increase noise and an inlet diameter substantially smaller than 2.5 mm was not feasible. Therefore the only convenient way to further reduce the interferences in the atomiser is to further increase the oxygen flow which suppresses the radical population interferences directly, and analyte decay interferences indirectly, by heating the T-tube.

2.4.2 Graphite furnace atomisation

Graphite furnaces have been used for hydride atomisation almost since the introduction of the hydride generation techniques. There are two approaches to using graphite furnace: in-situ trapping of hydrides and on-line atomisation.

2.4.2.1 In-situ trapping of hydrides

This approach uses commercial graphite furnaces for both steps as the trapping medium, and as the atomisation cell. Hydride purged from the generator is trapped in a heated graphite furnace usually in the 300 - 699 °C range, until evolution of the metal hydride is complete. The trapped analyte is subsequently atomised at temperatures above 2000 °C. Trapping temperatures should be optimised for each experimental set-up and the optimum temperature range usually does not extend down to the ambient temperatures[23].

Generated hydrides are introduced either via the internal gas line of commercial furnaces or to the sampling port of the graphite tube through an interface made either of graphite or quartz. Quartz interfaces have to be removed before the start of atomisation as metal hydrides trapped in the interface cannot be completely volatilised and atomised, therefore the efficiency of this particular system is relatively low. Similar difficulties could be encountered for metal hydrides introduced into the internal gas line, since there, the hydride comes in contact with metal components, graphite tube ends and graphite cylinders which are cold during both the trapping and the atomisation stages. Sturgeon et al.[35] investigated the hydride trapping and analyte atomisation mechanism for arsenic,

bismuth, antimony, selenium, tin and lead. The analyte was deposited in the preheated graphite furnace via thermal decomposition. Atomisation of arsenic, antimony, selenium and tin was found to be identical to that occurring when these elements were injected in an aqueous solution. Atomisation of lead and bismuth deposits was distinctly different than from their aqueous counter parts but this may have been due to volatilisation of the metal.

2.4.2.2 On-line atomisation

This approach utilises a direct transfer of metal hydride from the generator to the furnace, which is preheated to a high temperature, usually over 2200 °C. This arrangement is very simple but has the disadvantages as discussed for in-situ trapping where hydrides can be captured on cooler metal or graphite parts before reaching the furnace.

Sensitivity for on-line atomisation is generally lower than in-situ trapping. It is also lower than when quartz tube atomisers are used since the small dimensions of graphite furnaces and their high atomisation temperatures reduce the residence time of free analyte atoms and consequently sensitivity. Andrae et al.[36] reported sensitivity with commercial graphite furnaces, as 50 % lower for antimony than in flame-in-tube atomisers.

2.4.3 Diffuse flame atomisation

Diffuse flames were used mainly in the past as they have been shown to be inferior to other atomisers, with the sensitivity being lower due to a marked dilution of the hydride with flame gases[31]. The flame also has a high background absorption and its "flicker noise" results in poor limits of detection. In recent years diffuse flames have not been employed to any great extent and therefore have not been discussed in detail.

Hershey and Keleher[38] used an argon-hydrogen-entrained air flame system to investigate inter-element interference reduction studies utilising an ion exchange resin. Wagenen and Carter[39] also used a diffusion flame to study kinetic control of peak shapes in arsine generation whereas Hershey and Oostdyk[40] used an argon-hydrogen-entrained air flame to determine arsenic and selenium in environmental and agricultural samples. Diffuse flame hydride generation atomic absorption spectrometry is a very simple method for detection of volatile hydrides and certainly more sensitive than ordinary flame AAS, but with the availability of graphite furnace, flame-in-tube and externally heated tubes, diffuse flame finds little use.

2.4.4 Externally heated quartz tubes atomisation

In 1972 Chu et al.[31] introduced the electrically heated quartz tube for hydride atomisation. Today the electrically heated quartz tube is the most commonly used technique, because of its simplicity and because it offers many advantages over the other available techniques.

The design of externally heated quartz tubes is similar to flame-in-tube atomisers. They consist of a T-tube with its bar aligned in the optical path and the central arm of the T serving for the delivery of hydrides carried by a flow of gas from a generator. The bar tube is heated either by a chemical flame or more often electrically. The two bar-tube outlets are either open, similar to the flame-in-tube atomiser, or closed with optical windows. If closed, two outlet arms are fused to the bar-tube near its end to prevent ignition of hydrogen at the ends of the open ended system, which leads to noisy signals. A second approach adopted to prevent ignition is to leave the ends of the tube unheated and uninsulated or even to provide them with graphite rings. Petterson et al.[32] used an electrically heated quartz tube and left the ends unheated and uninsulated for the determination of selenium in bovine liver. Donker et al.[10] used an open end quartz tube provided with graphite rings at the end for cooling.

The cell temperature is limited by the thermal durability of quartz and of the resistance wire used for heating. Full sensitivity is reached at temperatures much lower than in a graphite furnace. Welz and Melcher[41] found temperatures around 800 °C to be optimum for the atomisation of arsenic, antimony, bismuth, selenium, tellurium and tin whereas temperatures of 1700 - 1800 °C are necessary to atomise arsine or selenium hydride in a graphite tube furnace. Tube material has a significant effect on the optimum atomisation temperature. Wang et al.[42] found that 1200 °C was necessary for atomisation to reach full sensitivity in an externally heated alumina tube. Chamsaz et al.[43] investigated the use of silica, alumina and graphite tubes of the same lengths and diameters. The graphite tube gave much lower sensitivity, even if impregnated with sodium tungstate or zirconium chloride. A silica tube gave 10 - 20 % higher sensitivity than an alumina tube for inorganic tin and organotin compounds, however the silica tube was subject to deterioration. Alumina was chosen for the analysis of

tin(IV) and organotin compounds in sea-water, as it did not deteriorate quickly and offered good sensitivity.

The sensitivity obtained in externally heated quartz tubes is in the same range as flame-in-tube atomisers and considerably higher than in diffuse flame or in graphite tube atomisers. The superior sensitivity of quartz tube atomisers is due to their large dimensions and to the low dilution of the hydride. Both these factors increase residence time and thus sensitivity. The length of the externally heated quartz tube atomisers is limited by the dimensions of the burner compartment of the instrument employed. The atomiser bar tube is most often around 150 mm long with a diameter typically over 10 mm[23].

There is no sharp division between externally heated quartz tube and flame-in-tube atomisers. Some electrically heated atomisers are actually air-hydrogen flame-in-tube atomisers with an electrically heated bar-tube, since oxygen is introduced into the atomiser and hydrogen is either used as a purge gas or introduced directly into the atomiser. Some others do not employ hydrogen as a purge gas, but some hydrogen is always present in the atomiser as a result of the decomposition of the reducing solution which usually is borohydride, they employ an additional flow of oxygen or air to the atomiser because it increases sensitivity. Evans et al.[44] reported that air introduced had no effect on sensitivity in the flame heated quartz atomiser that was used, but it did improve precision. Welz and Melcher[30] reported that in a closed electrically heated atomiser, a very small amount of oxygen which may be dissolved in the sample is necessary to reach optimum sensitivity for almost all volatile hydride forming elements. Parisi and Heyndrickx[45] report that oxygen has an effect on determination of volatile forming elements not only at low quartz cell atomisation temperatures but also at temperatures above 800 °C. Dedina[23] concluded that for optimum sensitivity there is a necessary minimum oxygen concentration

which depends, apart from hydride identity, on temperature. The higher the temperature the lower the oxygen concentration necessary. At low temperatures oxygen present in the system as a contaminant may not be sufficient for optimum sensitivity and thus additional oxygen may be required.

The quality of the inner quartz surface has an effect on sensitivity. Optimum sensitivity/performance is usually maintained by rinsing the tube in 40 % hydrofluoric acid. Grinding of the inner atomiser surface with alumina has also been employed. Welz and Melcher[41] studied the effect of the quartz cell surface on sensitivity. They found that a new untreated quartz cell gives a low response for volatile hydride-forming elements. Heating the cell for 24 hours at 1000 °C usually overcomes this problem but they found that rinsing the quartz cell with 40 % hydrofluoric acid for approximately 15 minutes is the most effective procedure for optimising sensitivity.

Basic processes in heated quartz tube atomisers have been investigated. Welz and Melcher[41, 46] concluded that in the externally heated quartz tube, hydrides are atomised by the same mechanism as in the flame-in-tube atomisers, that is by an interaction with H radicals. These radicals are generated by reactions between hydrogen and oxygen near the gas inlet to the heated portion of the atomiser. Bax et al.[47] confirmed that hydrogen is necessary for efficient hydride atomisation. Agterdenbos and Bax[48] demonstrated the role of free radicals. They used radical scavengers such as methane and iodine vapour which depressed the selenium signal in an electrically heated tube to 20 % and zero respectively. They stated that the radicals are not populated enough for reactions of the type:





to take place. They suggest the following reactions which are catalysed by H and OH radicals.:



Welz and Schubert-Jacobs[49] found that for arsine, in the absence of hydrogen, it is not atomised but thermally decomposed and the product (most likely an oxide) is retained in the heated quartz tube quantitatively and can be re-volatilised and atomised in part as soon as the hydrogen is fed into the heated quartz tube.

The nature of the analyte atom decay is not totally understood. Ageterdenbos and Bax[48] suggested that it takes place on the quartz surface. In the presence of hydrogen the following reaction could take place.



With all factors taken into account such as design, temperature, sensitivity, oxygen, hydrogen, surface of the tube and atomisation decay, externally heated quartz tubes are the most commonly used atomisers whether they are heated by flame or electrically.

2.5 INTERFERENCES IN HYDRIDE GENERATION AAS

There are basically two types of interference in hydride generation AAS: spectral interferences and nonspectral interferences.

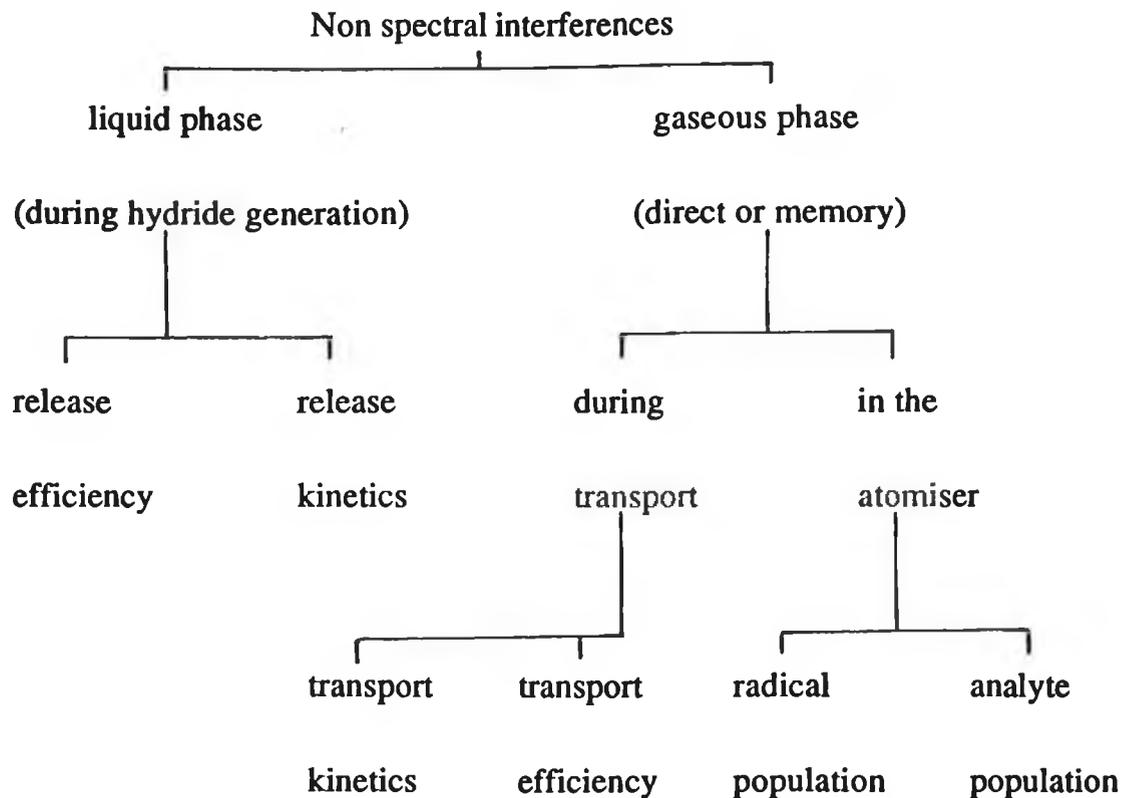
2.5.1 Spectral interferences

These are not very significant in hydride generation AAS since the analyte is separated from the matrix. Background absorption may occur usually in diffuse flame atomisation when the transparency of the flame may change when the hydride is purged into it. Dedina[37] found background interference by hydrides of arsenic, antimony and tin on the selenium 196 nm line with the flame-in-tube atomiser when background correction was not used.

Paris and Heyndrickx[45] considered background correction unnecessary for externally heated quartz tube atomisation. With the instability of the deuterium hollow-cathode lamp at wavelengths less than 200 nm, due to the intensity of the lamp being lower at this wavelength, this increases the baseline noise and detection limit and influences the precision of low-level measurements dramatically, compared with measurements made using the electrodeless discharge lamp alone they suggest that the deuterium lamp should not be used. Pacey and Ford[50] used graphite furnace for arsenic speciation determination and found that deuterium lamp background correction did not affect the quality of atomic absorption measurements and was therefore not used.

2.5.2 Non-spectral interferences

Nonspectral interferences are more common and they can be divided as follows;-



In principal nonspectral interferences occur either in the liquid phase during the hydride formation and its release from the liquid sample or in the gaseous phase during the hydride transport or in the atomiser.

2.5.2.1 Liquid phase interferences

The liquid phase interferences may be caused by changes in the hydride generation rate (generation kinetics interferences) and/or by a decreased fraction of analyte reduced and released from the sample solution (generation efficiency

interferences). These interferences fall into two basic groups : compound interferences and matrix interferences.

2.5.2.1.1 Compound interferences

When the analyte in the sample is not in the same form as that in the standard, rate of release of the hydride may be different from the sample than from the standard even when the standard is added to the sample. These interferences take place when the analyte sample is not completely decomposed during pretreatment or if the analyte is in the elementary form or in a valency which is converted to the hydride with a lower efficiency than the analyte valency in the standard.

Sinemus et al.[51] investigated the influence of valence state on the determination of antimony, arsenic, bismuth, selenium and tellurium. It was found that a substantial error would result if different oxidation states of antimony i.e. antimony III and antimony V, were found in samples. Antimony III gives about twice the sensitivity in peak height compared to antimony V. Potassium iodide may be used to reduce antimony V to antimony III in acid solution. There is also a significant difference in sensitivity between arsenic III and arsenic V. Brumbaugh and Walther[20] reported that the +5 state of arsenic has a slower rate of hydride formation than the +3 state.

Sinemus et al.[51] found that bismuth only occurs in the third valence state in natural waters. In the fifth valence state only a few salts of unstable bismuthic acid and bismuth pentoxide are known and no stable bismuth V compounds are offered by manufacturers of chemical reagents. Therefore the determination of bismuth only requires the samples to be acidified. The sensitivity difference between selenium IV and selenium VI is more pronounced than the difference between the two common valence states of antimony or arsenic. It is essential to

know the oxidation state of selenium in the sample and to reduce selenium VI to selenium IV for determination. This difference in sensitivity can be used to an advantage if the selective determination of both oxidation states of selenium is required.

The difference in sensitivity for the two oxidation states of tellurium is very similar to that of selenium. Tellurium VI cannot be determined effectively with the hydride technique using sodium borohydride as the reducing agent. Tellurium VI like selenium VI may be reduced to the fourth valence state by boiling with highly concentrated hydrochloric acid: the two oxidation states of tellurium can therefore be determined selectively[51].

It may be concluded, that it is vital that the oxidation state of the analyte be known and/or reduced or oxidised to a valence state that is determinable by the hydride technique.

2.5.2.1.2 Matrix interferences

Matrix interference may take place when the matrix affects the hydride release efficiency. The extent of the interference does not depend on the analyte concentration but only on the interferent concentration. The method of standard additions can alleviate these interferences.

The most commonly encountered class of matrix interferences are those of inorganic ions. Interferences due to the presence of nitrous oxides and/or chlorine may cause problems in selenium determinations[52, 53]. Procedures recommended for avoiding or decreasing matrix interferences have included

change of acidity of the solution, formation of complexes (masking) with the matrix interferents, and formation of precipitates of the interferences. Complexation reagents that have been reported include EDTA, potassium iodide, citric acid, thiosemicarbazide, 1,10-phenanthroline, oxalic acid and thiourea[17].

Aggett and Hayashi[54] investigated interferences of copper II, cobalt II and nickel II on the determination of arsenic. They found that the interferences of these elements are due to the formation of specific chemical species between arsenic and the interferent in lower than normal oxidation states. The species may be stabilised in the presence of excess sodium tetrahydroborate. Complexing agents such as thiourea and o-phenanthroline appear to prevent interference by forming complexes with the metal ions.

Agterdenbos and Bax[55], on investigating the generation of selenide hydride and the decomposition of tetrahydroborate, found that the presence of transition metal ions increased the decomposition rate of tetrahydroborate so that the formation of hydrogen selenide was incomplete. However iodide was found to catalyse the formation of hydrogen selenide and therefore even in the presence of much higher concentrations of interfering metal ions the hydride formation reaction was rapid enough to result in complete conversion to the metal hydride in the presence of iodide.

Yamamoto et al.[22] investigated the elimination of metal interferences on arsenic determination using sodium tetrahydroborate. They found that results were consistent with previously proposed mechanisms for interference by metal ions i.e. metal ions interfere in the hydride generation reaction after reduction to the elemental metals. They found that interferences decreased with decreasing sodium tetraborohydride concentration but this was only the case for some interfering elements e.g. aluminium, copper and magnesium. Other hydride

forming elements could not be eliminated by decreasing the concentration of sodium tetraborohydride solution. To eliminate these interfering elements perhaps the mechanism described by Aggett and Hayashi[54] and Agterdenbos and Bax[55] would be appropriate that is the use of complexing and catalysing agents.

The effect of iron as a releasing agent for metal interferences has been studied by many[56 - 58]. Bye[21] found that the releasing effect of iron III on the interference from nickel II in the determination of selenium was much more effective than alternative oxidants such as chromium VI, thallium III and nitric acid, in reducing the nickel interference. The reason for the favourable effect of iron, III on the nickel interference, is that iron III may be reduced to iron II by the tetraborohydride before the nickel ions. The effect is much lower for the other strong oxidants such as chromium VI, thallium III and nitric acid, probably for kinetic reasons. Iron III is not reduced to a precipitate as with some other oxidants, this is another advantage. Wickstrom et al[56] found iron III useful as an effective releasing agent, on the interference of copper, for the determination of selenium. Chan[57] investigated the effectiveness of iron III for controlling interferences and found that during the reduction process, the cations copper, nickel etc., are being partially reduced to the metallic form. By using 1,10-phenanthroline the cations are chelated and may be protected from this reduction. Otherwise 1,10-phenanthroline and iron(III) exhibit equal ability in controlling interferences.

Boampong et al.[58] investigated the use L-cystine in reducing interferences when determining arsenic. L-cystine is cheap and readily available and has much lower toxicity than thiourea which has also been used[17]. It was found that interferences from cobalt II, copper II, iron II and III, nickel II and zinc II at

concentrations up to 10,000 µg/ml are effectively eliminated. Interference from silver I, mercury II and platinum IV was eliminated up to levels of 1000 µg/ml.

Bye[59] determined selenium in copper and eliminated the interfering copper by removing it from solution rather than isolating the analyte. The copper was removed electrolytically from the sample solution using the traditional electrogravimetric method for the determination of copper[59]. It is necessary to ensure the selenium is in the +6 state prior to the electrodeposition as selenium VI cannot be electrochemically reduced. After the electrolysis was terminated, the platinum electrode was removed and the selenium in the solution was reduced back to the tetravalent state with hydrochloric acid, and thus may be determined by the hydride generation technique.

Hershey and Keliher[38] found that by using ion exchange resins inter-element interferences were reduced. Ikeda[60] used a mini column of a chelating resin to remove transition metal interferences. The method was successfully applied to the determination of selenium in standard copper alloy and nickel sponge. Chelating resins strongly adsorb transition metals whereas hydride forming elements (except bismuth and lead) and alkali and alkali earth metals are only slightly retained.

Interference effects can also be reduced by controlling the acidity. Yamamoto et al.[26] found that a weakly acidic medium was suitable for eliminating the interference of foreign ions of both arsenic and antimony. Hershey and Keliher[61] found that higher acid concentrations greatly improved the signal recovery for antimony, arsenic, bismuth and selenium when the following interferences were present: cobalt, copper, lead, molybdenum, nickel, palladium, and rhodium. Boampong et al.[58] found that 5 M hydrochloric acid has a negligible effect on the arsenic signal but improves the recovery of arsenic in the

presence of iron and copper compared with similar determinations in 1.4 M hydrochloric acid. Hydrochloric acid at high concentrations effectively eliminates interference from cadmium at the 1000 µg/ml level. Agett and Hayashi[54] also found a decrease in interference, with increasing hydrochloric acid concentration when determining arsenic. The interfering metals were nickel II, copper II and iron III. Agett and Hayashi[54] found that the greatest effect on interference is the concentration of interferent and the acidity of the solution when determining arsenic.

Most acids do not interfere with hydride generation, however, serious interference may occur with the analysis of samples decomposed in solutions containing larger amounts of hydrofluoric acid. Petrick and Krivan[62] investigated the interference of hydrofluoric acid in the determination of arsenic and antimony and found that up to a concentration of 1 % hydrofluoric acid does not effect the hydride formation of arsenic III and antimony III. In solutions with higher hydrofluoric acid concentrations, arsenic V forms $[\text{AsF}_5\text{OH}]^-$ ions which do not react with sodium tetrahydroborate. Antimony V is not hydrogenated in the presence of hydrofluoric acid.

Agterdenbos et al.[52] eliminated the interference of nitrous oxides (NO_x), when determining selenium, by using sulphamic acid. Nitrate ions do not interfere but copper ions catalyse their reduction to nitrite and therefore combined presence of nitrate and cupric ions gives more serious interference than cupric ions alone. The interference by nitrate is attributed to the reaction between selenium hydride and volatile nitrogen oxides.

Krivan et al.[53] found that when hydrogen peroxide is used as one of the reagents for sample decomposition, in the determination of selenium, chlorine is formed when boiling the sample solution in hydrochloric acid to reduce selenium

VI to selenium IV. The residual chlorine remaining in the sample solution oxidises selenium IV back to selenium VI at room temperatures. This can cause considerable errors as selenium VI cannot be converted to selenium hydride. This effect however can be eliminated by the removal of chlorine by bubbling a stream of nitrogen through the digestion during the boiling step.

Most metals have little or no influence on the generation of gaseous metal hydrides such as alkali and alkali earth metals, aluminium and silicon. Only those metals that can be reduced easily by sodium tetraborohydride, under the experimental conditions used, have been found to interfere with hydride generation e.g. cobalt and nickel, the elements of the copper group and noble metals of the palladium and platinum groups. With optimum conditions of acid and tetrahydroborate concentrations it is possible to extend the range of interference free determination by more than three orders of magnitude[26, 54, 61]. The use of releasing agents e.g. iron III and complexing agents e.g. 1,10-phenanthroline can also extend the range of interference free determination[17, 21, 56, 57].

2.5.2.2 Gaseous phase interferences

Gaseous phase interferences are caused by the presence of a volatile interferent. These interferences can take place on the surface or in the dead volume of the generator, the connective tubing and the atomiser. These interferences can either have a direct effect (if observed only in the presence of the volatile form of the interferent) or a memory effect (if they persist after the cessation of the interferent generation). Gaseous phase interferences may be divided into two

groups according to their origin: transport interferences and interferences in the atomiser.

2.5.2.2.1 Transport interferences

Transport interferences take place on route from the sample solution to the atomiser, causing delay (transport kinetics interferences) and/or loss (transport efficiency interferences) of the analyte hydride.

Hydride losses or delay become an interference only when their magnitude differs between sample and reference solutions. The interaction of the hydride with the surface manifests itself more often as a change in sensitivity with a dependence on changing surface properties of the apparatus rather than as an interference[23].

Parisis and Heyndrickx[45] found that using silanised glass or F.E.P (fluorinated ethylene propylene) tubing aids the transportation of gases and the maximum sensitivity can be obtained after a few determinations. The reproducibility is low using silicone-rubber and nylon tubing. The use of glass tubing increases sensitivity and reproducibility but hydrides may be adsorbed on the surface. Chamaz et al.[43] found that the use of small (2 - 5 mm) diameter carrier lines contribute to the overall high sensitivity as a result of the lower dead volume.

2.5.2.2 Interferences in the atomiser

Interferences in the atomiser depend on the hydride atomisation mechanism in the given type of atomiser.

2.5.2.2.1 Graphite furnace

Dittrich and Mandry[63] using a graphite paper atomiser found improvement for determining arsenic, antimony and selenium in the presence of other hydride forming elements. This was due to the increase in atomisation temperatures in the range 1600 - 2600 °C. This is direct proof that higher temperatures have a beneficial effect on suppressing interferences in the atomiser. The graphite paper has similar dimensions to those of commercial quartz tubes. It was found that the main cause of matrix interference in the gaseous phase was the formation of diatomic molecules between the trace and matrix elements e.g. AsSb, BiAs etc. Diatomic molecules are formed at temperatures below 1000 °C and are dissociated at 2000 °C, therefore, significant improvements in accuracy and relative sensitivity can be obtained by using graphite tube atomisers at temperatures > 2000 °C.

2.5.2.2.2 Flame-in-tube

Taking into account the mechanism of hydride atomisation as described previously, two types of interference in the atomiser may emerge: radical population interference and analyte decay interference.

Radical population interference occurs when the interferent reduces the H radical population in the radical cloud. If the reduced radical population is not sufficient to atomise the analyte fully a decrease in sensitivity is observed. An enhancement of the hydrogen radical population and suppression of the interference maybe achieved by an increase of oxygen flow to the flame as reported by Dedina[23].

Analyte decay interferences occur when an interferent accelerates the decay of free analyte atoms in the bar-tube. Faster decay leaves fewer atoms in the optical path and thus reduces the sensitivity.

Both the radical population and the analyte decay interferences in the flame-in-tube atomiser can take place either on the surface or in the gaseous phase. Dedina[37] studied interferences of tin, lead, arsenic, antimony, bismuth, tellurium and mercury (in concentrations of up to 125 µg/ml) in selenium determination with a flame-in-tube atomiser and found they exhibited strong gaseous phase interferences. The gaseous phase effects were due to an acceleration of decay of the free analyte atoms or due to a decrease in the level of hydrogen radical population. By use of a specially designed atomiser the interferences were reduced to a level 2 - 3 orders of magnitude lower than that of flameless electrically heated quartz tube atomisers.

2.5.2.2.3 Externally heated quartz tubes

Interferences in externally heated quartz tubes are similar to those obtained in flame-in-tube atomisers. Thus radical population and analyte decay interferences are expected. The radical population interferences are generally much higher in

externally heated quartz tubes than in small flame-in-tube atomisers because of the very low oxygen supply which can only produce a small population of hydrogen radicals. The analyte decay interference can be more severe because externally heated quartz tubes are generally larger than the flame-in-tube atomisers.

There are contrasting views as to the cause of interferences in externally heated quartz tubes. Dittrich and Mandry[64] conclude the interferences are due to the formation of stable diatomic molecules e.g. AsSe. Welz and Melcher[46] suggest the interferences of arsenic and selenium are due to competition of their hydrides for free radicals in the atomiser.

The magnitude of the interferences depends on the supply of oxygen, temperature of the atomiser, generation procedures and design of atomiser. Welz[27] suggests that interferences inside the atomiser can be caused only by containments that are first volatilised after reacting with sodium tetraborohydride and then carried to the heated quartz tube. Such interferences can be avoided if the interferents are not allowed reach the atomiser. One way to achieve this is to add to the sample solution a transition metal ion at a concentration that prevents the evolution of the interfering hydride from solution but does not effect the analyte hydride this may be achieved by adding a certain concentration of copper to a solution of selenium and arsenic. The copper suppresses the interference of selenium when determining arsenic.

Agterdenbos et al.[65] found that enough oxygen is present, to produce hydrogen radicals used in atomisation of hydrides, in the nitrogen, used as a carrier gas, and the sample solution provided the temperature is over 700 °C and the residence time of the gas in the absorption cuvette is not too short. The addition of more than the minimum required amount of oxygen hardly influences the

signal. However if the amount of oxygen added is approximately equivalent to or higher than the amount of hydrogen generated by the decomposition of sodium tetraborohydride, the absorption sharply drops to zero. Parisis and Heyndrickx[45] found that oxygen has an effect on the determination of volatile forming elements, not only at low quartz temperatures, but also at temperatures above 800 °C, possibly by accelerating the production of radicals that may take part in the atomisation of the hydrides.

Agterdenbos et al.[65] found that by decreasing the internal diameter of the absorption cuvette from 8 to 4 mm the signal found at high carrier gas flow rates was as expected from the difference in cuvette diameters. A low flow rate decreased the signal, probably due to dimerisation ($\text{Se} \rightarrow \text{Se}_2$) as the decrease is much greater than that which would be obtained from a difference in cuvette diameters. The results are explained by the assumption that the dimerisation reaction is favoured by the quartz wall but the atomisation reaction is not.

Welz and Melcher[41] studied the influence of the quartz cell surface on the sensitivity. It was found that rinsing in 40 % hydrofluoric acid for approximately 15 minutes is effective in obtaining optimum sensitivity immediately for each hydride forming element. Inserting a small untreated quartz tube into the heated quartz cuvette, conditioned with hydrofluoric acid, resulted in 75 % decrease in an arsenic signal. Inserting the same quartz tube after it was treated with hydrofluoric acid had no influence and gave the full sensitivity for arsenic.

2.6 CONCLUSION

Hydride generation AAS is a fast, sensitive and convenient technique, but like all techniques in analytical science it is not without its interferences. Optimisation of a hydride generation system depends on the analyte being determined. Various factors come into play such as valence of the analyte, acidity of the solution, amount of reducing agent, the need for masking/releasing agents.

The various methods of hydride generation have their advantages and their limitations. Direct mode which is simple and fast includes continuous flow, flow injection (both are easily automated) and batch (better sensitivity but it does not lend itself to automation). Collection mode which has better sensitivity than batch mode, but is limited with regard to unstable hydrides, includes pressure and cold trap methods.

Atomisation techniques include, diffuse flame which is not used much today and graphite furnace. With the introduction of graphite paper atomisation, higher temperatures and a decrease in interferences can be achieved using this technique. Flame-in-tube and externally heated quartz tube techniques basically have the same mechanism of atomisation but externally heated quartz tubes are by far the most common mode of atomisation used because the technique is simple and sensitive.

Interferences are a problem in hydride generation. Transition metal interferences are the most serious as they cause decreases in efficiency. Interferences from other hydride forming elements are also common. There are also differences in sensitivity between different oxidation states of the analyte, although this has the advantage that it can be used for their selective determination. Various methods are available to overcome these interferences and these include: changes in

acidity, control of reducing agent, use of masking or releasing agents, precipitation reactions, design of atomiser, control of temperature and oxygen content and use of ion exchange resins for separation of the analyte from interferences.

Hydride generation can be used to determine only a few elements, but these are of great importance and have to be determined frequently at low levels in many samples. Hydride generation offers high sensitivity, low cost, reliability, high speed, convenience and simplicity. With a greater understanding of interferences and the incorporation of automated instrumentation hydride generation has become a popular technique.

2.7 DETERMINATION OF ARSENIC IN COAL BY FLOW INJECTION HYDRIDE GENERATION ATOMIC ABSORPTION SPECTROMETRY

2.7.1 Introduction

The accurate determination of arsenic is important because of the toxicity of this element and its wide distribution in different forms in the environment. Many analytical techniques are available to detect arsenic in environmental samples[66 - 72]. Flame AAS is still a popular method for arsenic analysis. The conventional atomic absorption method of aspirating an arsenic solution into an air-acetylene flame has limited effectiveness due to the inherent insensitivity of the technique. A significant increase in analytical sensitivity is obtained when arsenic is analysed by flame AAS following conversion to the volatile arsine, a technique known as hydride generation AAS[2]. Hydride generation AAS has become well established for the determination of several hydride forming elements because of its high sensitivity and simplicity. The technique allows the selective separation of the analyte from the matrix enabling most interfering species to be avoided and pre-concentration of the analyte to occur.

The majority of work performed previously with the hydride generation technique used batch systems. Although these systems offer the required precision and sensitivity, many of them have to be dismantled after a single analysis and therefore require considerable operator manipulation. The use of continuous flow methods in combination with the technique reduces the need for this requirement and allows greater sample throughput to be achieved. Continuous flow techniques, however, suffer from the disadvantage that a large volume of sample solution is required for analysis[73].

In this study, a flow injection hydride generation system was first optimised for four separate arsenic species i.e. arsenite, arsenate, MMA and DMA, and then

applied to the determination of total arsenic in coal. The method described here using a flow injection method for sample introduction offers the advantage of the reproducible use of small sample volumes and the ability to achieve rapid sample throughput. The method also maintains acceptable detection limits and levels of precision.

The accuracy of the technique was tested by participation in an inter-laboratory comparison with the BCR programme of the Commission of the European Communities.

2.7.2 Experimental

2.7.2.1 Reagents

Unless otherwise stated, all reagents were of analytical reagent grade. Deionised water was obtained by passing distilled water through a Millipore Milli-Q water purification system. All glassware was washed for four hours in 10 % nitric acid, rinsed and soaked in deionised water until used.

Standards of arsenite, arsenate, MMA and DMA were obtained as part of the BCR programme on arsenic speciation. The arsenite standard was received as arsenic trioxide (As_2O_3) along with a procedure for dissolution (sodium hydroxide), as arsenite in solution may be subject to oxidation. 50 mg of arsenite was dissolved in sodium hydroxide (4 %) and made up to 50 cm^3 to give a 1 mg ml^{-1} solution of arsenite (As_2O_3). The solution was stored at 4°C in the dark.

For the hydride generation studies, stock solutions 1000 ng ml^{-1} of arsenite (As_2O_3), arsenate (as As_2O_5), MMA and DMA (obtained as part of the BCR programme on arsenic speciation), were prepared daily, by diluting a standard solution containing 1 mg ml^{-1} of the appropriate arsenic compound. For arsenite

and arsenate, aliquots were diluted with 1 M hydrochloric acid, and for DMA and MMA aliquots were diluted with 0.5 M hydrochloric acid, to obtain appropriate working reference solutions for calibration. A 100 ng ml⁻¹ standard was used for arsenite, MMA and DMA and a 200 ng ml⁻¹ standard was used for arsenate for optimisation work. Sodium tetrahydroborate solution (1 % m/v) was prepared by dissolving sodium tetrahydroborate powder in 1 % sodium hydroxide solution. The solution was prepared fresh each day and filtered before use through a 0.45 µm filter. For initial optimisation of the hydride generation system, arsenite (BDH, Poole, Dorset, UK) was used. Standard anthracite coal was obtained from a local supplier.

2.7.2.2 Equipment

For the hydride generation studies, an Instrumentation (IL) Laboratory Model 357 atomic absorption spectrometer was used with suitable burner modifications to allow a silica atomisation cell to be supported in an air-acetylene flame approximately 5 mm above the slot of a 5 cm single-slot burner. The atomisation cell, consisted of a T-shaped silica tube (150 x 2 mm i.d.).

Before analysis, the atomisation cell was allowed to warm up until the atomisation cell reached equilibrium. The signal from the spectrometer was displayed on a chart recorder (Linseis 650). A 1nm band pass was used and an arsenic hollow-cathode lamp (S&J Juniper and Co, Harlow, Essex, UK) was operated at a lamp current of 8 mA and a wavelength of 193.7 nm. Air and acetylene flow-rates of 8.5 and 1.9 L min⁻¹ respectively were used.

The flow injection system shown in figure 2.2 consisted of a peristaltic pump (Watson-Marlow 501U), a four-way rotary valve (Tecator 5001) with an external loop for the sample injection, a Kel-F mixing T (plasma-Therm, London, UK)

and a gas/liquid separator (Plasma-Therm). A sample loop consisting of Teflon tubing of 1 mm i.d. and a volume of 334 μl was used for analysis. This tubing was also used throughout the flow injection system. The tube lengths from the rotary valve to the T-piece, and from this to the gas/liquid separator were 15 and 6.5 cm, respectively. The sodium tetrahydroborate solution and 1 M hydrochloric acid were pumped at 4.0 and 4.8 ml min^{-1} , respectively.

Sample digestions were carried out in standard Erlenmeyer flasks, which were heated on a hot-plate.

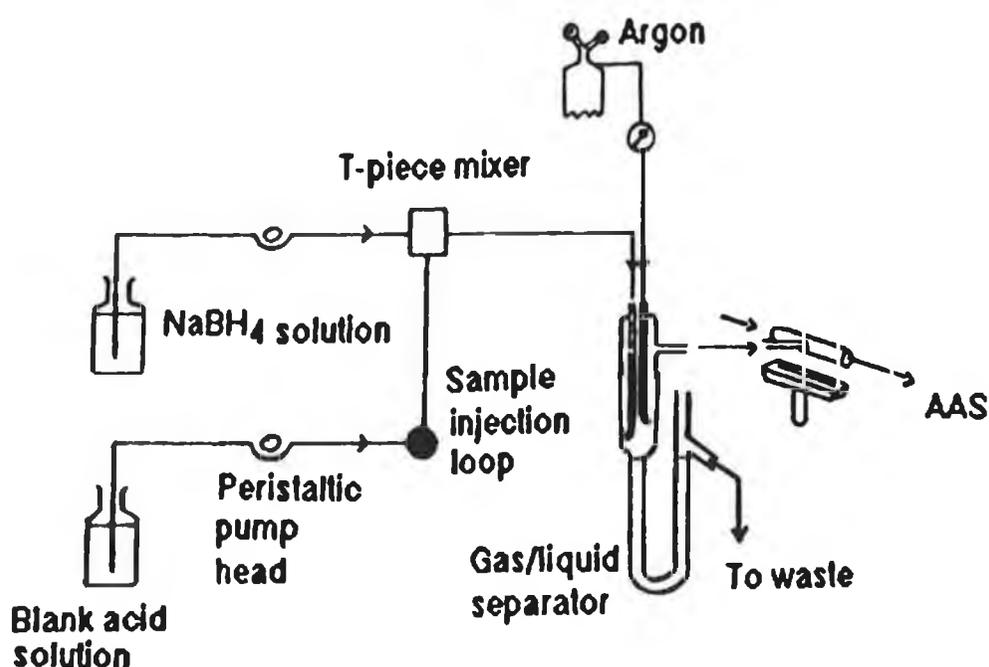


Fig. 2.2 Schematic diagram of the flow injection hydride generation atomic absorption spectrometer system.

2.7.2.3 Methods

In the hydride generation system, reduction of arsenic by sodium tetrahydroborate occurs at the T-piece, and the reaction is completed by the time the flow reaches the gas/liquid separator (figure 2.2). At this point, the liquid products flow via a U-tube to a free running drain, while the gaseous products are purged by argon (99 % purity) into the atomisation cell. In the operation of the system, two sampling cycles are used. In the first, the acid and sodium tetrahydroborate streams are allowed to mix at the T-piece, and the peak height signal is measured over a 20 second integration time and recorded. During this period, the hydrogen generated enables a blank level to be monitored. The second period occurs immediately after the first and involves the injection of the sample via a four-way rotary valve into the acid carrier stream. Once again the peak height signal is measured over a 20 second period and recorded. At the end of this period, the rotary valve is switched back to the injection position and the cycle is repeated. This sequence of events does not include a specific time period for washing the system, as experimentation had shown that, in the period immediately after the analysis, the signal had returned to the baseline. This ensured that the blank level was achieved between each cycle, and that within each cycle, the analyte was measured above the blank level.

2.7.2.4 Digestion procedure

A digestion procedure used by McLaughlin et al.[74] to analyse selenium in blood plasma was used to digest coal. Good recoveries (> 95 %) were obtained. Larger volumes of acid were used in order to digest the coal samples but the ratio between the acids remained the same. A digestion mixture consisting of nitric, sulphuric and perchloric acids (5/2/1 v/v/v) was used. The use of sulphuric acid prevents the digestion flask from drying out, but care must also be taken as its use

increases the risk of charring which may result in the losses of arsenic through volatilisation. Potassium iodide was used to ensure all the arsenic was in the +3 oxidation state as the +5 oxidation state gives a lower response in hydride generation AAS.

In the digestion procedure, a 0.4g sample of ground coal was measured accurately into a 100 ml Erlenmeyer flask and 10 ml of 16 M nitric acid were added. Subsequently, two pre-cleaned glass beads were added to the flask and the mixture was placed on a hot-plate. The temperature was raised over a 20 minute period to 120 °C and maintained at this temperature for 20 minutes. The flask was cooled, 5 ml of 18 M sulphuric acid and 2 ml of 11.6 M perchloric acid were added, and the temperature was slowly raised again to 120 °C and maintained at this temperature for 15 minutes. The temperature was then raised over a further 15 min period to 205 °C and maintained at this temperature until white fumes of perchloric acid were evident. The flask was cooled, 10 ml of 5 M hydrochloric acid were added and the mixture was heated to 95 °C for 30 minutes. After cooling, the contents of the flask were transferred to a 50 ml volumetric flask and 20 ml of 40 % (v/v) potassium iodide added. The contents were diluted to 50 ml and 334 µl aliquots were taken for arsenic measurements.

2.7.3 Results and discussion

2.7.3.1 Effect of carrier gas flow rate

In addition to transporting the hydrogen arsenide to the atomisation cell, the carrier gas also expels any air present in the system, hence allowing precise measurements to be made in the far ultraviolet region[75]. In this study, the carrier gas flow rate was varied over the range 0.2 - 1.4 L min⁻¹ (figure 2.3.1, 2.3.2, 2.3.3 and 2.3.4) for the analysis of arsenite, arsenate, DMA and MMA. It

was found that the sensitivity increased as the flow rate of the carrier gas was decreased for all four analytes. At flow rates less than 0.6 L min^{-1} the signal did not return to baseline within a 20 second integration time. 0.6 L min^{-1} was chosen as the optimum flow rate for this work as it gives good sensitivity while the signal returns to baseline within a 20 second integration time. To achieve higher sensitivity, a lower flow rate could have been chosen but the analysis time would have been increased.

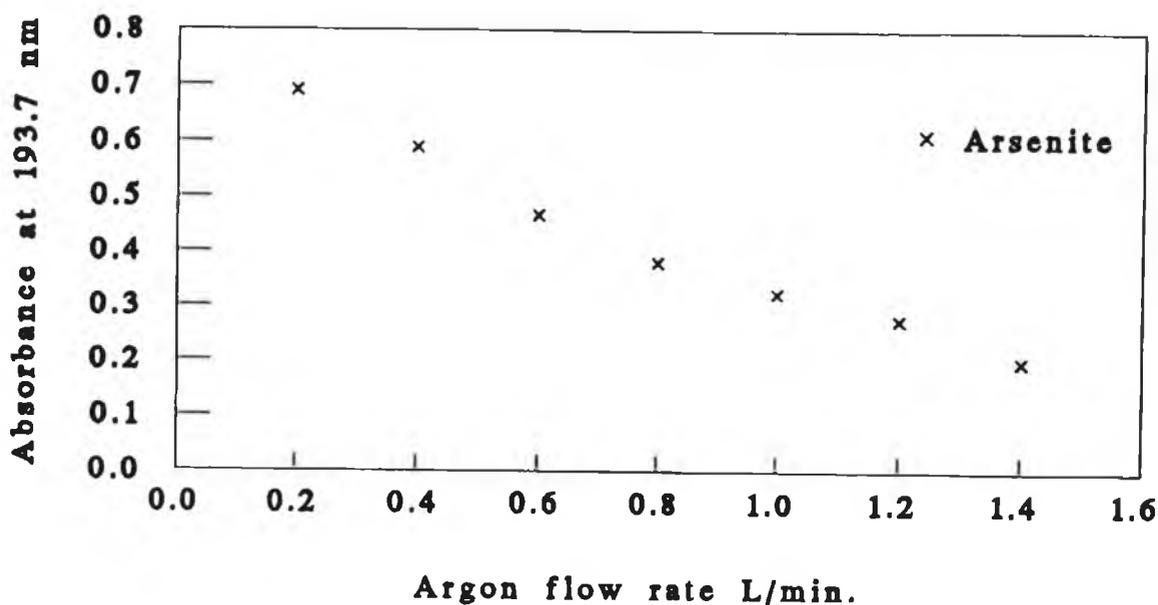


Fig. 2.3.1. Effect of carrier gas flow rate on 100 ppb arsenite using 1 % NaBH_4 and 1 M HCl.

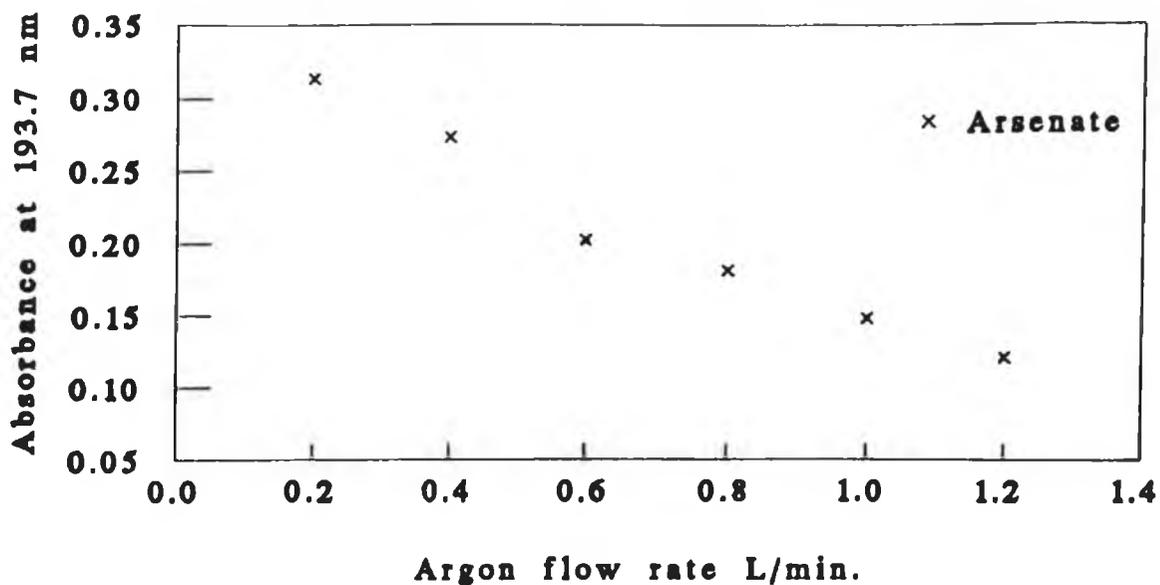


Fig. 2.3.2. Effect of carrier gas flow rate on 200 ppb arsenate (as As_2O_5) using 1 % NaBH_4 and 1 M HCl .

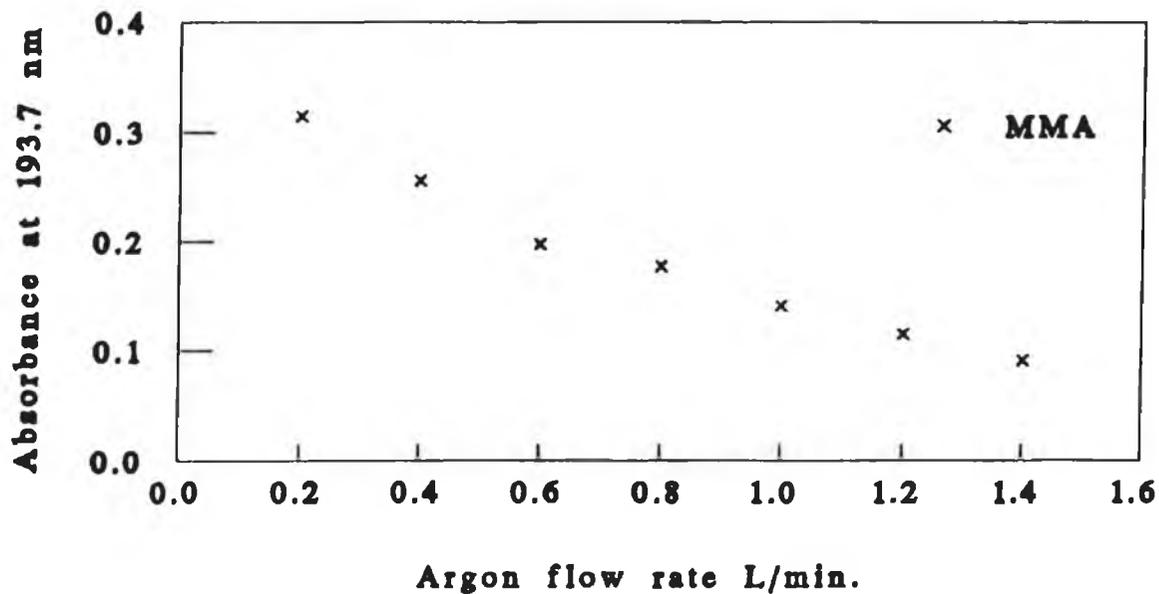


Fig. 2.3.3. Effect of carrier gas flow rate on 100 ppb monomethylarsonic acid (MMA) using 1 % NaBH_4 and 0.5 M HCl .

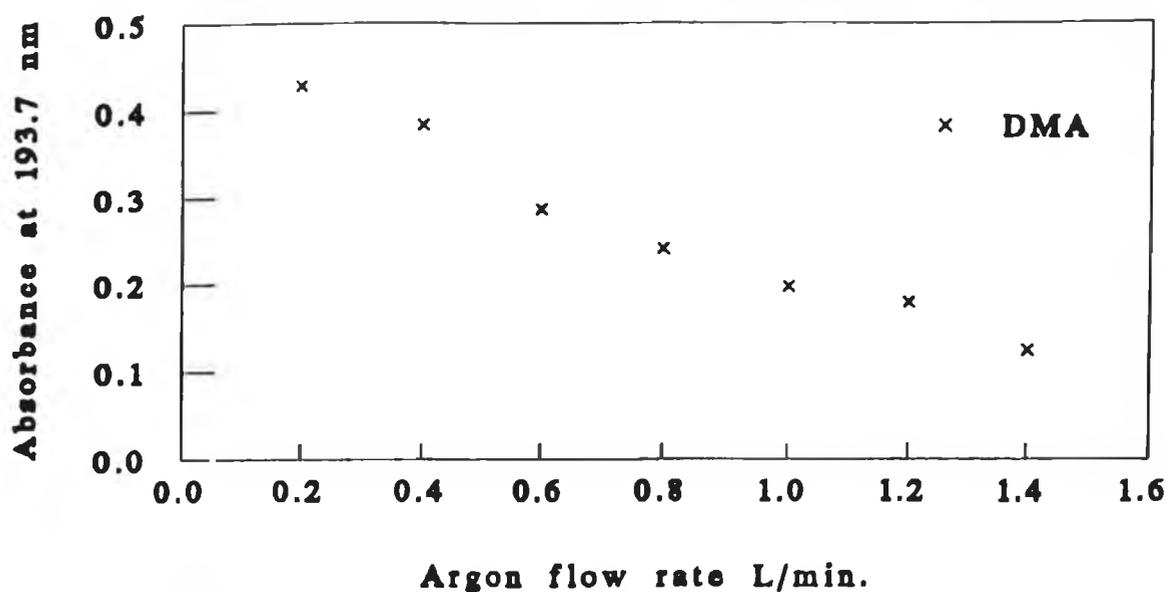


Fig. 2.3.4. Effect of carrier gas flow rate on 100 ppb dimethylarsinic acid (DMA) using 1 % NaBH_4 and 0.5 M HCl .

2.7.3.2 Effect of acid concentration

The effect of the acid concentration on the sensitivity of arsenite, arsenate, MMA and DMA is shown in figure 2.4.1, 2.4.2, 2.4.3 and 2.4.4 respectively. It was decided to use 1 M acid for arsenite and arsenate analysis, although not the optimum for arsenate, it does give adequate sensitivity. Higher acid concentrations are unsuitable for use with the apparatus employed due to its corrosive properties. The maximum sensitivity for MMA and DMA was obtained using 0.5 M hydrochloric acid.

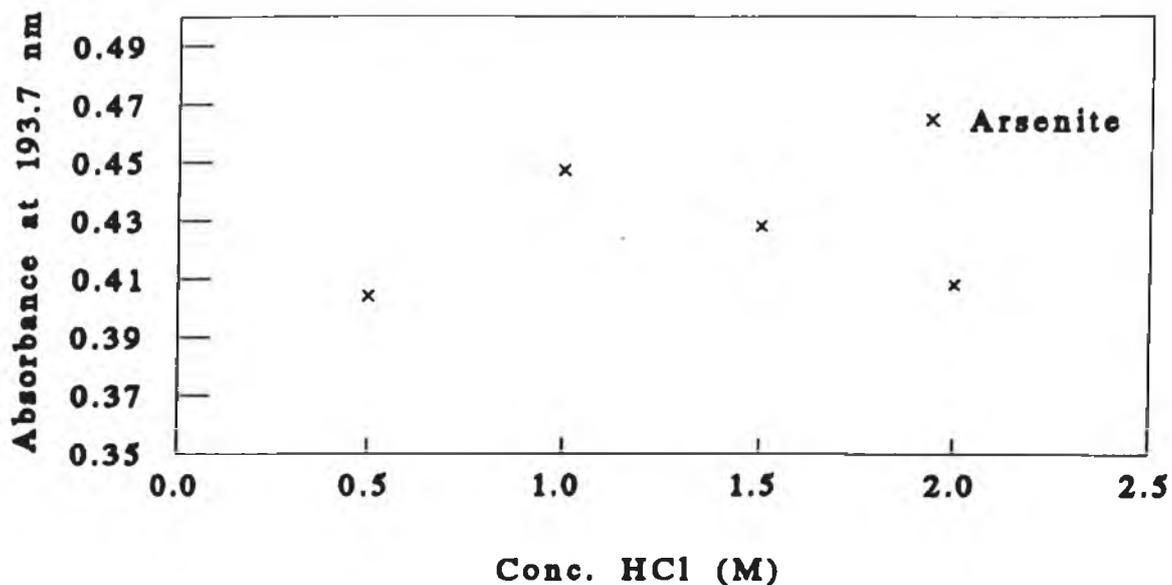


Fig. 2.4.1. Effect of acid concentration on 100 ppb arsenite using 1 % NaBH₄ and 0.6 L/min. argon.

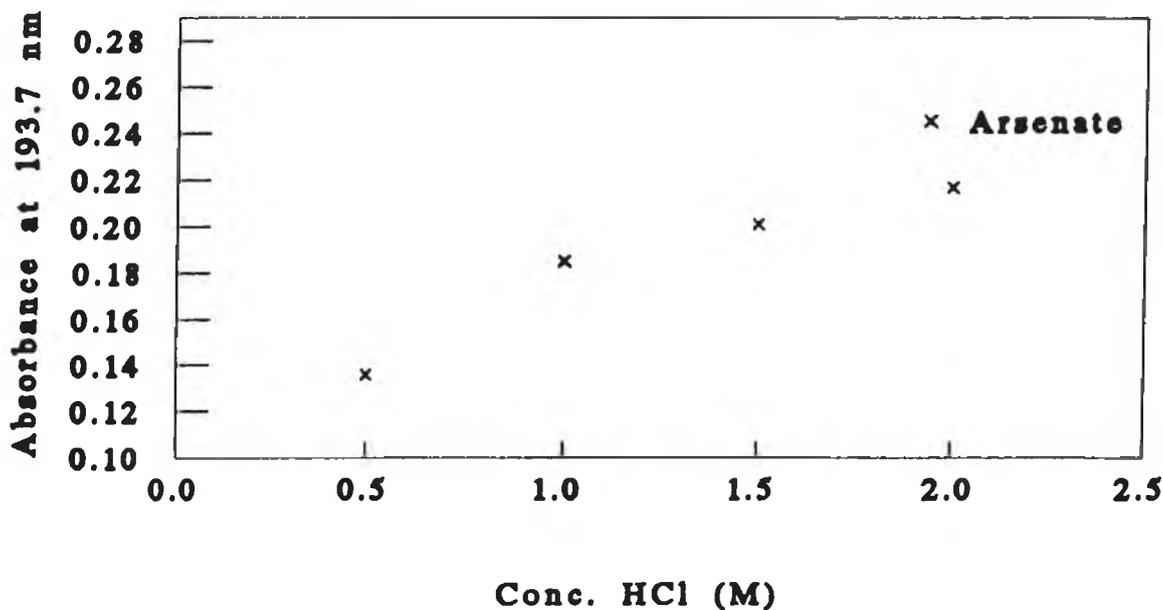


Fig. 2.4.2. Effect of acid concentration on 200 ppb arsenate (as As₂O₅) using 1 % NaBH₄ and 0.6 L/min. argon.

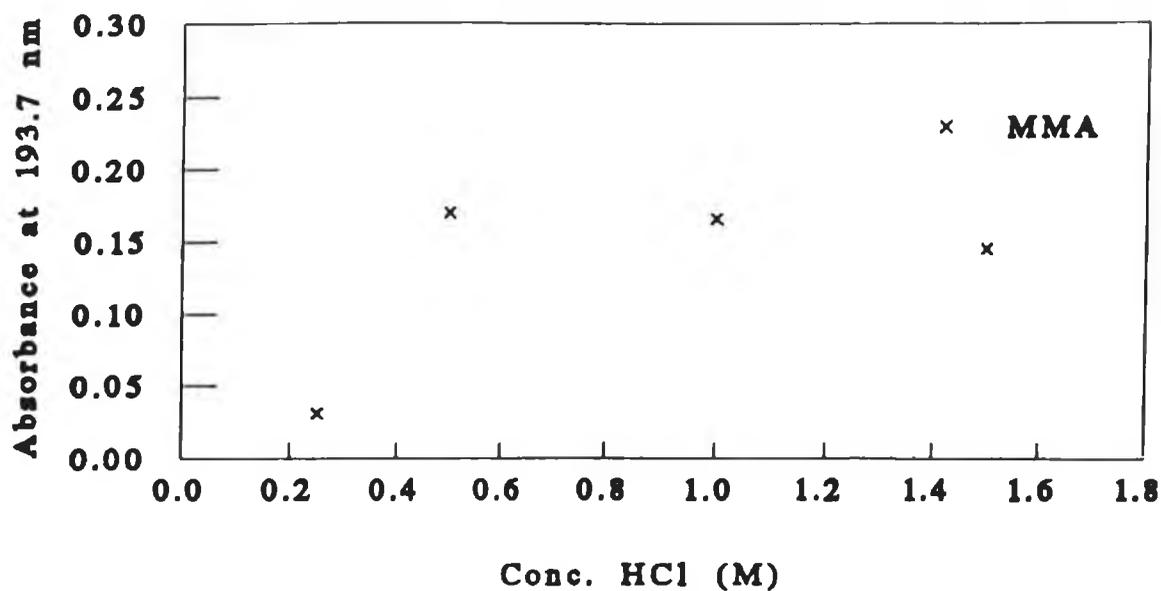


Fig. 2.4.3. Effect of acid concentration on 100 ppb monomethylarsonic acid (MMA) using 1 % NaBH₄ and 0.6 L/min. argon.

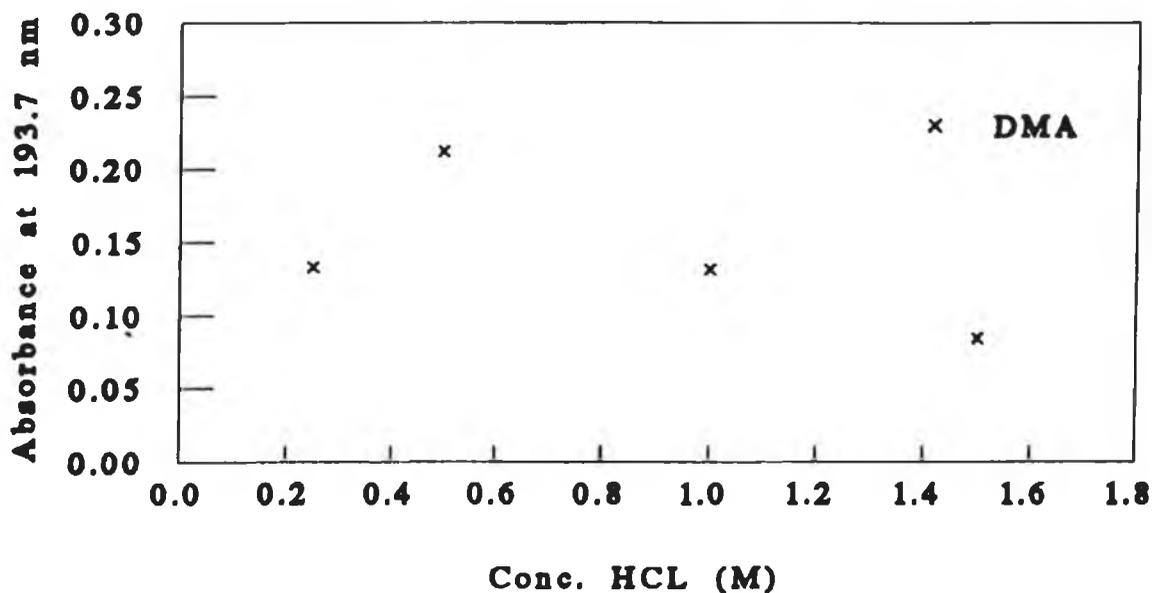


Fig. 2.4.4. Effect of acid concentration on 100 ppb dimethylarsonic acid (DMA) using 1 % NaBH₄ and 0.6 L/min. argon.

2.7.3.3 Effect of sodium tetrahydroborate concentration

The effect of the concentration of sodium tetrahydroborate on the sensitivity of arsenite, arsenate, MMA and DMA analysis is shown in figure 2.5.1, 2.5.2, 2.5.3 and 2.5.4 respectively. A concentration of 1 % (m/v) was chosen for further work. At higher concentrations the reaction between sodium tetrahydroborate and the acid is more vigorous which resulted in a loss of reproducibility making such concentrations unsuitable for use. At lower concentrations the sensitivity was reduced in each case.

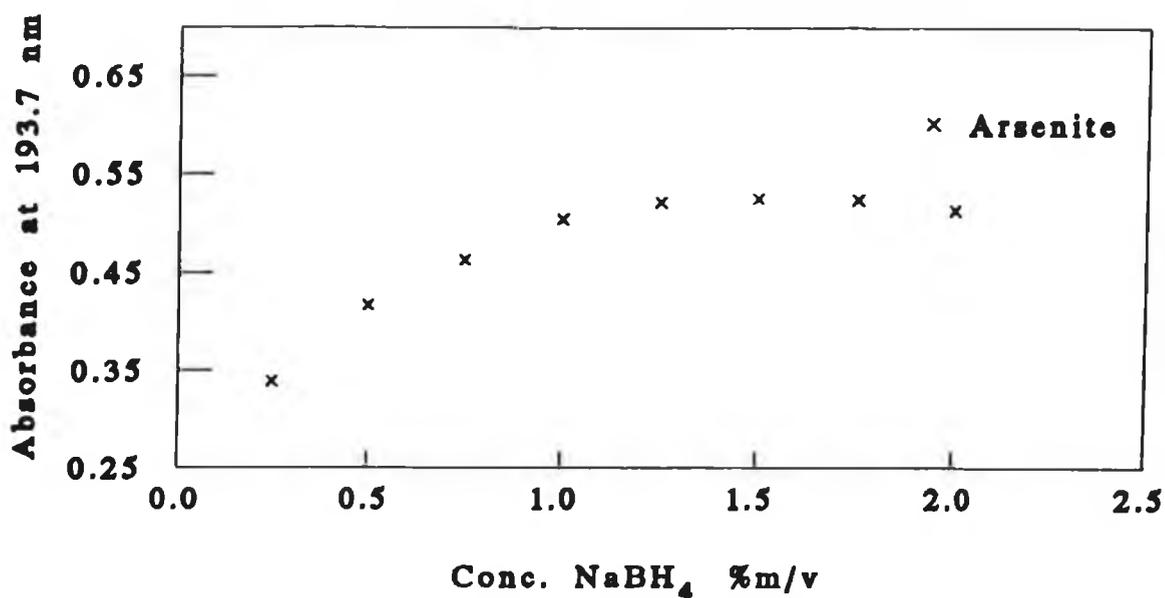


Fig. 2.5.1. Effect of NaBH₄ on 100 ppb arsenite using 1 M HCl and 0.6 L/min. argon.

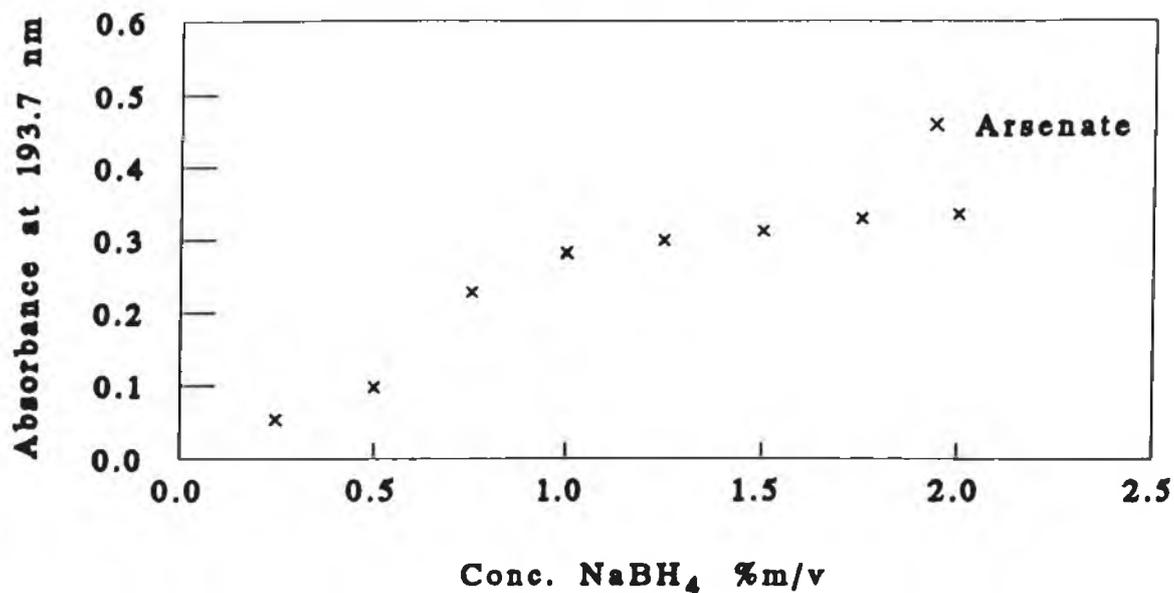


Fig. 2.5.2. Effect of NaBH₄ on 200 ppb arsenate (as As₂O₅) using 1 M HCl and 0.6 L/min. argon.

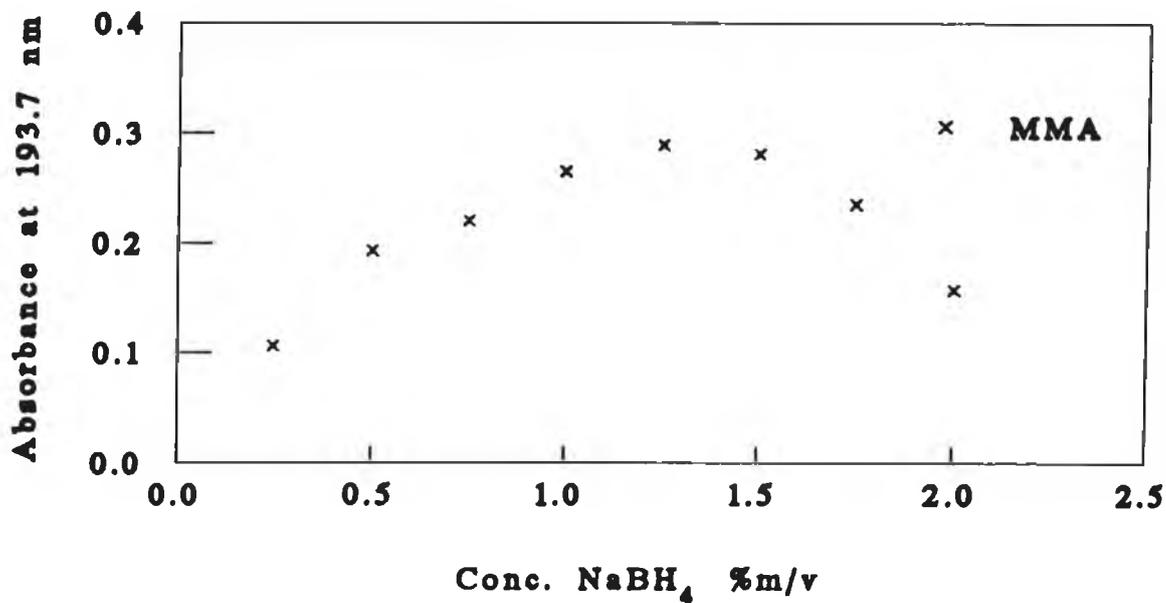


Fig. 2.5.3. Effect of NaBH₄ on 100 ppb monomethylarsonic acid (MMA) using 0.5 M HCl and 0.6 L/min. argon.

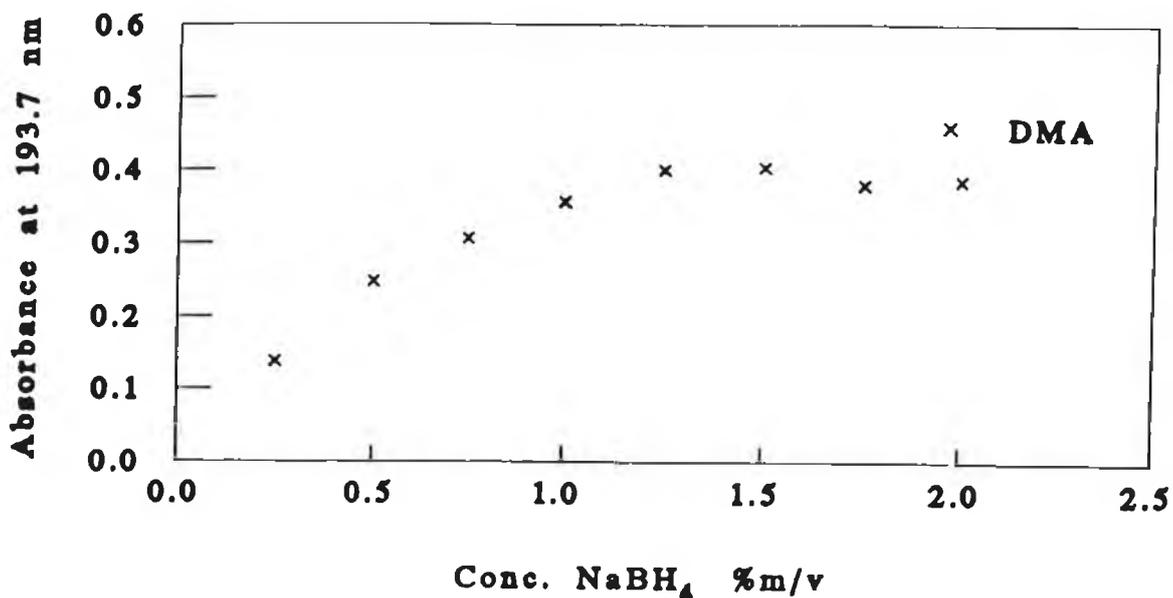


Fig. 2.5.4. Effect of NaBH₄ on 100 ppb dimethylarsinic acid (DMA) using 0.5 M HCl and 0.6 L/min argon.

2.7.3.4 Effect of oxidant/fuel ratio

By varying the oxidant/fuel ratio the atomisation cell temperature was varied. The oxidant/fuel ratio was varied between 3.75 and 5.00 (table 2.1) by varying the inlet gauges. At higher ratios there was noise in the system due to the oxidant ratio being too high. At lower ratios the fuel level was very low and the flame was found to quench. No significant difference was found using other oxidant/fuel ratios so it was decided to continue using a ratio of 4.50 for all further work. This was equivalent to using air and acetylene flow rates of 8.5 and 1.9 L min⁻¹ respectively.

Oxidant/fuel ratio	Absorbance
3.75	Flame quenching
4.00	0.431
4.25	0.425
4.50	0.432
4.75	0.429
5.00	High background noise

Table 2.1 Effect of oxidant/fuel ratio.

2.7.3.5 Precision and accuracy

To assess the validity of the proposed method, the detection limit, sensitivity, within-run and between-run precisions and recovery were calculated.

The detection limit for arsenite, arsenate, MMA and DMA, defined as twice the standard deviation of the blank measurements, and the sensitivity, defined as the concentration equivalent to an absorption of 0.0044 units, are shown in table 2.2.

Species	Limit of Detection (ng/ml)	Sensitivity (ng/ml)
Arsenite	6.2	1.1
Arsenate	15.3	3.5
MMA	15.9	3.0
DMA	16.0	3.3

Table 2.2 Limit of detection and sensitivity for arsenite, arsenate, MMA and DMA using flow injection hydride generation AAS.

Calibration curves are shown in figure 2.6.1, 2.6.2, 2.6.3 and 2.6.4 for arsenite, arsenate, MMA and DMA respectively.

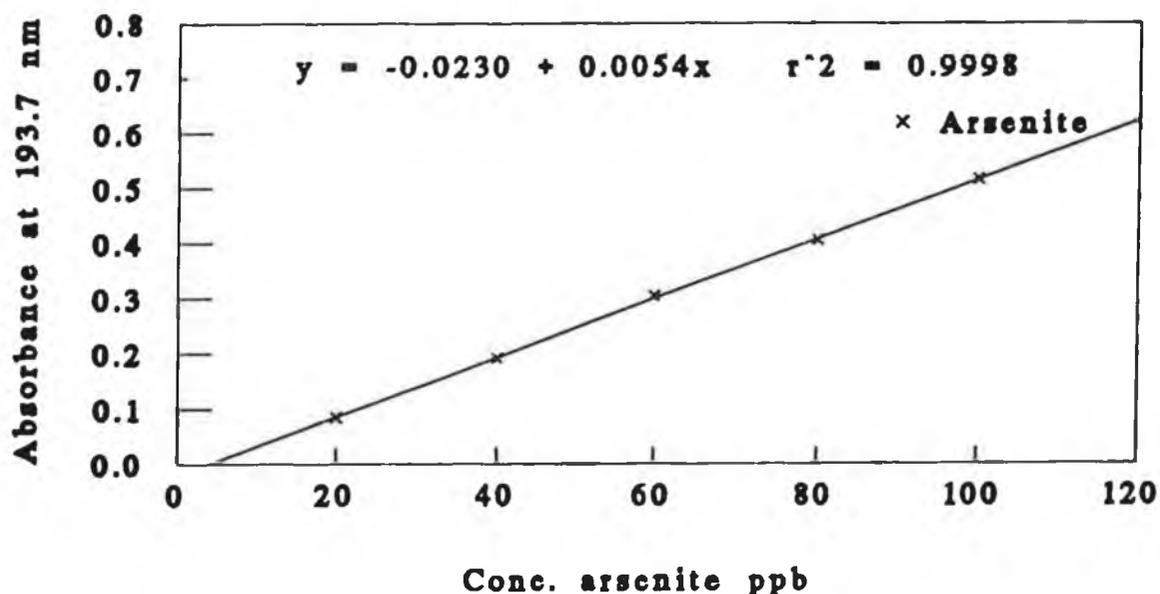


Fig. 2.6.1. Calibration graph of arsenite using 1 M HCl, 1 % NaBH₄ and 0.6 L/min. argon.

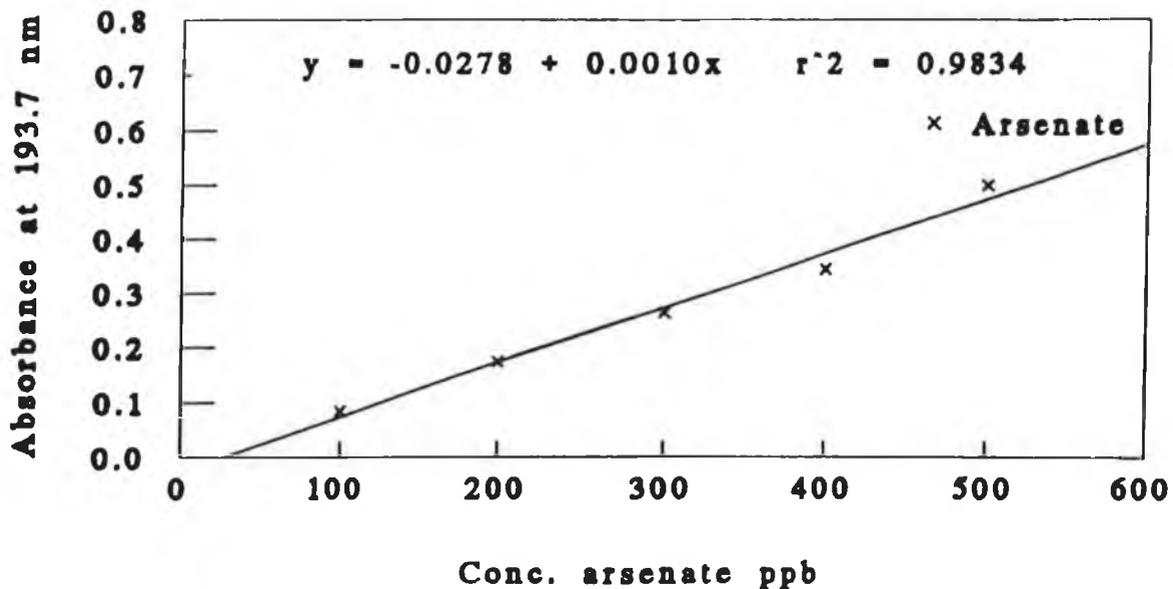


Fig. 2.6.2. Calibration graph of arsenate (as As_2O_5) using 1 M HCl, 1 % NaBH_4 and 0.6 L/min. argon.

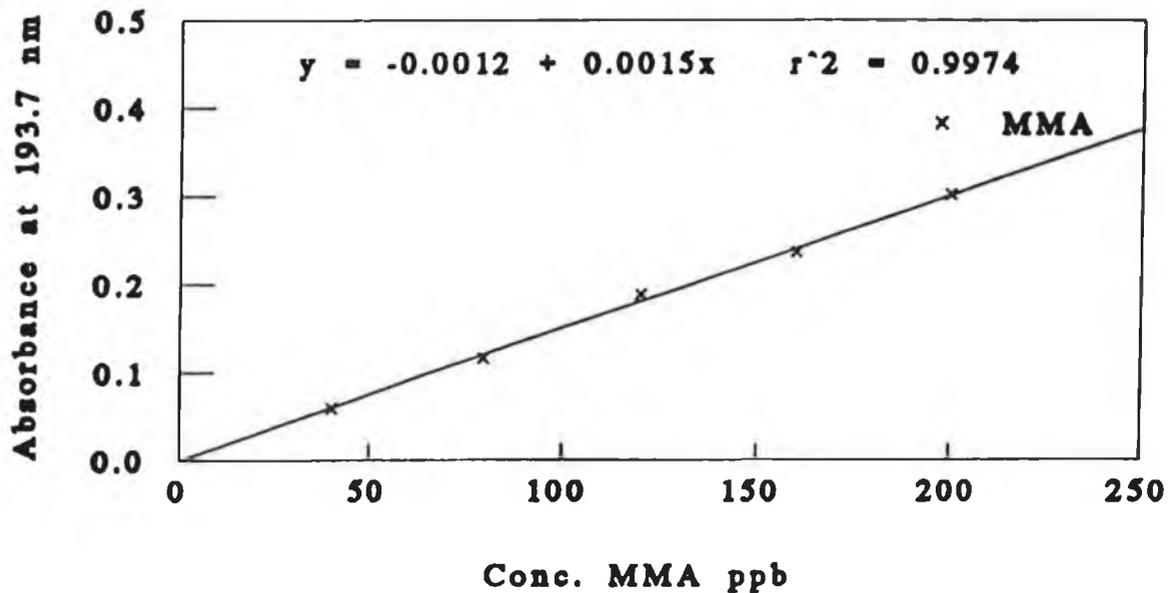


Fig. 2.6.3. Calibration graph of monomethylarsonic acid (MMA) using 0.5 M HCl, 1 % NaBH_4 and 0.6 L/min. argon.

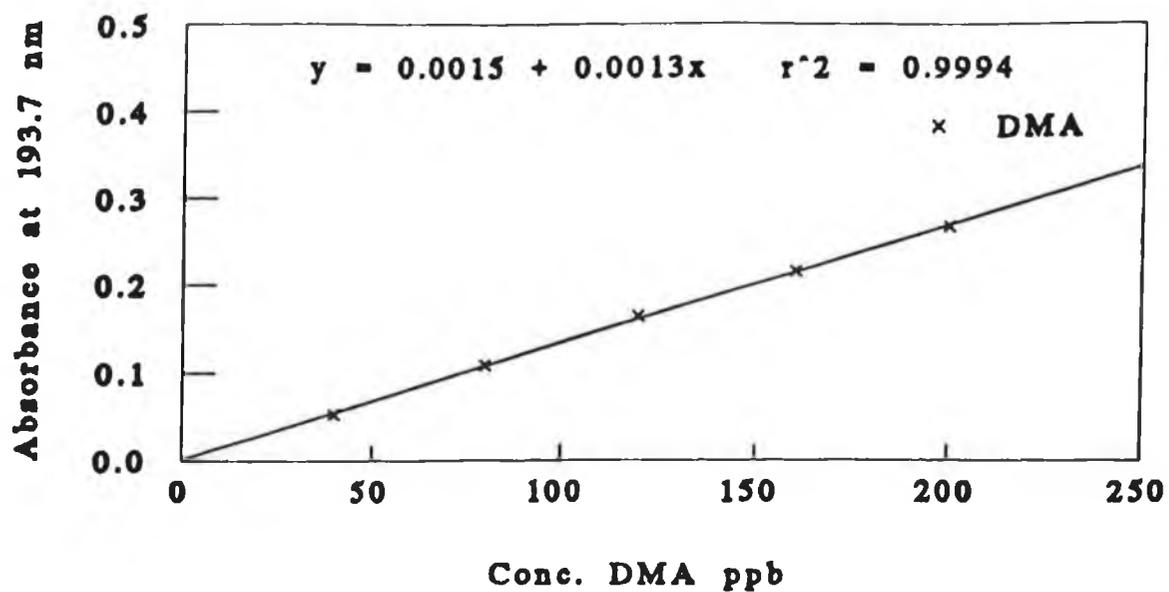


Fig. 2.6.4. Calibration graph of dimethylarsinic acid (DMA) using 0.5 M HCl, 1 % NaBH₄ and 0.6 L/min. argon.

The within-run and between-run precision results are shown in table 2.3. As can be seen from the results the method is very reproducible for all four species analysed.

Species	Precision				
Arsenite	Conc. ppb	20	40	60	80
	Within-run (n = 6)	3.9%	2.3%	1.3%	1.0%
	Between-run (n = 4)	3.7%	1.5%	1.6%	0.6%
Arsenate	Conc. ppb	100	150	200	250
	Within-run (n = 6)	3.0%	3.1%	1.1%	3.3%
	Between-run (n=4)	4.0%	1.5%	3.1%	1.3%
MMA	Conc. ppb	50	80	100	150
	Within-run (n = 6)	2.7%	1.5%	2.2%	1.0%
	Between-run (n = 4)	4.0%	1.7%	2.0%	1.7%
DMA	Conc. ppb	50	80	100	150
	Within-run (n = 6)	2.7%	1.6%	1.1%	2.5%
	Between-run (n = 4)	0.8%	4.2%	4.1%	3.9%

Table 2.3 Within-run and between-run precision.

The method was validated further by participation in an inter-laboratory comparison with the Commission of the European Communities. This involved the determination of arsenite (as As_2O_3), arsenate (as As_2O_5), MMA and DMA in unknown samples (table 2.4). Results showed excellent agreement with the other participating laboratories for the analysis of all four species.

Arsenic	Sample No.	Target value (ppm)	Conc. found (ppm) (n = 5)	Std. dev.
Arsenite	1	1.00	1.04	0.03
Arsenate	2	12.50	12.63	0.24
MMA	3	7.50	7.29	0.14
DMA	4	10.00	10.30	0.12

Table 2.4 Inter-laboratory comparison on arsenic part one.

The flow injection technique was applied to the determination of arsenic in coal. Assessment of the recovery of arsenic during the digestion method was made by a comparison with digested arsenic standards. Recoveries ranged from 96 to 109 % indicating that the digestion procedure used was efficient. Standard anthracite coal was digested using the procedure outlined and on average ($n = 11$) was found to contain 3.59 mg Kg^{-1} arsenic with a coefficient of variation of 5.0 %.

2.7.4 Conclusion

The detection limit of 6.2 ng ml^{-1} (arsenite) for the flow injection method described here compares favourably with other systems used[76, 75]. This could be further lowered using lower carrier gas flow rates and larger injection volumes, although analysis time would be increased.

When optimising the working conditions using arsenite, the pattern obtained for the variation of sodium tetrahydroborate concentration was similar to that found by Yamamoto M. et al.[75], who also used flow injection, with the sensitivity increasing rapidly initially and then remaining almost constant.

By varying the hydrochloric acid concentration Anderson et al.[17] found that the response for the arsenite increased rapidly up to 1 M hydrochloric acid and then remained constant. A similar pattern was found in this research up to a concentration of 1 M hydrochloric acid when analysing arsenite but above this concentration the response started to decrease rather than remain constant. The variation of the hydrochloric acid concentration when analysing MMA and DMA gave rise to similar patterns to those found by Anderson et al.[17]. with the optimum sensitivity reached at 0.5 M in each case.

The effect of carrier gas flow-rate on sensitivity, found by Yamamoto et al.[75] was similar to that found in this research where a loss in sensitivity was noted at higher gas flow rates. Increased sensitivity could be achieved at low flow rates but with an accompanying increase in analysis time.

Fugita and Takada[76] varied the quartz tube temperature by varying the acetylene/air flow-rates. They found the sensitivity did not change dramatically and the maximum sensitivity was reached at 860 °C. In the system used here the actual temperature was not measured but the oxidant/fuel ratio was varied. There was little or no difference found in sensitivity over the range investigated but an optimum of 8.5 L min⁻¹ air and 1.9 L min⁻¹ acetylene (ratio of 4.50) was used.

Due to differences in matrix not all the calibration graphs go through the origin. A sampling rate of 90 injections per hour can be achieved using this system. This is a significant improvement over a direct method of analysis and allows for rapid analysis of liquid samples. The analysis time is reduced even in the analysis of coal, where the majority of time is taken up by the digestion procedure (4 hours).

The flow injection system described here allows a rapid and economical analysis to be carried out. It is easily assembled and requires minimum operator manipulation and expertise. It offers an alternative, precise and sensitive approach for the trace determination of arsenic species if present in a single form.

This flow injection technique is suitable for the determination of total arsenic present in complex matrices but is not suitable for the analysis of individual species in the presence of each other. To determine the actual forms of arsenic present rather than total arsenic a separation technique coupled with the flow injection hydride system is required. The combination of HPLC with hydride generation AAS overcomes such a problem. This approach will be discussed in the next chapter.

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CHAPTER 3

Separation of arsenic species by HPLC and preconcentration of arsenate and MMA using column switching HPLC.

3.1 SEPARATION OF ARSENIC SPECIES BY HPLC

3.1.1 Introduction

A variety of techniques have been used for the speciation of arsenic. Gas chromatography[1], spectrophotometry[2], voltammetry[3], and selective hydride generation AAS[4] have been demonstrated as effective techniques for the analysis of one or more arsenicals. So far the most successful separation of arsenic species has been by HPLC using ion exchange columns[5 - 9]. Arsenite, arsenate, MMA and DMA are the four arsenic species most commonly found in the environment which are of concern. HPLC provides a simple and efficient technique for their separation compared to the other techniques mentioned above.

Ebdon et al.[5] determined arsenite, arsenate, MMA and DMA using an ODS 3 - 5 μm column with 1.8×10^{-10} M sulphuric acid as the eluent. Due to the uncertainty in reproducibility a resin-based strong anion exchange (SAX) 5 μm column with ammonium carbonate (0.1 M) as the eluent was subsequently used. However, arsenite was not retained. The additional use of a precolumn packed with Zipax, a silica-based anion exchange material (40 μm), and a step elution system consisting of 10^{-4} % sulphuric acid - 0.1 M ammonium carbonate resulted in a pre-concentration step. The Zipax also acted as a guard column. Hydride generation flame AAS, flame atomic fluorescence spectrometry and ICP AES were used and compared as detectors. Hydride generation flame AAS and flame atomic fluorescence spectrometry had slightly better limit of detection. Hydride generation flame AAS was preferred due to its simplicity and well understood operation but ICP AES was preferred for multi-element studies. Branch et al.[10] used an ODS 5 μm column connected in series to a 5 μm SAX column. A phosphate buffer was used as the eluent. Low concentrations of phosphate were required to separate arsenite, MMA and DMA but under these conditions arsenate was totally retained. At higher concentrations the arsenate was eluted

but resolution between arsenite, MMA and DMA was lost. A step gradient was required to overcome these problems. However after several weeks, efficiency of the silica based anion exchange column rapidly deteriorated and all reproducibility was lost. Hence the study was switched to a resin-based exchanger.

A column packed with 7 -10 μm anion exchange resin was used with sulphate mobile phase. Sulphate was used instead of a phosphate mobile phase with a view to using ICP MS as the mode of detection as it was felt that phosphate might effect the sampler and skimmer cones. A 0.001 M potassium sulphate, pH 10.5, was used for the column equilibration and on injection the mobile phase was switched to 0.05 M potassium sulphate, pH 10.5. The mobile phase, containing the lower concentration of sulphate, remaining in the connecting tubing enabled the separation of arsenobetaine, DMA, arsenite and MMA and the mobile phase with the high concentration of sulphate then eluted the arsenate. The method gave good reproducibility and rapid separation. Using ICP-MS for detection , limits of 5 - 10 ng ml^{-1} for each species of arsenic were obtained.

Tye et al.[6] used a SAX resin 5 μm column to separate arsenic species. A Zipax ion exchange resin, 40 μm acted as a guard column and enabled pre-concentration of arsenate, MMA and DMA. Step elution with sulphuric acid 10⁻⁴ % and ammonium carbonate 0.1 M was carried out. Arsenite, arsenate, MMA and DMA were separated and detected by hydride generation flame AAS. Arsenite was not retained. Hydride generation flame AAS was used as the detector and limits of 2 ng of arsenic for arsenite, arsenate and MMA and 1 ng for DMA were achieved. Spall et al.[7] used a column of anion exchange resin of 15 μm diameter with linear gradient elution from water to 0.5 M ammonium carbonate to separate arsenite, arsenate, MMA and DMA from neutral arsenic containing compounds. ICP AES was used as detection. Detection limits ranged from 60 to 400 ng per injection depending on the species. The retention order of

the compounds was as anticipated with the singly charged arsenite and DMA eluting before the doubly charged MMA and arsenate.

Chana and Smith[8] used a SAX 10 μm column and phosphate buffer as the eluent, pH 6.2 - 6.3 for the four arsenic species. Hydride generation AAS was used as detection. There was some loss in resolution between DMA and arsenate after approximately 400 injections but by decreasing the buffer concentration to 0.025 M from 0.03 M the life of the column was extended without any significant loss in sensitivity. Rauret et al.[9] used a silica-based anion exchange column with 5 μm packing with phosphate buffer 0.05 M, pH 6.75, as the eluent to separate arsenite, arsenate, MMA and DMA. Again loss in resolution appeared with time and could be retrieved by diluting the buffer. The silica columns lifetime was limited to approximately 150 injections. Hydride generation ICP AES was used for detection.

Cation exchange columns have also been used for the separation of arsenic species. Morita et al.[11] used a cation exchange column on which, arsenite and arsenate were found to elute together followed by MMA, DMA and arsenobetaine when using a phosphate buffer mobile phase. Morita et al.[11] also used an anion exchange column with a phosphate buffer mobile phase and separated arsenite, arsenate, MMA, DMA and arsenobetaine with ICP AES detection. They found that this method was better as all five species were separated. Pacey and Ford[12] used anion and cation columns for the separation of arsenic species. The cation column was used to isolate DMA using acetic acid as the mobile phase while the other arsenic species passed through the column. The DMA is strongly bound to the cation exchange column probably as $(\text{CH}_3)_2\text{AsO}_2\text{H}\text{NH}_4^+$ in this case. The DMA was then eluted with 1 M ammonia solution and detected by graphite furnace AAS. An anion column was used to retain arsenate and MMA using a 0.01 M ammonium acetate/acetic acid buffer while the arsenite and DMA passed through the column. The arsenate and MMA

were eluted with 0.5 M acetate buffer[12]. Using this method four arsenic species could be analysed but two columns were required and the analysis was not carried out online.

Many different columns have been used for the analysis of arsenic species but in general ODS columns have been shown to have uncertain reproducibility. Anion exchange columns have been found to be more suitable for separation of arsenite, arsenate, MMA and DMA than cation or ODS in this regard. Silica based anion exchange columns deteriorate with loss of reproducibility relatively quickly and therefore have been replaced by resin based anion exchange columns. These seem to give few problems and are usually used with ammonium carbonate or phosphate buffer as the mobile phase. The resolution may deteriorate with continuous use of phosphate buffer but using a lower concentration of the buffer reduces this problem.

The procedure described in this chapter allows the separation of arsenite, arsenate, MMA and DMA by HPLC using an anion column and a phosphate buffer mobile phase and detection of the separated arsenic species was achieved by continuous hydride generation atomic absorption spectrometry.

3.1.2 Experimental

3.1.2.1 Reagents

Unless stated otherwise, all reagents were of analytical reagent grade. Deionised water was obtained by passing distilled water through a Millipore Milli-Q water purification system. All glassware was washed for four hours in 10 % nitric acid, rinsed and soaked in deionised water until used.

Standards of arsenite, arsenate, MMA and DMA were obtained as part of the BCR programme on arsenic speciation. The arsenite standard was received as As_2O_3 (Aldrich). 50 mg of arsenite (as As_2O_3) were dissolved in sodium hydroxide (4 %) and made up to 5 ml to give a 1 mg ml⁻¹ solution of arsenite. Arsenate (as As_2O_5), MMA and DMA standards were received as solutions containing 1 mg ml⁻¹ of the appropriate arsenic compound. All solutions were stored at 4 °C in the dark.

Stock solutions of arsenite (As_2O_3), arsenate (As_2O_5), MMA and DMA of 0.2, 0.5, 0.2 and 0.2 mg ml⁻¹, respectively, were prepared daily, by diluting a standard solution containing 1 mg ml⁻¹ of the appropriate arsenic compound. For all arsenic compounds aliquots were diluted with water to obtain appropriate working reference solutions for the calibration graphs. Concentrated sulphuric acid was diluted to 1 M with purified water. Sodium tetrahydroborate solution (1 % m/v) was prepared by dissolving sodium tetrahydroborate powder in 1 % sodium hydroxide solution. The solution was prepared fresh each day and filtered before use through a 0.45 µm filter. The mobile phase was phosphate buffer, prepared from 0.03 M sodium dihydrogen phosphate, and its pH adjusted to 5.8 with 0.03 M disodium hydrogen phosphate before filtration through a 0.45 µm filter.

3.1.2.2 Equipment

For the hydride generation studies, an Instrumentation (IL) Laboratory Model 357 atomic absorption spectrometer was used with suitable burner modifications to allow a silica atomisation cell to be supported in an air-acetylene flame approximately 5 mm above the slot of a 5 cm single-slot burner. The atomisation cell, consisted of a T-shaped silica tube (150 x 2 mm i.d.).

Before analysis, the atomisation cell was allowed to warm up until the atomisation cell reached equilibrium. The signal from the spectrometer was displayed on a chart recorder (Linseis 650). A 1 nm band pass was used and an arsenic hollow-cathode lamp (S&J Juniper and Co, Harlow, Essex, UK) was operated at a lamp current of 8 mA and a wavelength of 193.7 nm. Air and acetylene flow-rates of 8.5 and 1.9 L min⁻¹ respectively were used.

The HPLC system as shown in figure 3.1 consisted of a solvent delivery system (Waters 501) equipped with a Rheodyne injection valve fitted with a 2 ml sample loop. Separation of the arsenic species was achieved using a 3 µm SAX resin (Altech Associates) laboratory packed in a 3 cm x 2 mm i.d. column in series with a 10 µm Hamilton PRP-X100 anion column (Hamilton Company) with a 250 x 4.1 mm i.d. The eluent from the column at 1 ml min⁻¹ was merged with the 1 M sulphuric acid flowing at 3.8 ml min⁻¹. This solution went on to mix with 1 % sodium tetrahydroborate flowing at 3.2 ml min. Minimum tube lengths were used in order to minimise band spreading. A injection volume of 200 µl was used.

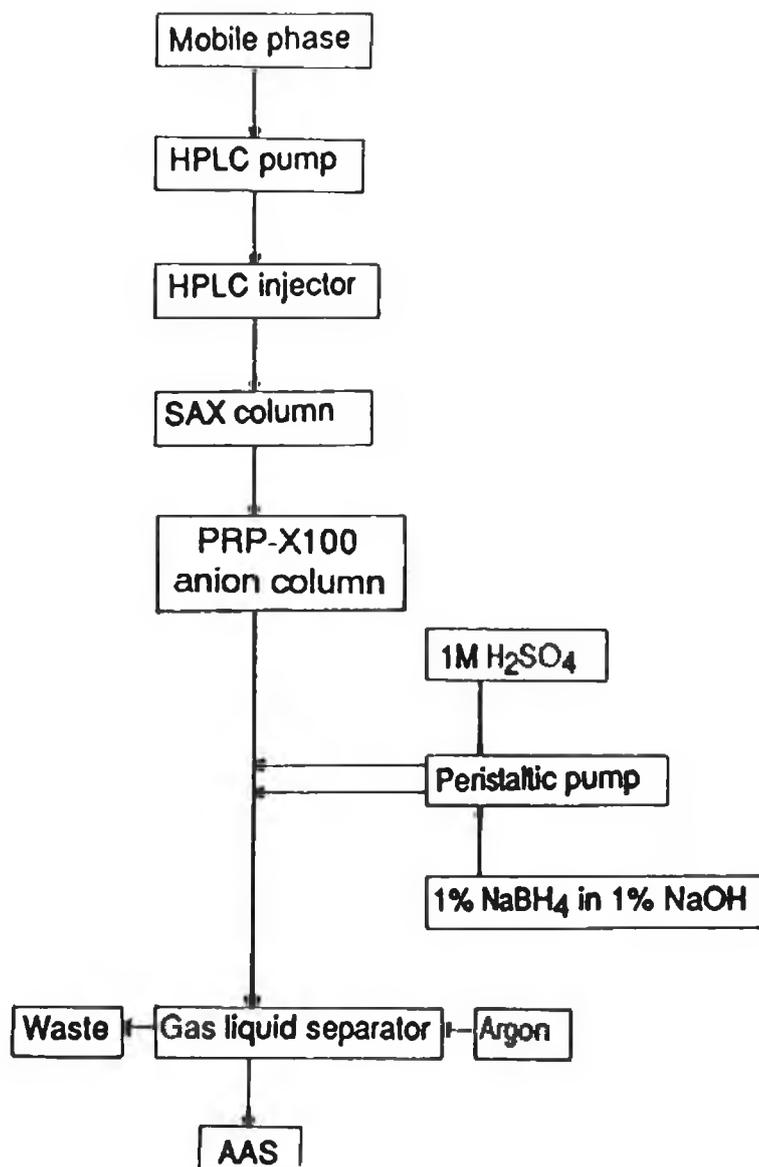


Fig. 3.1 Schematic diagram of HPLC hydride generation AAS.

3.1.3 Results and discussion

3.1.3.1 Optimisation of HPLC system

1 M sulphuric acid was used for the HPLC-hydride generation AAS analysis of arsenic as it gave a slightly better response for arsenite, arsenate, MMA and DMA than the previously reported hydrochloric acid[chapter 2]. The flow-rates of the sulphuric acid and sodium tetrahydroborate were varied with 3.8 ml min⁻¹ and 3.2 ml min⁻¹ for sulphuric acid and sodium tetrahydroborate, respectively, being found to be optimum. It was found that at lower flow-rates the sensitivity dropped and at higher flow-rates no increase in sensitivity was observed.

The pH of the phosphate mobile phase was varied between 5 and 9 as shown in table 3.1. At the higher pH's the components eluted as one peak. At the lower pH's the elution time was too long. A pH = 5.8 was chosen for all further work as it gave good separation and an elution time of eleven minutes for all four arsenic species i.e. arsenite, arsenate, MMA and DMA. The concentration of the phosphate mobile phase was varied between 0.01 and 0.05 M as shown in table 3.2. 0.03 M was chosen as the optimum as at lower concentrations there was excellent separation but the elution time was too long and at higher concentrations the opposite was true. An initial mobile phase flow rate of 1 ml min⁻¹ was set and was found to be suitable.

The sample volume was varied between 50 and 400 µl. 200 µl was chosen as optimum, as at higher volumes the peaks were broader and separation was poorer and lower injection volumes resulted in a loss in sensitivity as expected.

pH	Retention time (min.)				Comment
	Arsenite	DMA	MMA	Arsenate	
5.0	2.4	3.2	5.0	8.7	Excellent separation, elution time for four arsenic species too long.
5.8	2.5	3.1	4.4	7.9	Excellent separation and good elution time for four arsenic species.
7.0	2.6	3.1	3.3	6.8	MMA and DMA not resolved.
8.0	2.7	3.0	3.3	6.1	Very poor separation of arsenite, MMA and DMA.

Table 3.1 Effect of pH of phosphate mobile phase on the separation of arsenite, arsenate, MMA and DMA.

Conc. phosphate buffer (M)	Retention time (min.)				Comment
	Arsenite	DMA	MMA	Arsenate	
0.01	2.6	4.1	6.8	18.8	Elution time too long.
0.03	2.5	3.3	3.9	7.7	Excellent separation.
0.05	2.5	3.0	3.3	5.5	Poor separation of arsenite, MMA and DMA

Table 3.2 Effect of concentration of phosphate in the mobile phase on the separation of arsenite, arsenate, MMA and DMA.

3.1.3.2 Separation of arsenic species

Complete separation of arsenite, DMA, MMA and arsenate was achieved using the optimum conditions described above. A typical chromatogram is shown in figure 3.2. The limit of detection defined as twice the noise was found to be 100, 300, 240 and 460 ppb for arsenite, DMA, MMA and arsenate respectively. Results for the second and third intercomparison on arsenic speciation carried out for the BCR of the Commission of the European Communities using the HPLC-hydride generation AAS method are shown in table 3.3 and 3.4. The results compared favourably with the target values.

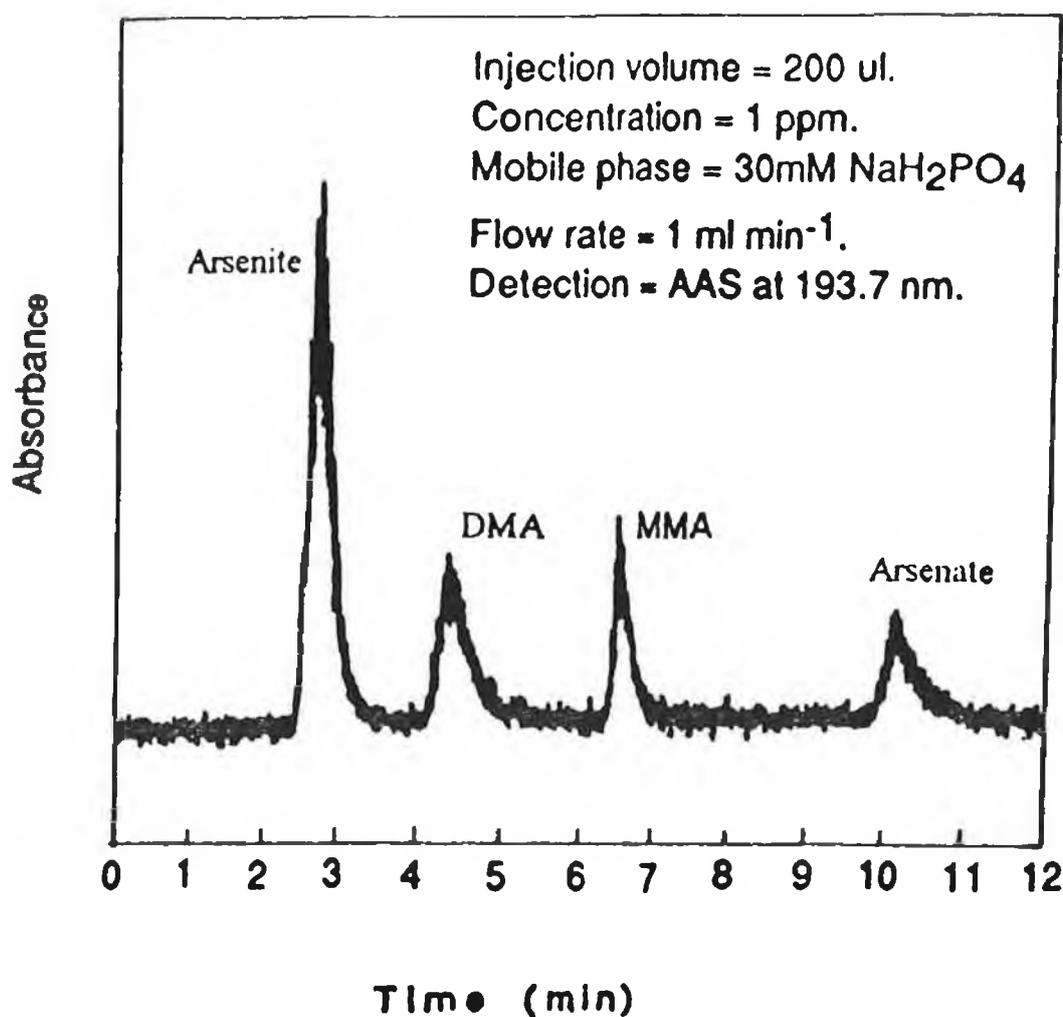


Fig. 3.2 Typical chromatogram obtained for the separation of arsenic species using 0.03 M phosphate buffer at a flow rate of 1 ml min.⁻¹.

Arsenic species	Sample No.	Target value (ppm)	Conc. found (ppm) n = 5	Std. Dev.
Arsenite	1	5.00	5.30	0.33
	3	1.00	1.07	0.16
	4	2.00	2.01	0.16
Arsenate	1	5.50	6.62	0.51
	2	10.00	10.72	0.44
	5	2.00	2.19	0.18
MMA	1	5.00	5.56	0.43
	3	1.00	1.02	0.09
	5	1.20	1.15	0.92
DMA	1	5.50	5.44	0.48
	2	10.00	11.48	0.68
	3	10.00	10.67	0.71
	4	0.85	0.93	0.10

Table 3.3 Inter-laboratory comparison on arsenic species part two.

Arsenic species	Sample No.	Conc. found (ppm) n = 5	Std. Dev.
Arsenite	1	0.293	0.027
	2	0.420	0.050
	3	0.510	0.043
Arsenate	1	0.495	0.018
	2	0.714	0.044
	3	0.999	0.055
	4	0.883	0.074
	5	3.377	0.216
MMA	4	0.511	0.032
	5	0.572	0.034
DMA	2	0.342	0.018
	4	0.406	0.038
	5	0.782	0.093

Table 3.4 Inter-laboratory comparison on arsenic species part three (target results not available).

3.1.4 Conclusion

After optimisation the coupling of HPLC to hydride generation AAS achieved the desired separation and detection of arsenite, DMA, MMA and arsenate. The reproducibility and accuracy of the system developed was demonstrated by participation in the European inter-laboratory comparison. The detection limits obtained for the species are acceptable for many applications, however with the increasing demand for lower detection limits especially in the analysis of environmental samples a reduction in these limits is desirable. To achieve this the arsenic species require preconcentration prior to separation and analysis. This was investigated using both on-line and off-line preconcentration techniques as discussed in the next section.

3.2 PRECONCENTRATION OF ARSENIC SPECIES

Methods based on the direct chromatographic separation of the different species of arsenic offer the advantage of being fast and reliable, however, due to the high dilution factors associated with these methods sensitive detection techniques such as graphite furnace AAS[12, 13] or hydride generation AAS[5, 6] are required to compensate for the dilution. Some of the arsenic species occur at such low levels that even graphite furnace AAS or hydride generation cannot provide sufficient sensitivity, therefore, pre-concentration techniques have to be coupled with chromatographic separation in order to achieve the required detection power. Evaporation of water samples has been investigated in an attempt to pre-concentrate arsenic species but it resulted in losses of arsenic due to evaporation and also due to redox reactions between inorganic arsenic species[13].

Hydride generation is in effect a pre-concentration technique where the elements that form hydrides are separated and concentrated to a gaseous phase while other compounds go to waste as liquid. Only with the formation of excess hydrogen will the hydride be diluted. To counteract this the hydrides may be concentrated by cold trapping. Arenas et al.[14] used cold trapping hydride generation to pre-concentrate arsenic, where, the trap was held at $-190\text{ }^{\circ}\text{C}$ which collected the arsines produced, it was then heated and the arsines transported to an AAS detector with a carrier gas of helium. Argon is normally used as a carrier gas in arsenic determinations, however it was found that argon was condensed, frozen and clogged the tubes due to low temperature in the arsine trap. A more volatile carrier gas such as helium (m.p. $-269.7\text{ }^{\circ}\text{C}$) or hydrogen (m.p. $-259.2\text{ }^{\circ}\text{C}$) had to be used. By using cold trapping it was possible for Arenas et al.[15] to achieve a 30 fold enhancement of peak height. However, cold trapping has not found widespread use because it is relatively slow and not very precise.

Mentassti et al.[15] pre-concentrated arsenic using a co-precipitation method. The reaction of sodium tetrahydroborate coupled with a carrier (iron or palladium) permits the separation of several metal ions by co-precipitation as the tetrahydroborate or as the metal in the zero oxidation state. The method was modified by replacing iron with indium carrier to pre-concentrate and separate arsenite and arsenate, as the presence of iron increases the intensity of emission lines for arsenic. A 1000 fold pre-concentration factor could be achieved. Metassti et al.[15] also used a polydithiocarbamate resin to pre-concentrate arsenite. Solutions of arsenic were passed through the resin at the desired pH. Recovery of the arsenic was achieved by treating the resin with concentrated nitric acid - hydrogen peroxide (3 + 2 v/v). For this system arsenate must be reduced to arsenite for pre-concentration to occur.

Hata et al.[16] converted arsenic to arsenomolybdate and collected it on a nitrocellulose membrane filter as an ion associate with the tetrapentylammonium ion. The filter, and the arsenomolybdate on the filter, were dissolved in a small volume of concentrated sulphuric acid and diluted to 2 - 10 ml to reduce the viscosity and analysed by ICP AES. Enrichment factors of up to 250 may be attained. Arsenate may only be determined in this way, arsenite must be oxidised to arsenate prior to the pre-concentration method.

Terada et al.[17] used thionalide (2-mercapto-N-2-naphthylacetamide) on silica gel for differential pre-concentration of arsenite and arsenate. Arsenite was retained on the gel from a solution of pH 6.5 - 8.5 but arsenate and organic arsenic compounds were not retained. The retained arsenic was completely eluted with 0.01 M sodium borate in 0.01 M sodium hydroxide containing 10 mg L⁻¹ iodine at pH 10. The arsenic was determined spectrophotometrically after complexation with silver diethyldithiocarbamate. Arsenate could subsequently be determined after reduction to arsenite.

Sperling et al.[18] used an on-line separation and pre-concentration step for the determination of arsenite and total arsenic. The arsenite can be quantitatively extracted using sodium diethyldithiocarbamate as the complexing agent and C₁₈ reverse phase packing as the column material for solid phase extraction. Arsenate must be reduced to arsenite prior to extraction. A 7.6 - fold enhancement in peak area compared to direct injection was obtained after a 60 second pre-concentration step.

The above procedures mentioned for pre-concentration are concerned with pre-concentrating total arsenic as arsenite or arsenate. Speciation studies involve differentiating between arsenite, arsenate and organoarsenicals. The organic forms have not been studied in great detail. However there are some on-line pre-concentration methods available with which the preconcentration of organic arsenicals have been investigated. Tye et al.[6] pre-concentrated arsenate, MMA and DMA on a pellicular anion exchange column by loading with 10⁻⁴ % sulphuric acid. After loading, the eluent was switched to ammonium carbonate which eluted the arsenicals and separated them on a strong anion exchange column. Ebdon et al.[5] used a similar procedure to preconcentrate arsenate, MMA and DMA. Only 1 ml was loaded in each of the above cases and up to 200 fold enrichment was achieved. In an attempt to use larger volumes which would allow lower concentrations of arsenic species to be detected a technique known as column switching was investigated for arsenic preconcentration and speciation in this work. This technique is on-line and offers the advantage of being less tedious to carry out while allowing large enrichment factors to be achieved. These methods also have the advantage being easier to automate and reduce analysis time thus allowing faster sample throughput.

3.2.1 Column switching

Column switching is a technique used to transfer selectively fractions of the mobile phase from the outlet of one column to the inlet of another column. A typical instrument arrangement (incorporating a six-port switching valve) is shown in figure 3.3. The sample may be introduced via the injection port or for larger volumes a pump may be used to introduce the sample. The sample is swept onto the precolumn by pump A and the analytes of interest retained while the rest flows to waste. At the same time the mobile phase is passed by pump B through the analytical column. On switching the valve the analytes on the precolumn are swept in a back-flush mode onto the analytical column where they are separated. This column switching technique has been applied to GC and HPLC and used for sample clean-up, heart-cutting and trace enrichment[19 - 21].

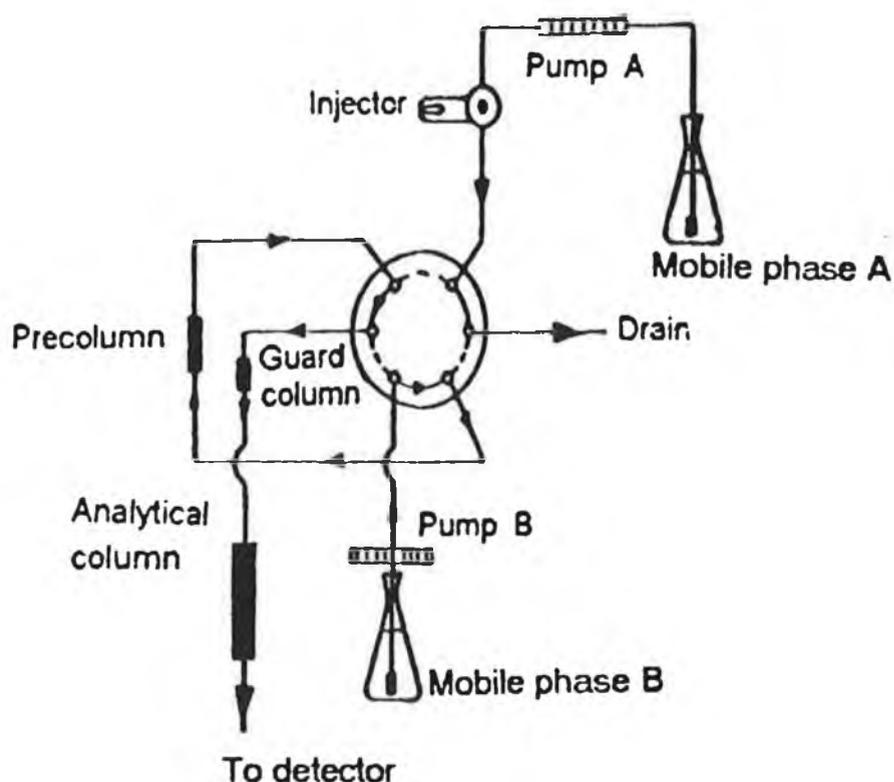


Fig. 3.3 Column switching assembly incorporating a six-port switching valve.

3.2.1.1 Sample clean-up

A sample is transferred to a column which retains the analyte of interest and allows other components, i.e. impurities, to flow to waste. Following this a switching valve is turned starting a back-flushing of the analyte with a different mobile phase which carries the analyte onto a second column where separation and detection take place. Smith et al.[19] incorporated column switching to clean up plasma samples. Plasma samples containing propranolol and its 4-hydroxy derivative were loaded onto a pre-column of Hypersil MOS 30 μm with 0.1 M phosphoric acid. Proteaceous and other contaminants went to waste. On switching the valve a mobile phase of 0.1 M phosphoric acid - methanol (1/1) eluted the components of interest onto a Hypersil MOS 5 μm analytical column. Column switching in this case allows unnecessary components to flow to waste which may otherwise interfere with separation or bind irreversibly to the analytical column and thus shorten the life of the column.

3.2.1.2 Heart cutting

With complex samples a single column may not fully resolve all the components of interest. Even with an elaborate elution program separation may be prolonged by slowly eluting components. Heart cutting techniques utilizing column switching may overcome such problems. Using this technique the sample may be partially separated on the first column and the eluent monitored. At an appropriate point the eluent stream containing the unresolved components of interest are switched onto a second column and further elution of this column gives an improved separation without interference from the other components in the sample. Low et al.[20] applied column switching in HPLC to arsenic speciation with ICP AES detection. On an anion exchange column under certain solvent conditions arsenobetaine eluted with arsenite. On a reverse phase C_{18}

column under certain solvent conditions arsenobetaine could be separated from arsenite but arsenite, arsenate, MMA and DMA could only be resolved into two peaks. Therefore switching of the co-eluting peaks from the anion column to the reverse phase column would facilitate their complete resolution. To achieve this a mobile phase which is compatible with both columns must be chosen. In this case an ammonia buffer was selected and the unresolved arsenite and arsenobetaine were diverted with the switching valve from the anion column to the reverse phase column. The valve was switched back and allowed the other species to separate and finally the mobile phase was switched to the reverse phase column again to allow separation of arsenite and arsenobetaine. Optimisation of the switching technique was shown to significantly reduce analysis time and the system may be completely automated.

3.2.1.3 Trace enrichment

Trace enrichment allows on-line concentration of analyte. A large volume of sample is passed through a concentration column under chromatographic conditions where the analyte does not elute and is adsorbed onto the column. After concentration the column is switched on-line with a different solvent so the analyte is rapidly eluted onto a second column where separation can take place. Trace enrichment may also concentrate other material from the sample, to overcome this problem, trace enrichment should be combined with sample clean-up. Kelly et al.[21] preconcentrated several drugs from plasma samples on a reverse phase C₁₈ column. The plasma samples were loaded onto the reverse phase column with water. Ammonium nitrate/ammonia solution pH 8 - methanol (1/4) was used to elute the retained analytes onto a silica column 5 µm where they were separated. The column switching procedure allowed clean up and preconcentration of the drugs. This type of system has been applied to inorganic

analysis by Ryan et al.[22]. Aluminium, copper and iron were preconcentrated from waste water from a mine and beverage samples on a C₁₈ precolumn. The samples were loaded with water/acetonitrile (9/1) and eluted with acetonitrile (containing 8-hydroxyquinoline) and acetate buffer (containing potassium nitrate) onto a C₁₈ column for separation. The samples were mixed with the loading mobile phase before preconcentration.

3.2.1.4 Conclusion

Column switching improves sample throughput, separation efficiency and loadability of the chromatographic system. On-column concentration extends the working range of the detection technique by effectively increasing the sensitivity through the use of higher sample loadings on the pre-column.

The procedure described in this thesis uses column switching coupled with hydride generation AAS for the pre-concentration and analysis of two arsenic species. A precolumn is used to achieve pre-concentration and the species are separated by ion exchange HPLC before being detected by hydride generation AAS. The two species studied were arsenate and MMA and using this on-line pre-concentration system detection limits of 5 and 10 ppb respectively could be achieved. This is an improvement of approximately 50 - fold on the detection limits achievable without the incorporation of the on-line pre-concentration step.

3.2.2 Experimental

3.2.2.1 Reagents

Unless otherwise stated, all reagents were of analytical reagent grade. Deionised water was obtained by passing distilled water through a Milli-pore Milli-Q water purification system.

Arsenite and arsenate were obtained from BDH Poole, Dorset, UK. Monomethylarsonate (MMA) and dimethylarsinate (DMA) were obtained as part of a BCR programme on arsenic speciation. Phosphate buffer was prepared from 0.01 M sodium dihydrogen phosphate (E. Merk, D-6100 Darmstadt, F.R. Germany) and was adjusted to pH of 5.8 with 0.01M disodium hydrogen phosphate (Riedel-de Haen A.G., D3016 Seelze 1) before filtration through a 0.45 µm filter. Concentrated sulphuric acid (Riedel-de Haen A.G., D3016 Seelze 1) was diluted to 1M with water. 1% sodium tetrahydroborate (Aldrich Chemicals Co. Ltd., Gillingham, Dorset, England) solution was prepared by dissolving sodium tetrahydroborate powder in 1 % sodium hydroxide (BDH, Poole, Dorset, UK) solution. Argon was obtained from Air Products PLC Molesey Rd., Walton-on Thames, UK.

3.2.2.2 Equipment

A six port, two way switching valve (Rheodyne 7000), two HPLC pumps (Waters 501) equipped with a Rheodyne 7125 injection valve and fitted with a 2ml sample loop were used in the switching system (figure 3.4). A stainless steel pre-column, 10 x 1.5 mm i.d., packed with Vydac anion exchange material and housed in a stainless-steel cartridge, was incorporated in the switching system for the preconcentration step. A Dionex Ionpac CG5 guard column and Dionex

Ionpac CS5 analytical column (Dionex Corporation) were used for separation of the arsenic species. A peristaltic pump (Watson Marlow 501U) was used to pump the sulphuric acid and sodium borohydride for hydride generation.

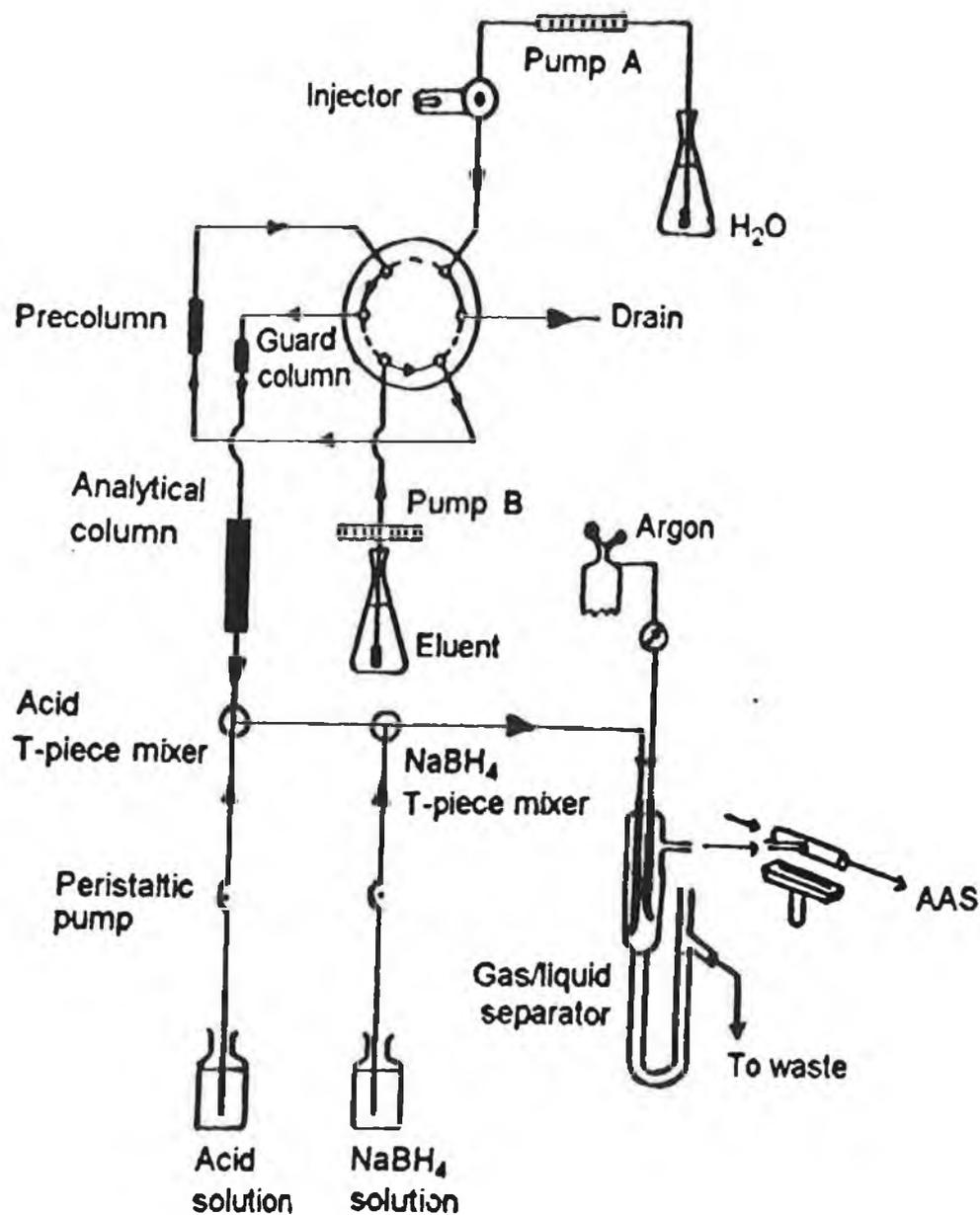


Fig. 3.4 Schematic diagram of column switching system.

The detection system consisted of an atomic absorption spectrophotometer (Instrumentation Laboratory model 357) with suitable burner modifications to allow a silica atomisation cell to be supported on an acetylene flame, approximately 5 mm above the slot of a 5mm single slot burner. The atomisation cell consisted of a T-shaped silica tube (150 x 2 mm i.d.). Before analysis the atomisation cell was allowed to warm up until the atomisation cell reached equilibrium. The signal from the spectrophotometer was displayed on a chart recorder (Philips). A 1 nm band pass was used and an arsenic hollow-cathode lamp (S &J Juniper and Co, Harlow, Essex, UK) was operated at a lamp current of 8 mA and a wavelength of 193.7 nm. Air and acetylene flow rates of 8.5 and 1.9 L min⁻¹ respectively were used.

3.2.2.3 Procedure

3.2.2.3.1 Chromatography

Initially the samples were introduced into the system via a rheodyne injection loop. The arsenic species in the injection loop were loaded onto the precolumn (which was packed with the anion exchange material) by pump A with a mobile phase of water. the arsenic species were retained while the remainder of the sample went to waste. After switching the six port valve a mobile phase of 0.01 M sodium dihydrogen phosphate adjusted to pH of 5.8 was pumped in the opposite direction through the precolumn and onto the analytical column. As a result the arsenic species were eluted from the pre-column onto the analytical column where separation took place. The eluent from the column at 1 ml min⁻¹ merged with the 1 M sulphuric acid flowing at 2.1 ml min⁻¹. This solution went on to mix with the 1% sodium tetrahydroborate flowing at 1.8 ml min⁻¹. The resultant reaction produced hydrogen and volatile arsines. A gas/liquid separator through which argon carrier gas was passed at 0.6 L min⁻¹ stripped the gaseous

components from the eluate. The gases were passed to the flame heated quartz tube for analysis by atomic absorption spectrometry (figure 3.4). In later studies larger volumes of samples were pumped directly onto the pre-column for preconcentration without the use of an injection port.

3.2.2.3.2 Hydride generation

Sodium borohydride was used as the reductant for the arsenic species. A concentration of 2 % was found to be optimum for simultaneous analysis of both arsenate and MMA. Sulphuric acid was also used in the reduction step. Sulphuric acid at a concentration of 1.25 M was found to give optimum readings for arsenate, above this concentration the response dropped rapidly. A similar profile was obtained for MMA but a slight loss in sensitivity was noted above 1 M. Therefore 1 M was chosen for all further work.

After optimisation the flow rates of sulphuric acid and sodium tetraborohydride were maintained at 2.1 and 1.8 ml min⁻¹, the maximum flow-rates permissible without exerting too much pressure on the tubing. Argon was used as the carrier gas, at a flow-rate of 0.6 L min⁻¹ which allowed a good response and return of the signal to the baseline in an appropriate time. In addition to transporting the hydrogen arsenide to the atomisation cell, the carrier gas also expels any air present in the system, hence allowing precise measurements to be made in the far UV region. The tube lengths were kept to a minimum (figure 3.4) to reduce peak broadening.

3.2.3 Results and discussion

3.2.3.1 Pre-column selection

Several Bond Elute sep paks were used off-line to find a packing that would retain the arsenic species in order that preconcentration of the species could be carried out. A table of the packings investigated and the results obtained are listed in table 3.5. These studies were carried out by preconcentrating from water. The arsenic species in the eluent was analysed to investigate if retention had taken place. The results show that arsenite was not fully retained by any of the packings used. DMA was retained by the cation exchange material only and arsenate and MMA were retained by the aminopropyl and anion exchange materials. It was decided to investigate the use of the on-line column switching technique using an anion exchange material for the preconcentration of arsenate and MMA. A stainless-steel pre-column (10 x 2 mm i.d.) was chosen as the concentration column which was packed with the packing material chosen.

Packing (Bond Elute)		As ₂ O ₃	As ₂ O ₅	DMA	MMA
Octadecyl	C ₁₈	not retained	not retained	not retained	not retained
Phenyl	PH	not retained	not retained	not retained	not retained
Diol	2OH	not retained	not retained	not retained	not retained
Silica	Si	not retained	not retained	not retained	not retained
Aminopropyl	NH ₂	not retained	retained	not retained	retained
Benzenesulfo -nylpropyl	SCX	not retained	not retained	retained	not retained
SAX (Vydac)	SAX	not retained	retained	not retained	retained

Table 3.5 Bond Elute sep paks used off-line for the preconcentration of arsenic species.

3.2.3.2 Mobile phase selection

Compatible mobile phases of different eluotropic strengths were needed ; one to concentrate the arsenic species on the precolumn and the second to elute them off the pre-column and onto the analytical column for separation to take place. The mobile phases must be compatible as the preconcentration system is on-line and the last few mls of mobile phase used to concentrate the species are mixed with the first few mls of mobile phase used to elute the species. Incompatible mobile phases cause the packing in the preconcentration column to swell to different extents and this leads to a build up of pressure on the column. It also effects the life of the column packing material. Phosphate buffer was investigated as the mobile phase to elute the arsenic species from the pre-column as this phosphate mobile phase had been used previously to separate arsenic species on an anion exchange column by Chana and Smith[8]. As the arsenic species had to be separated after elution this mobile phase had a dual purpose; it eluted the species from the pre-column and separated them on the analytical column. This selection proved successful and was used in further studies. The solvent selected for loading and concentrating the analyte on the pre-column had to have poor elution capability for the arsenic species to ensure maximum preconcentration. Water was found to have the least eluotropic strength and it was miscible with the phosphate buffer so it was used for loading the species onto the pre-column.

3.2.3.3 Switching techniques

The column switching arrangement incorporating a six port switching valve is shown in figure 3.4. A back-flushing technique was employed to transfer the arsenic species from the pre-column onto the analytical column. In this mode, the arsenic species were introduced via the injection port and swept onto the pre-column with water by pump A. Meanwhile the eluent was passed by pump B through the analytical column thus maintaining equilibrium on this column. Switching the valve causes the eluent to flow in a back-flush mode through the pre-column from which the retained arsenic species are desorbed and swept onto the analytical column for separation.

3.2.3.4 Metal preconcentration

3.2.3.4.1 Optimisation

The preconcentration conditions were optimised using a 2 ml loop, 500 ppb MMA and 200 ppb arsenate. The conditions which were optimised were breakthrough volume, equilibrium wash volume and elution volume.

3.2.3.4.2 Optimisation of breakthrough volume

Breakthrough volume is defined as the amount of mobile phase A i.e. water, with which it is possible to wash the precolumn without causing elution of the retained analytes due to the washing effect of the solvent. The breakthrough volume was found by varying the volume of mobile phase A. A fixed flow-rate of 1 ml min^{-1} was used and the volume was varied by changing the time of the wash rather than the flow-rate, as flow-rate has been shown to effect the retention. Figure 3.5 shows breakthrough curves for arsenate and MMA. Arsenate is very strongly

retained by the Vydac packing material and breakthrough does not occur at the volumes used i.e. arsenate is not displaced even due to the washing effects of 5 mls of mobile phase A. However for MMA breakthrough occurred at 2.75 mls.

At higher flow-rates not all the species were retained i.e. the flow was too fast to allow total interaction between analyte and packing and as a result some of the analyte went through the preconcentration column to waste. At lower flow rates the magnitude of the response did improve for MMA i.e. more of the MMA was retained but the loading time had to be increased if the same volume of sample was to be loaded. For the simultaneous analysis of arsenate and MMA an optimum flow-rate of 1 ml min^{-1} was chosen as it gave an appropriate response and minimised loading time. A loading volume of 2.5 mls was used for further studies as this ensured that all the MMA and arsenate were washed onto the column while losses due to breakthrough were minimised.

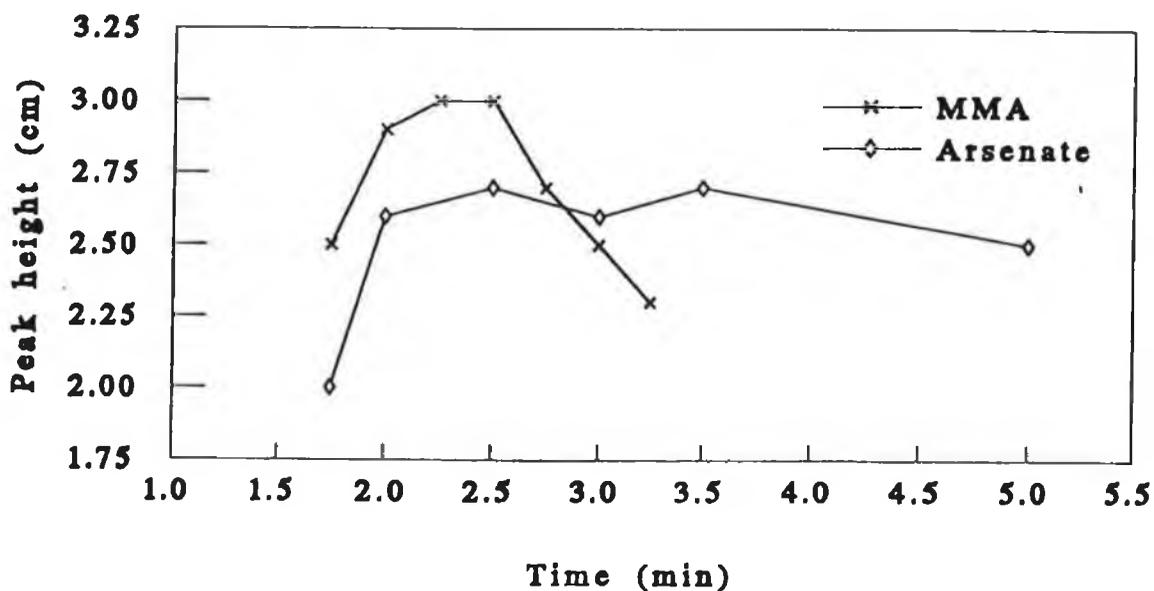


Fig. 3.5 Breakthrough curve recorded for arsenate and MMA. Injection volume 2 ml.

3.2.3.4.3 Optimisation of equilibrium wash volume

Equilibrium wash volume is the volume of mobile phase A required to re-equilibrate the pre-column following the elution of retained analytes using mobile phase B i.e. phosphate buffer, before another sample can be preconcentrated. Volumes from 0.25 to 1.5 mls were investigated and no difference in peak heights were observed as shown in table 3.6. Therefore the pre-column was found to be ready for use after only washing with 0.25 ml of water.

Volume mobile phase A (ml)	Peak height (mm)	
	Arsenate (200 ppb)	MMA (500 ppb)
0.25	20	21
0.50	19	21
0.75	19	21
1.00	18	21
1.25	19	20
1.50	20	20

Table 3.6 Equilibrium wash volume following 1 ml elution of arsenate and MMA.

3.2.3.4.4 Optimisation of elution volume

The elution volume for both species was also measured. This is the minimum volume of mobile phase B which will wash the analyte from the pre-column to the analytical column. The elution volume was varied from 0.1 to 2.0 ml with both arsenate and MMA being eluted with a minimum of 0.25 mls of phosphate buffer as shown in table 3.7.

Volume mobile phase B (ml)	Peak height (mm)	
	Arsenate	MMA
0.10	17	13
0.20	17	17
0.25	19	18
0.50	20	18
0.75	19	19
1.00	21	19
1.50	18	19
2.00	19	20

Table 3.7 Elution volume of arsenate and MMA.

3.2.3.4.5 Effect of loadability

Loadability refers to the maximum concentration of arsenic that can be loaded onto the preconcentration column. When a 2 ml aliquot containing varying concentrations of both species were injected a linear relationship between peak height and concentration was achieved with concentrations up to 3 ppm. When the concentration was increased further no corresponding increase in peak height was noted. This would suggest that the capacity of the column had been exceeded. This was further investigated by loading different volumes of the same concentration onto the preconcentration column and it was found that up to 5 ml of both species could be loaded if the associated concentration did not exceed the preconcentration column capacity which was 3 ppm. It was also noted that larger volumes did not cause any adverse affects in the case of arsenate but in the case of MMA increased volumes at the same flow-rate resulted in a loss in sensitivity. However, a reduction in the flow rate was found to once again restore the response expected suggesting that at higher flow rates the washing effect of mobile phase A was too great causing loss of MMA from the column.

3.2.3.5 Preconcentration and analysis

A linear calibration curve was obtained by (a) keeping the volume constant and increasing the concentration (figure 3.6.1) (b) keeping the concentration constant and increasing the volume (figure 3.6.2). At very low concentrations a limit is reached whereby if the volume is increased the response does not increase. At this cut-off volume if the concentration is increased a linear relationship is still obtained. The lowest levels of arsenic species detected loading 5 mls were 5 ppb arsenate and 10 ppb MMA. Figure 3.7 shows separation of these species. The within-run ($n = 5$) coefficient of variation for 3 mls of 25 ppb arsenate and 3 mls of 50 ppb MMA was 5.1 % and 3.6 % respectively.

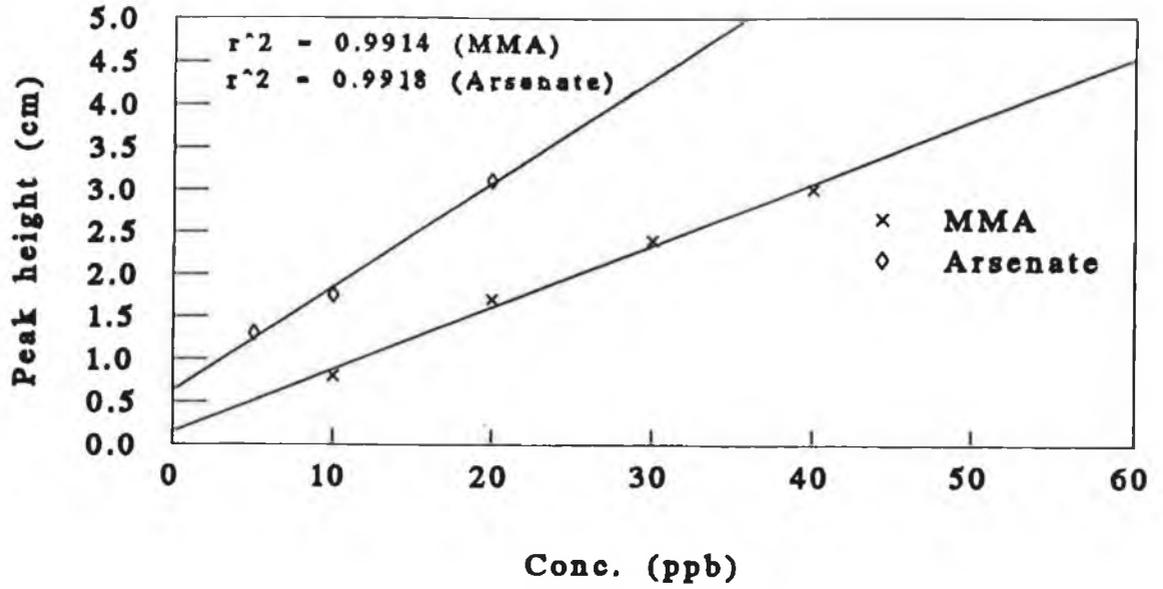


Fig. 3.6.1 Calibration graph for 5 mls of arsenate and MMA at different concentrations.

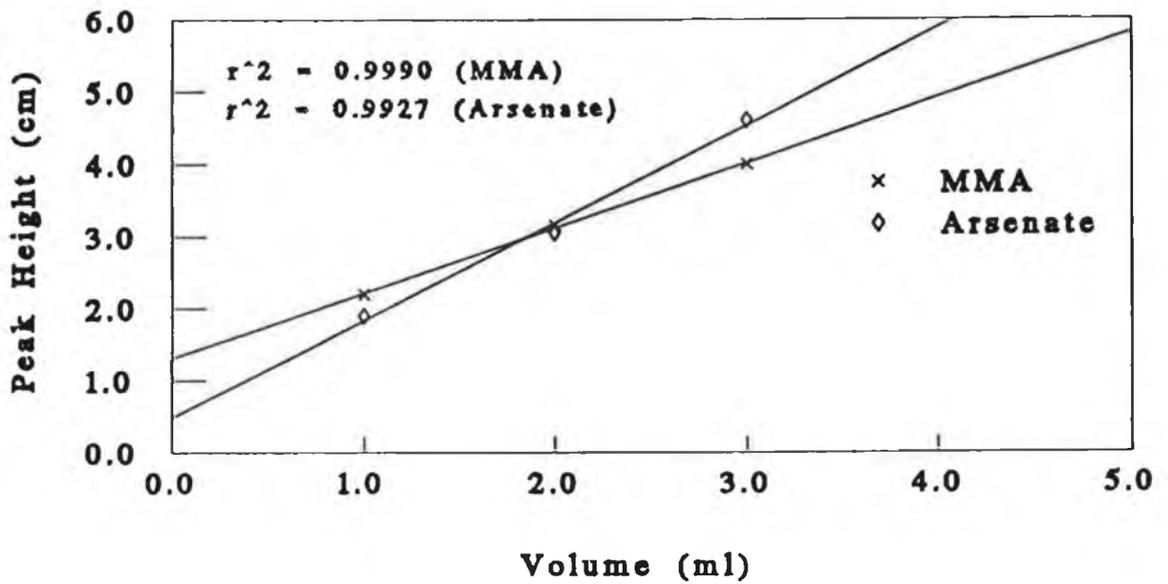


Fig. 3.6.2 Calibration graph for 25ppb arsenate and 50 ppb MMA at different volumes.

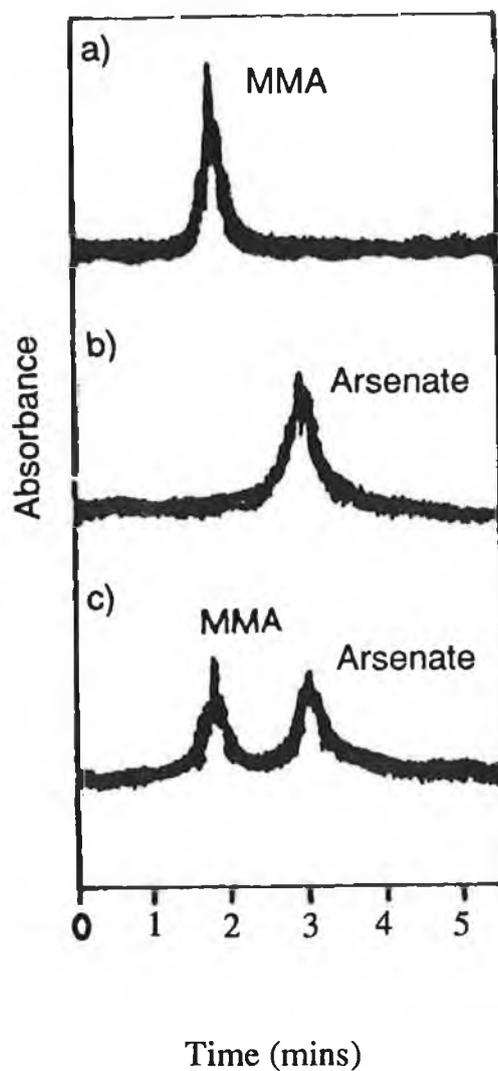


Fig. 3.7 Chromatogram of a) 5 mls of 30 ppb MMA b) 5 mls of 10 ppb arsenate and c) 5 mls of 5 ppb arsenate and 10 ppb MMA. A mobile phase A of water and mobile phase B of 0.01 M sodium dihydrogen phosphate adjusted to pH 5.8 with 0.01 M disodium hydrogen phosphate.

3.2.4 Conclusion

The column switching method described has achieved preconcentration and separation of arsenate and MMA on-line. The detection limits are improved i.e. 5 and 10 ppb for arsenate and MMA respectively using column-switching compared to 300 and 240 ppb for arsenate and MMA respectively using HPLC alone. Linear plots maybe obtained by changing the concentration or volume of the species loaded. The method achieves high sample throughput, low detection limits, good reproducibility and allows high volumes to be loaded. The method also enables on-line sample clean-up i.e. interferences flow to waste and only material retained and eluted from the pre-column go onto the analytical column and thus prolonging the life of the analytical column. The other arsenic species i.e. arsenite and DMA are not retained and are therefore separated from arsenate and MMA.

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CHAPTER 4

Matrix solid phase dispersion isolation and liquid chromatographic determination of arsenate, MMA and DMA.

4.1 INTRODUCTION

4.1.1 Digestions procedures

Extraction of arsenic from numerous matrices has been carried out using various methods[1 - 9, 12 - 18]. Early work involved either wet or dry digestions[1 -9]. Wet digestions are carried out with strong acids at elevated temperatures. Dry digestions involve ashing the samples at high temperatures in the presence of an ash-aid, magnesium nitrate. In either of these processes the organic matrix is destroyed so that only total arsenic may be determined.

For wet digestions the acids normally used include hydrochloric, nitric, perchloric and hydrofluoric acids. Several mixtures of these acids have also been used[2 -4, 8]. Hydrofluoric acid has been found to be necessary for the decomposition of silicate matrices. Van der Veen et al.[1] reported that the presence of hydrofluoric acid appears to cause losses of arsenic due to volatilisation. Perchloric acid carries the danger of explosion, and contact dermatitis, and requires the use of special venting facilities[1]. Nitric acid alone is a poor solvent but can act as an oxidising agent. Mixtures of acids have been found to be preferred because a single acid is not usually sufficient to digest samples. Each acid has an advantage and when combined with another acid a better digestion is achieved. Care must be taken to avoid loss of the analyte by volatilisation as this is a common occurrence at high temperatures but by using slow temperature methods this problem may be partially or totally eliminated.

Kuldevere[2] found that hydrochloric acid alone was not sufficient to quantitatively extract arsenic, antimony, bismuth and selenium from geological samples. An oxidative treatment was needed as these elements occur, at least in part, as free elements. He found that nitric and hydrochloric acid mixtures are sufficient for the extraction of these elements with arsenic being oxidised to the +5 state. Arsenic may be lost from vessels during reaction with acids at elevated

temperatures but if the reaction is carried out in sealed tubes, decomposition bombs or autoclaves these losses of evaporation can be avoided[2]. Welz and Melcher[3] compared three digestion procedures for extraction from marine biological tissue. Pressure decomposition with nitric acid in a closed polytetrafluoroethylene vessel resulted in low recoveries. However, this decomposition followed by sulphuric and perchloric acid digestion resulted in good recoveries. Destruction with sulphuric and perchloric acid at a maximum temperature of 310 °C yielded good recoveries but occasional and non-reproducible losses were found. Combustion in a stream of oxygen was also applied and gave good results. This technique was however relatively slow and was not applicable to a large number of samples.

Webb and Carter[4] determined total arsenic in biological samples using nitric and sulphuric acid with potassium dichromate in the digestion procedure and subsequently reducing to arsenite with sodium iodide. The procedure was effective in the recovery of MMA, DMA and inorganic arsenic. Potassium dichromate used with nitric and sulphuric acid in the recovery of DMA as an inorganic acid was found to be effective, as the dichromate is a strong oxidative catalyst and DMA is very resistant to decomposition. Vanadium pentoxide is another catalyst often used for decomposition instead of potassium dichromate[5]. However many digestions are carried out without strong oxidative catalysts and report good recoveries have been reported[2 - 4, 8].

Dry ashing techniques usually employ magnesium nitrate as an ashing aid. Cervera et al.[6] determined arsenic in tomato products with a dry ashing technique using magnesium nitrate - magnesium oxide as an ashing aid at 450 °C. The ash was dissolved in hydrochloric acid. Cervera et al.[7] also determined arsenic in beer under the same conditions. Good recoveries and precision were reported in both. Maher[8] investigated both wet and dry digestion of marine samples and found that ashing with magnesium nitrate was unsatisfactory as

foaming and charring of samples often occurred during heating. Wet procedures were also unsatisfactory as the presence of nitric acid suppressed the arsine signal. The addition of perchloric acid however removed these interferences and gave quantitative recoveries. The use of nitric, sulphuric and perchloric acid for the digestion of marine organisms was shown to convert organoarsenic compounds into inorganic arsenic and in all cases recoveries were > 93 %.

Brumbaugh and Walther[9] used a combined wet chemical and dry ash digestion for the determination of arsenic in fish tissue. A nitric acid wet digestion before ashing helped to disperse the sample evenly in the magnesium nitrate "cake" and eliminated the fluffing out of the sample during ashing. It also provided for additional oxidation at low temperatures that might reduce losses of volatile analyte forms. The addition of magnesium oxide was eliminated as it offered no improvement in recoveries and was often contaminated with arsenic.

In general a combination of acids for digestion is preferred. Nitric, sulphuric and perchloric seem to give best results but a wet chemical digestion followed by dry ashing has been found to be equally as good. Other combinations seem to lack reproducibility or fail to convert the organic arsenic to inorganic arsenic and thus give rise to peak broadening in arsine generation, which is usually used for detection. However these wet or dry digestions are only suitable for total arsenic determinations.

4.1.2 Solvent extraction techniques

Solvent extraction has been used to some extent for the extraction of arsenic from various samples [12 -18]. Various solvents have been used including chloroform, benzene, cyclohexane, methanol and methyl isobutyl ketone, to extract complexes of arsenic[10 -18]. In some cases extraction with methanol is

sufficient to remove essentially all organoarsenicals from marine samples[10]. Extraction with chloroform is not effective even though arsenicals are slightly soluble in this solvent[11].

Donaldson and Leaver[12] separated arsenic from ores by cyclohexane extraction of arsenic xanthate from 8 - 10 M hydrochloric acid. Recoveries were good and a detection limit of 0.1 µg of arsenic per g of ores and related materials was achieved. Aneva and Iancheva[13] extracted lead and arsenic from petrol by converting them to water soluble iodides by reaction with a solution of iodine in toluene followed by extraction into dilute nitric acid before determination by graphite furnace AAS. A detection limit of 5.6 µg arsenic per L was achieved. Kanke et al.[14] converted arsenic to arsenomolybdic acid in 1 M hydrochloric acid and extracted it quantitatively into methyl isobutyl ketone. The extraction was applied to steel, mine water and river water samples. Arsenite must be oxidised to arsenate as only arsenate forms a complex with the molybdate. An iodine solution was used for the oxidation. When applying the extraction to steel, a digestion with nitric, perchloric and hydrochloric acid was carried out before the extraction was applied. Recoveries were good.

Suzuki et al.[15] investigated various systems for the extraction of arsenite, arsenate, MMA and DMA. Halides (chloride, bromide and iodide), diethylammonium diethyldithiocarbamate, didodecyltindichloride and pyrogallol/tetraphenylarsonium chloride were used as extractants. An aqueous solution of the arsenic species was shaken with an equal volume of an organic solvent in the presence of an appropriate amount of an extractant. In the halide system the arsenic species were extracted into benzene from sulphuric acid. In the diethyldithiocarbamate system the arsenic species were extracted into carbon tetrachloride from sulphuric acid. In the didodecyltin system a mixture of benzene and methyl isobutyl ketone (1:1) was used from sulphuric acid. If a benzene solution of didodecyltin was used an insoluble hydroxide formed when

shaken with water. Extraction of the arsenic species was from a sulphuric acid solution, containing pyrogallol, into chloroform containing tetraphenylarsonium chloride. In the halide and diethyldithiocarbamate systems the arsenite species were selectively extracted as complexes. Only arsenate was quantitatively extracted and readily separated from other species in the pyrogallol/tetraphenylarsonium chloride system. MMA was separated in the iodide system. Therefore the four species can be mutually separated by combining these extraction systems.

Some solvent extraction techniques use acid digestion prior to the solvent extraction procedure. This destroys speciation information unless the acid digestion is carried out under very mild conditions. Korenaga[16] digested a sample of acrylic fibre with a mixture of concentrated nitric, perchloric and sulphuric acids. Titanium chloride was added to reduce arsenate to arsenite. The arsenite was extracted into benzene from a sulphuric and hydrochloric acid mixture and further back-extracted into water. This solvent extraction step eliminates interference from antimony as it is not extracted into benzene. Antimony oxide is present in acrylic fibres as a fire retarding agent and arsenic may be present as an undesirable toxic impurity. Holak and Specchio[17] used acid treatment before solvent extraction when determining total arsenic, arsenite and arsenate in foods. Total arsenic was determined with an acid digestion of nitric, perchloric and sulphuric acid. Arsenite and arsenate were determined by using a less drastic digestion procedure using 70 % perchloric acid (used to solubilise the arsenic species) and iron(III) sulphate (a mild oxidant to prevent the reduction of arsenate and not oxidise the arsenite) and finally adding hydrochloric acid. Arsenite was extracted with chloroform and back-extracted into 1 M hydrochloric acid. Following the extraction of arsenite, arsenate was reduced to arsenite with hydrazine sulphate and hydrogen bromide and extraction was carried out as for the arsenite. Arsenite exists as neutral arsenic trichloride

in strong hydrochloric acid and thus is extractable with an organic solvent. Re-extraction of arsenite into water or dilute hydrochloric acid is possible due to hydrolysis of arsenic trichloride to H_2AsO_3 . The total arsenic determination gave no problem assuming the sample was completely digested and with no loss of arsenic. On the other hand determination of arsenite and arsenate may be sample dependent due to protein binding and possible oxidation state changes during the sample treatment which may account for some incomplete recoveries reported.

Takamatsu et al.[18] determined arsenite, arsenate, DMA and MMA in soil using an acid and solvent extraction procedure. The soil was extracted with 1 M hydrochloric acid by mechanically shaking for 1 hour at 30 °C. The arsenite was selectively extracted by adjusting the hydrochloric acid extract to 10 M and extracting with benzene and back-extracting into water. The other arsenic species remained in the 10 M hydrochloric acid solution. To this 10 M solution potassium iodide was added and the concentrated hydrochloric acid was adjusted to 5 M. All the arsenic compounds were extracted with benzene and back-extracted into water containing hydrogen peroxide. The arsenic species were separated on an anion exchange column and determined. Recoveries ranged from 88 to 98 %.

Solvent extraction is usually carried out with the aid of a complexing agent which is specific for one species of arsenic. The arsenic is usually converted to this species for extraction and hence total arsenic is determined rather than the amount of individual species present. For speciation studies several extraction steps are required which increase the risk of losing information on the species present. For many samples an initial acid digestion or treatment is required to obtain a liquid sample and this immediately puts the speciation information at risk unless a very mild procedure is used with carefully controlled conditions. This may still result in oxidation state changes and incomplete recoveries.

4.1.3 Direct analysis

In some cases no rigorous digestion or extraction procedure is necessary unless the sample is highly polluted. Direct analysis may be applied to water and in some cases urine samples where in some cases partial clean-up may be achieved on-line[19, 20]. Tye et al.[19] analysed water samples directly, whereby arsenate, MMA and DMA were preconcentrated on an anion column where arsenite was not retained. A preconcentration step was necessary in order to detect ppb levels of arsenic. Chana and Smith[20] determined arsenic in urine. Samples were introduced directly, without sample pretreatment, onto reverse phase C₁₈ guard column, which removed most of the organic components from the urine that would otherwise bind irreversibly to the packing material in the anion exchange column used to separate the arsenic species. This precolumn was back-flushed between each sample run. Filtration of the sample was the only sample preparation required.

Direct analysis avoids lengthy procedures which are not easily automated and are therefore not suited to routine monitoring. However these direct methods may only be applied to relatively clean liquid samples and in many instances the sample is not in this form.

4.1.4 Solid phase extraction

The use of solid phase extraction is very convenient and amenable to automation by use of disposable and on-line extraction columns[21 -29]. These disposable columns are usually polypropylene cartridges containing a chromatographic material. The samples are applied to the columns under low pressure or vacuum and the desired analyte selectively eluted from the column for further analysis. Solid phase extraction has found widespread application in drug analysis[21 - 25]

and some use in the analysis of metals[26 - 28]. To date only Van-Elteren et al.[29] used solid phase extraction for arsenic analysis. Two types of stationary phase were used, a C₁₈ bonded silica with hexadecyltrimethylammonium-pyrrolidinedithiocarbamate or a strong anion exchange resin converted from the quaternary ammonium form into the pyrrolidinedithiocarbamate form. Only arsenite was investigated and retained as arsenite-trispyrrolidinedithiocarbamate.

The main advantage of solid phase extraction is that on-line analysis of liquid samples may be carried out thus cutting down on analysis time and lending itself to automation. For non-liquid or highly polluted liquid samples, which obviously have to undergo a pretreatment step, solid phase extraction may be useful as a preconcentration step.

4.1.5 Matrix solid phase dispersion

Digestion procedures are suited to total arsenic determinations but are not suitable when information or speciation is required. Solvent extractions require a liquid sample. For a more efficient and less laborious method than these classical methods solid phase extraction offers a partial solution to the problem in which a supernatant may be added to a solid phase extraction column followed by elution of the sample with solvent to isolate a specific compound or class of compounds. This process reduces volumes of solvent required to isolate a given compound when compared to classical methods. For tissue analysis the homogenisation and removal of cell debris, and often, proteins and lipids, is required prior to application to the column. This is performed in order to prevent plugging of the column or overloading of the stationary phase. However, as with acid pretreatment, in this preliminary clean-up step the analyte may be lost or its oxidation state changed. In order to overcome this problem a matrix solid phase dispersion technique has been applied to tissue analysis for the on-line extraction

and determination of arsenic species.

The matrix solid phase dispersion technique employs the use of a solid phase packing material which is blended with the tissue yielding a semi-dry tissue-coated matrix which can be packed into a column and eluted with solvents to clean the sample and elute the analyte. By adding whole tissue to C₁₈ packing in a porcelain mortar and gently grinding the material for 30 seconds, one obtains a near homogeneous mix of tissue cell membranes "dissolved" into the solid phase packing material. This provides a semi-dry material which can be packed into a column from which compounds may be eluted based on their solubilities in the polymer/tissue matrix. The entire sample is exposed to the extraction and the processes of classical solvent extraction, homogenisation, centrifugation, precipitation, digestion and overall sample manipulation are eliminated.

Matrix solid phase dispersion is based on the classical idea of dissolving cell membranes so as to access components and to completely disrupt the cell structure allowing access to internal cellular components. Matrix solid phase dispersion has been successfully applied to the isolation of drug residues[30 - 39] from milk[31, 32, 35, 36, 38], infant formula[34] and animal tissue[30, 33, 37, 39]. The drug residues include organophosphates[30], beta-lactams[30], benzimidazoles[31,37], sulphonamides[33, 34, 38], tetracyclines[36], chlorosulfuron[32], chloramphenicol[35] and furazolidone[39].

Long et al[36] isolated and determined oxytetracycline, tetracycline and chlortetracycline in milk. Tetracyclines are antibacterial compounds used for the prevention/treatment of diseases in life-stock production. Fortified milk samples were blended with C₁₈ packing material. EDTA was added to the packing to release the tetracyclines which would otherwise bind with inorganic ions. The matrix was washed with hexane and the tetracyclines were eluted with ethyl acetate : acetonitrile, 1 : 3 v/v, and analysed by liquid chromatography with UV

detection. Recoveries varied from 63.5 to 93.3 % for the concentration range 100 to 3200 ng ml⁻¹.

Long et al[38] determined sulfonamides in milk. Sulfonamides are antibiotics used widely in the life-stock producing industry. Fortified samples were mixed with C₁₈ packing. The mixture was washed with hexane and the sulfonamides eluted with methylene chloride. Analysis was by HPLC with UV detection. Recoveries ranged from 73.1 to 93.7 % for the concentration range 62.5 to 2000 ng ml⁻¹ with a detection limit of 62.5 ng ml⁻¹ for a 20 µl injection. Long et al[34] also isolated sulfonamides from infant formula using a similar procedure. Recoveries ranged from 75.9 to 112.0 % over the concentration range 62.5 to 2000 ng ml⁻¹.

Benzimidazole anthelmintics in pork muscle tissue were also investigated[37]. Benzimidazole anthelmintics are used in swine and beef production to prevent or eliminate certain worm parasites. Fortified pork muscle tissue samples were blended with C₁₈. The matrix was washed with hexane and the benzimidazoles eluted with acetonitrile. This eluate was purified by passing it through an activated alumina column. The benzimidazoles were analysed by liquid chromatography with UV detection. Recoveries ranged from 85 to 98 % over the concentration range 100 to 3200 ng g⁻¹.

Long et al[39] determined furazolidone in pork muscle tissue. Furazolidone is an antimicrobial agent, as a feed additive it can increase animal vigor and aid in pork muscle growth promotion. Fortified pork muscle tissue was blended with C₁₈. It was washed with hexane and followed by elution of the furazolidone with ethylacetate. The extract was passed through an activated alumina column and was analysed by liquid chromatography with UV detection. The recovery averaged 89.5 % for the concentration 7.8 to 250 ng g⁻¹.

The MSPD process is similar for all extractions with the eluent varying

depending on the analyte being extracted. The MSPD technique has been applied to the extraction of organic species to date it has not, however, been applied to the extraction of inorganic ions. In this work MSPD has been applied to the extraction and analysis of arsenate, MMA and DMA from fish tissue. Extracts were analysed by HPLC with hydride generation AAS detection.

4.2 EXPERIMENTAL

4.2.1 Reagents

Unless otherwise stated, all reagents were of analytical grade. Deionised water was obtained by passing distilled water through a Milli-pore Milli-Q water purification system.

Arsenite and arsenate were obtained from BDH, Poole, Dorset, England. MMA and DMA were obtained as part of a BCR programme (Commission of the European Communities) on arsenic speciation. Phosphate buffer was prepared from 0.01 M sodium dihydrogen phosphate (E. Merk, D-6100 Darmstadt, F.R. Germany) and was adjusted to pH 5.8 with 0.01 M disodium hydrogen phosphate (Riedel-de Haen A.G., D3016 Seelze 1). For HPLC use, this buffer was filtered through a 0.45 μm filter. Concentrated sulphuric acid (Riedel-deHaen A.G., D3016 Seelz 1) was diluted to 1 M with water. 1 % sodium tetraborohydride (Aldrich Chemicals Co. Ltd., Gillingham, Dorset, England) solution was prepared by dissolving sodium tetraborohydride powder in 1 % sodium hydroxide (BDH, Poole, Dorset, England) solution. Argon was obtained from Air Products PLC Molesey Rd., Walton-on Thames, England.

C₁₈ solid phase extraction packing 30 - 70 μm was obtained from Altech Associates Inc., 205 Waukegan Rd., Deerfield IL 60015, England. This was washed with methanol (Fisons Scientific Equipment, Bishop Meadow Rd., Loughborough LE11ORG, England) and then water. Hexane (Lab-Scan Ltd., Stillorgan Industrial Estate, Co. Dublin, Ireland) was used in the sample clean-up. The sample used was cod fish obtained from a local supermarket.

4.2.2 Equipment

A HPLC pump (waters 501) equipped with a Rheodyne 7125 injection valve, a Dionex Ionophore CG5 guard column and Dionex Ionpac CS5 analytical column (Dionex Corporation) were used for the separation of the arsenic species. The eluent from the column at 1 ml min^{-1} was merged with $1 \text{ M H}_2\text{SO}_4$ flowing at 2 ml min^{-1} . This solution went on to mix with 1% NaBH_4 flowing at 1.6 ml min^{-1} . A peristaltic pump (Watson Marlow 501U) was used to pump the sulphuric acid and sodium borohydride. The resultant reaction produced hydrogen and volatile arsines. A gas/liquid separator through which argon carrier gas was passed at 0.6 L min^{-1} stripped the gaseous components from the eluate. The gasses were passed into a flame heated quartz tube for detection. The detection system consisted of an AAS (Instrumentation Laboratory Model 357) with suitable burner modifications to allow a quartz atomisation cell to be supported in an acetylene flame approx. 5 mm above the slot of a 5 cm single slot burner. The atomisation cell consisted of a T-shaped silica tube ($150 \times 2 \text{ mm i.d.}$). Before analysis the atomisation cell was allowed to warm up until it reached equilibrium. The signal from the spectrophotometer was displayed on a chart recorder (Philips). A 1 nm band pass was used and an arsenic hollow-cathode lamp (S & J Juniper and Co., Harlow, Essex, England) was operated at a lamp current of 8 mA and a wavelength of 193.7 nm . Air and acetylene flow rates of 8.5 and 1.9 L min^{-1} respectively were used.

A flow injection system was used instead of the HPLC system for the optimisation of the extraction. This consisted of a peristaltic pump (Watson and Marlow 501U) used for pumping the sulphuric acid and sodium borohydride, a four way rotary valve (tecator 5001) with an external loop for the sample injection, a Kel-F mixing T (Plasma-Therm London, England) and a gas/liquid separator (Plasma-Therm). A sample loop consisting of teflon tubing 1 mm i.d. with a volume of $500 \mu\text{l}$ was used for the analysis. Other equipment used for the

extraction procedure included a mortar and pestle, 2 ml plastic syringe barrels, oven and 0.2 μm disposable syringe filters.

4.2.3 Procedure

Cod fish samples were obtained from a local supermarket. 0.04g of the fish tissue was placed in a mortar. An aliquot of arsenic standard was added to the fish. 0.2g of C_{18} prewashed with methanol and then water, were added. Samples were blended for 30 seconds approx. with a pestle until the mixture was homogenous in appearance. The resultant mixture was placed in a 2 ml plastic syringe barrel containing a paper filter disc. The resulting column was washed with hexane (gravity flow). When the flow ceased most of the excess hexane was removed from the column by drawing it through with another syringe. The column was placed in an oven (70 - 80 $^{\circ}\text{C}$) for 1.5 hours to remove all the hexane. The arsenic was eluted with 0.01 M sodium dihydrogen phosphate adjusted to pH 5.8 with disodium hydrogen phosphate. The resultant extract was made up to 10 ml with the buffer. When analysing by HPLC all samples were filtered using 0.2 μm disposable filters before injection onto the column. The phosphate buffer was also used as the mobile phase. An injection volume of 200 μl was used.

Comparison of spiked sample peak heights to peak heights of pure standards analysed under identical conditions gave percentage recoveries. Inter-assay variability was determined as follows: The mean of three samples at each concentration 100, 200, 300, 400, and 500 ppb was calculated. Standard deviation (SD) corresponding to each mean was divided by its respective mean which resulted in the coefficient of variation (CV) for each concentration. The mean of these CV's was calculated along with its standard deviation, multiplied by 100 and defined as inter-assay variability plus or minus SD. Intra-assay

variability was determined as the coefficient of variation (standard deviation of the mean divided by the mean) of the mean peak height of three replicates of the same sample. Between-day variability was also investigated. The coefficient of variation for the mean of three samples analysed on separate days was calculated.

4.3 RESULTS AND DISCUSSION

4.3.1 Matrix solid phase dispersion

Classical techniques utilised for isolation of arsenic can be labour and material intensive. Multiple sample manipulations to the sample or extract can lead to inconsistent assays. Classical isolation techniques that require heating in acid solutions may lead to degradation of the analyte. A method developed for the isolation of drug residues in milk[31, 32, 35, 36, 38], infant formula[34] and animal tissue[30, 33, 37, 39] overcomes many of the problems associated with classical techniques. Here we have investigated this method for the isolation of inorganic species in our case arsenic species from fish samples. Matrix solid phase dispersion isolations are accomplished by blending the sample, in this case fish, with C_{18} packing material. A column made from the C_{18} /fish matrix is then treated with an experimentally determined solvent sequence. In this matrix solid phase dispersion technique the sample is dispersed over a large surface area and exposes the entire sample to the extraction process. Even though the extracting volume is only 8 ml, approximately, the process is exhaustive whereby a large volume of solvent is passed over an extremely thin layer of sample.

4.3.2 Extraction

4.3.2.1 Fish/packing ratio

Modifications have been made to the matrix solid phase dispersion technique for the extraction of the arsenic species. The packing/sample ratio was investigated and 1/5 fish/packing ratio was required to obtain a semi-dry mixture. The recoveries remained the same when higher packing ratios were used as shown in figure 4.1. At lower ratios of fish/packing the mixture becomes moist due to the fish sample. This mixture is not as easy to handle and all the fish is not in contact with the packing. Therefore the recovery of the analyte is effected. The moist mixture also causes problems when packed onto the column as it tends to block the column and frits and therefore elution of the analyte becomes impossible.

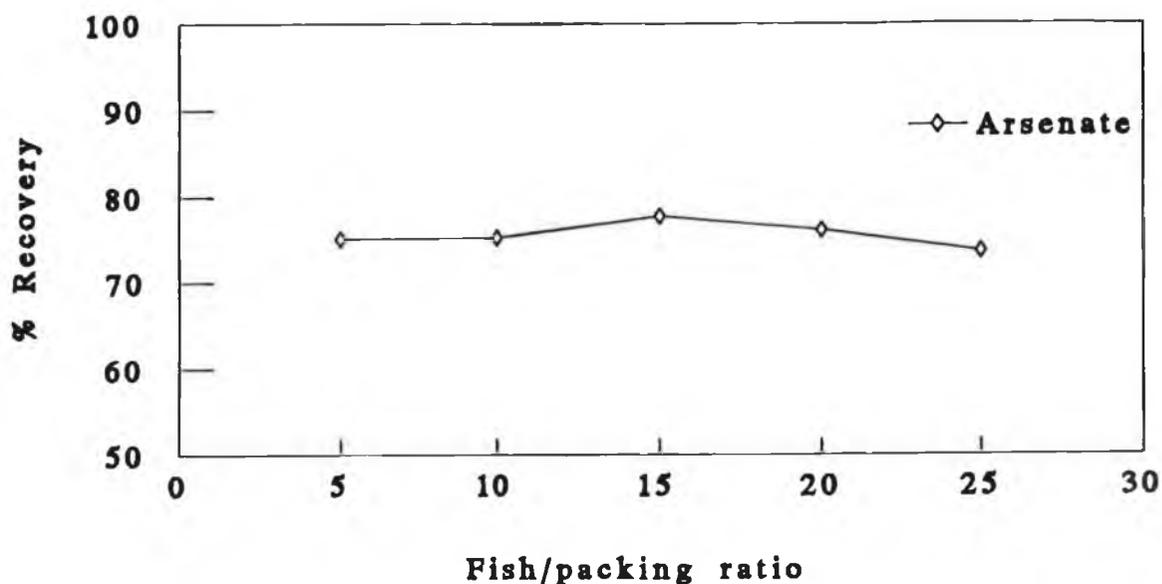


Fig. 4.1 Graph of fish/packing ratio versus recovery of arsenate.

4.3.2.2 Optimisation of column wash

Hexane was used to wash the fish/C₁₈ column. Hexane removes lipids and other non-polar compounds which would interfere with the arsenic analysis, whereas, other more polar compounds remain on the column. Any excess hexane remaining on the column was removed by drying at 70 - 80 °C for 1.5 hours. This drying procedure was effective at removing the hexane and it left the matrix free from organic solvent and ready for elution of the arsenic compounds with aqueous phosphate buffer. Drying times for the hexane removal were varied and a minimum of 1.5 hours was required to ensure all the hexane was removed. If this drying step was not included interference from the hexane was evident during the analysis step.

4.3.2.3 Optimisation of elution buffer

The arsenicals were removed from the fish/C₁₈ column with phosphate buffer. This solvent was used to separate the species using the optimised HPLC system developed previously and was therefore the obvious choice of solvent for the elution of the arsenicals from the fish/C₁₈ column. A 0.01 M buffer concentration at pH 5.8 was used for the separation by HPLC and on investigation this was also found to quantitatively elute the species from the fish/C₁₈ extraction column. Higher concentrations of buffer reduced the signal and the chromatographic separation was also affected.

4.3.2.4 Investigation of packing materials

An anion solid phase extraction material was also investigated. This method was also successful but required a higher concentration of buffer to elute the

arsenicals. It was decided to continue using the C₁₈ material for extraction as recoveries were similar and the weaker buffer could be used resulting in greater compatibility with the analysis using the chromatographic system.

4.3.3 Chromatographic separation

A Dionex column was used in this analysis which could separate MMA or DMA from arsenate. Representative chromatograms of arsenate and DMA standards and arsenate and DMA spiked fish sample are shown in figure 4.2. Separation of arsenite, arsenate, MMA and DMA may be achieved using a PRPX-100 anionic column (Hamilton Co., Nevada, 89510 USA) using phosphate buffer as the eluent.

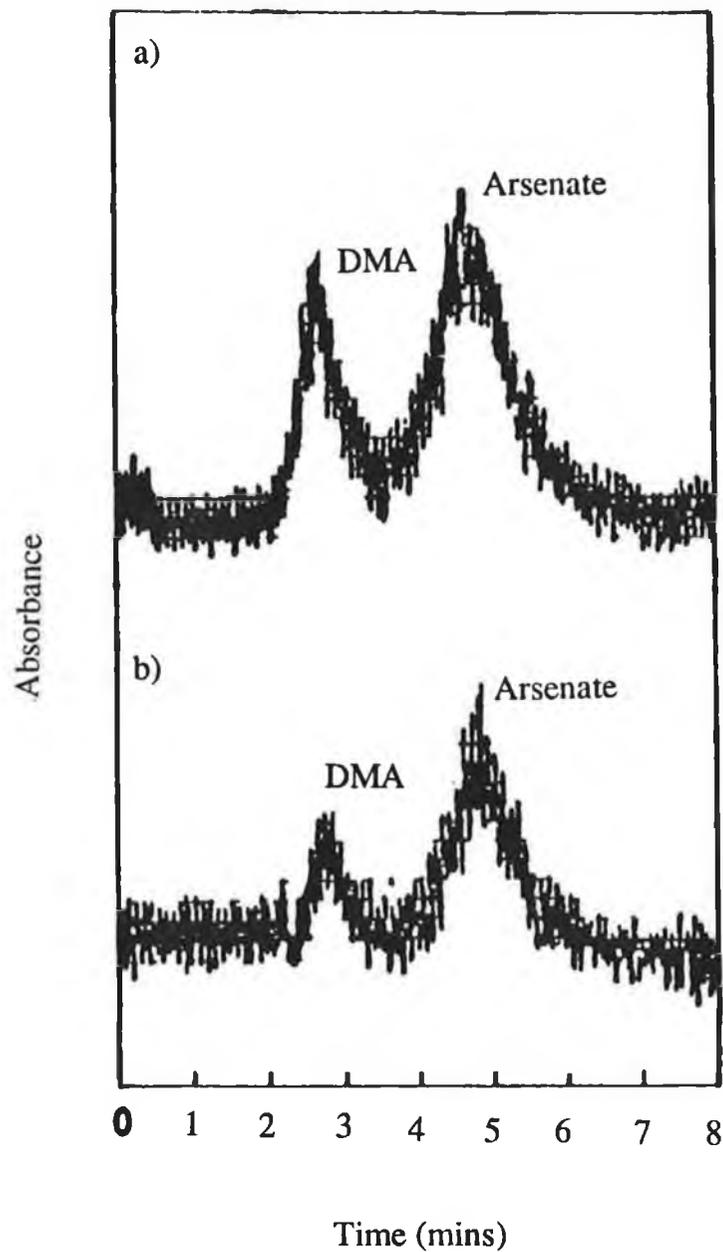


Fig. 4.2 Representative chromatograms of 1.0 ppm arsenate and DMA, using 0.01 M phosphate buffer and a flow rate of 1 ml min.⁻¹, a) standards and b) fortified fish sample.

4.3.4 Evaluation of results

The isolation of the arsenic species using the matrix solid phase dispersion method gave extracts that were linear with respect to increasing concentration of arsenate, MMA and DMA in fortified fish samples as shown in figures 4.3.1 - 4.3.3. Table 4.1 shows the concentrations examined, correlation coefficients, percent recoveries, inter- and intra-assay and between-day variabilities of arsenate, MMA and DMA isolated from spiked fish samples. Arsenite gave poor recoveries and therefore is under further investigation. The recoveries averaged 73.3 %, 66.2 % and 52.0 % for arsenate, MMA and DMA respectively. Overall the variability was good and is reflected in the average inter-assay variability in table 4.1. The intra-assay variability in table 4.1 is representative of the same sample. Between-day variabilities, also on table 4.1, are representative of different samples on different days.

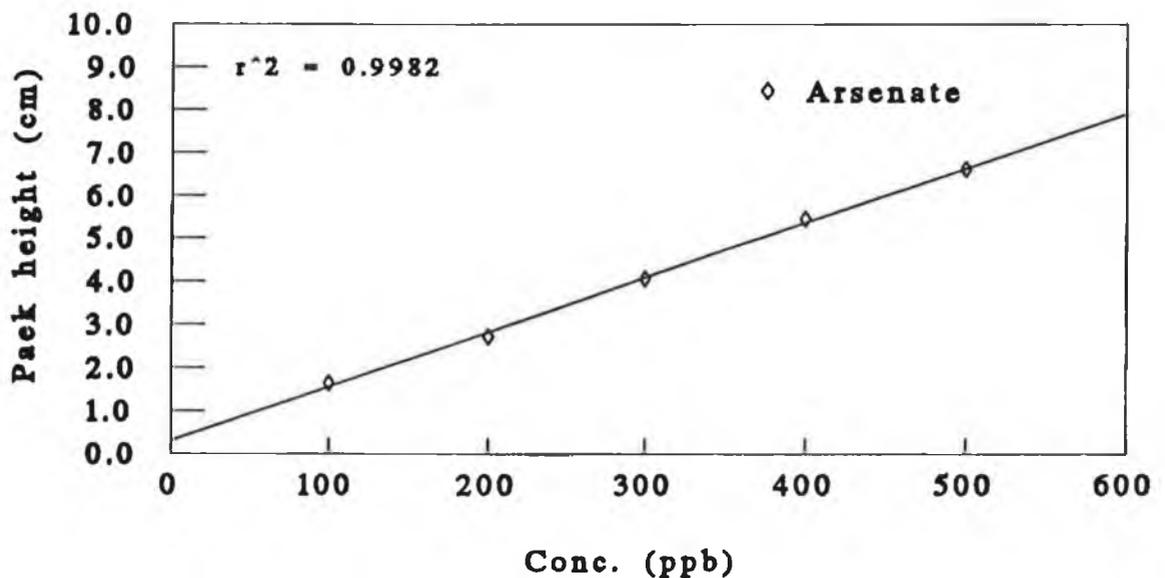


Fig. 4.3.1 Peak height of arsenate from fish versus fortified concentration.

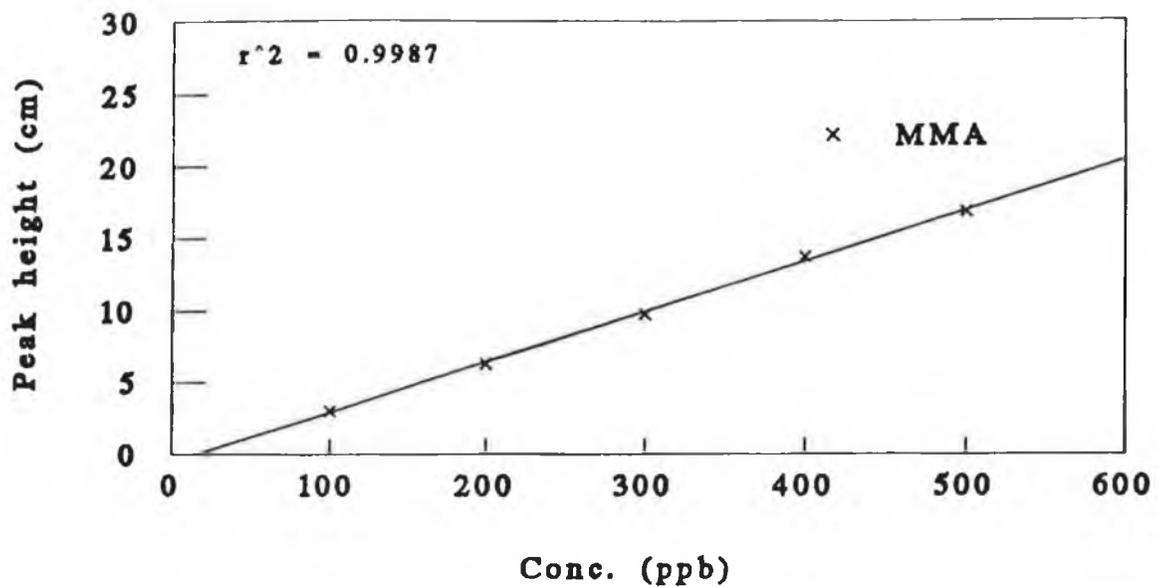


Fig. 4.3.2 Peak height of recovered MMA from fish versus fortified concentration.

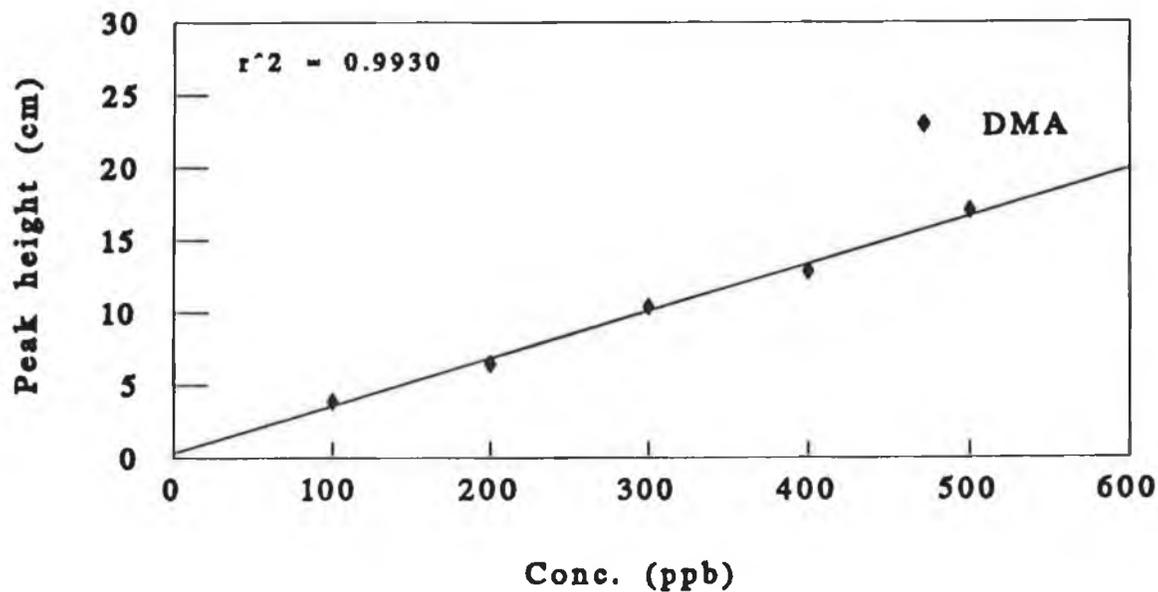


Fig. 4.3.3 Peak height of recovered DMA from fish versus fortified concentration.

Conc. (ppb) ^a	Recovery \pm SD, %		
	Arsenate	MMA	DMA
100	74.3 \pm 3.0	67.1 \pm 2.2	55.1 \pm 4.1
200	69.1 \pm 1.7	64.4 \pm 4.9	48.4 \pm 1.7
300	73.3 \pm 2.1	64.1 \pm 4.1	53.2 \pm 0.7
400	75.5 \pm 3.9	68.1 \pm 3.9	49.8 \pm 2.0
500	74.3 \pm 2.7	66.5 \pm 3.3	53.4 \pm 0.8
Corr. Coeff.			
Std. Curve	0.998	0.999	0.993
Inter-assay variability, %	3.6 \pm 1.1	5.6 \pm 1.6	3.5 \pm 2.5
Intra-assay variability, %	2.4	3.3	3.9
Between-day variability, %	4.4	1.4	1.4

^an = 6 at each conc.

Table 4.1 Correlation coefficients, percentage recoveries, inter- and intra-assay and between-day variabilities of arsenate, MMA and DMA isolated from fortified fish samples.

4.4 CONCLUSION

The purpose of this study was to examine the use of matrix solid phase dispersion for the isolation of arsenic species from fish tissue. This new technique had only been applied to organic extractions ie drug residues. Matrix solid phase dispersion offers a new approach for the isolation of analytes from complex matrices not only for organic but from this work it has been shown to be suitable for inorganic extractions also. In this work this extraction procedure has been applied successfully to the extraction of arsenic species from fish samples. The matrix solid phase dispersion isolation of arsenic species from fish uses small sample size and low volume of washing and extracting solvents. The results obtained are consistent, with inter and intra-assay variabilities achievable being very low. Extraction efficiencies of between 50 % and 70 % were achievable for the species studied. Even though the efficiency could be improved the extractions have been shown to be very reproducible with coefficient of variations up to 5.0 %. This method offers the major advantage of being able to extract individual species. As the technique does not involve the use of oxidative or reducing reagents the information obtainable should be very representative of the speciation within the sample in the case of fish. Matrix solid phase dispersion is an attractive alternative method to the classical approaches which are labour and material intensive, may require multiple manipulations and can result in inconsistent assays. It is sufficiently rapid compared to other extracting techniques which result in accurate information.

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CHAPTER 5

Multimycotoxin detection and clean-up method for aflatoxins, ochratoxin and zearalenone in animal feed ingredients using HPLC and gel permeation chromatography.

5.1 INTRODUCTION

Mycotoxins are toxic substances produced by moulds which cause disease in animals and man. The term mycotoxin comes from the Greek word "mykes" meaning fungus and the Latin word "toxicum" meaning poison or toxin or literally means fungus poison or fungus toxin[1]. Acute diseases caused by mycotoxins are called mycotoxicosis. Some mycotoxins are mutagenic, capable of causing mutagens in susceptible organisms. They can also be hepatotoxic, nephrotoxic, neurotoxic, hemorrhagic, dermatotoxic, genotoxic or teratogenic[2].

5.1.1 History

Several human disease outbreaks and animal poisonings thought to be mycotoxicosis have been recorded[3]. In Japan in the late 1800's and early 1900's "yellow rice" caused serious liver damage in animals and was associated with acute cardiac beri beri in humans. The yellow rice contained a number of *Penicillium* species[1].

In 1960 a severe toxic outbreak occurred in England, which became known as "Turkey X Disease" because of its involvement of a large numbers of turkey poults[4]. Peanut meal, which had been heavily infested with the common storage mould *Aspergillus flavus* was the cause of the disease. An outbreak of trout hepatoma was observed in the U.S. about the same time[5]. This was related to aflatoxin contaminated cotton-seed meal used in the fish food.

As far back as the middle ages outbreaks of ergotism have been recorded in Europe. This disease is also known as "St. Anthony's fire" and it killed thousands of people in France in 943 A.D.[3]. The disease is caused by a group of toxins produced by the fungus *Claviceps purpurea* commonly known as ergot, which grows on rye and other grasses.

During the 1930's and World War II a human disease known as Alimentary Toxic Aleukia occurred in Russia[3]. The disease was caused by eating overwintered mouldy grain and resulted in severe dermal necroses, haemorrhaging, leucopenia (abnormal decrease in leucocytes) and bone marrow degeneration. Several moulds were found to be involved in the disease including *Fusarium poae*, *Fusarium sporotrichoides* and several *Cladosporium* species.

Most foods are susceptible to invasion by moulds during some stage of production, processing, transport or storage. Fortunately the mere presence of toxic mould in food does not automatically mean the presence of mycotoxins on the other hand the absence of toxicogenic moulds does not guarantee that the commodity is free of mycotoxins, since the toxins may persist long after the moulds have disappeared.

To date many mycotoxins have been isolated and characterised. The significance of mycotoxins as causes of human disease is difficult to determine because there is no direct evidence of such involvement in terms of controlled experiments with man. But the various effects of mycotoxins on numerous animal species would make it difficult to believe that humans would not similarly be affected.

5.1.2 Production of mycotoxins

Moulds which have potential to produce mycotoxins include members of the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Trichothaeum*, *Cladosporium*, *Byssochlamys* and *Sclerotinia*. These organisms are capable of growth on a variety of substances and under a diversity of conditions of moisture, pH and temperature[3]. Many toxic compounds have been isolated from mould cultures. However for this discussion only those toxins which may be considered to pose the greatest potential risk to human health as food contaminants are

included. These toxins include aflatoxins, ochratoxin A and zearalenone and are more commonly found in cereals and grains.

5.1.2.1 Aflatoxins

Aflatoxins are secondary metabolites produced by the moulds *Aspergillus flavus* and *Aspergillus paraciticus*[6]. These metabolites were discovered in the 1960's and found to be a potent carcinogen[7]. These moulds grow on corn, peanuts, milo, rice and many other grains and nuts under appropriate conditions[6]. There are four main aflatoxins:- B₁, B₂, G₁, and G₂ plus two others that are of significance M₁ and M₂. The M toxins were first isolated from the milk of lactating animals fed aflatoxin preparations; hence the designation M[1]. Aflatoxins B₁ and B₂ fluoresce blue and aflatoxins G₁ and G₂ fluoresce green under long-wave ultraviolet light. The B and G designations of the toxins refer to the colour of fluorescence[3].

Of all the mycotoxins, aflatoxins are considered the most potent. They are highly toxic and potentially carcinogenic[1]. The most potent of the naturally occurring aflatoxins is aflatoxin B₁[8]. The toxins may be lethal when consumed in large doses; sub-lethal doses produce a chronic toxicity and low levels of chronic exposures results in cancers, primarily liver cancer. Mould growth and aflatoxin production are favoured by warm temperatures and high humidity of tropical and sub tropical regions[1].

In general mycotoxins are complex molecules containing one or more oxygenated acyclic rings.

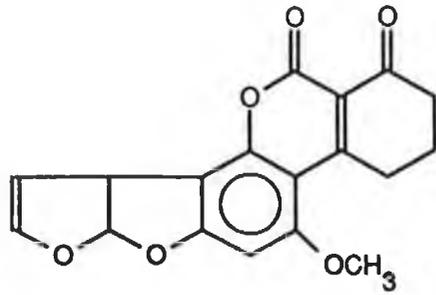


Fig. 5.1 Chemical structure of aflatoxin B₁.

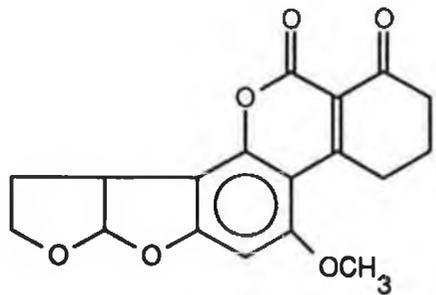


Fig. 5.2 Chemical structure of aflatoxin B₂.

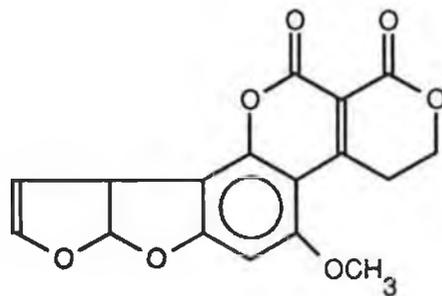


Fig. 5.3 Chemical structure of aflatoxin G₁.

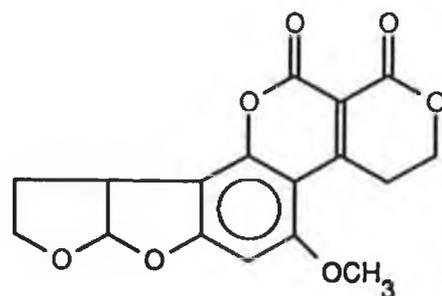


Fig. 5.4 Chemical structure of aflatoxin G₂.

5.1.2.2 Ochratoxin

Ochratoxins are secondary metabolites of several fungal species belonging to the genera *Aspergillus* and *Penicillium*[9]. The most extensively studied compound of this group, ochratoxin A, is considered to be the most toxic. Ochratoxin has been detected in commercial corn, barley, in feed grains and mixed feeds of low quality. Ochratoxin has also been found in dried beans, mouldy peanuts and oats[3].

The first reported natural occurrence of ochratoxin A was in 1969; approximately 150 ppb of ochratoxin A was found in a sample of corn that was infected with *penicillium* and *fusarium* species[10]. Ochratoxin A causes kidney damage in rats, dogs and swine and ochratoxin is thought to be involved in a disease of swine in Denmark known as porcine nephropathy which was associated with the feeding of mouldy barley[3].

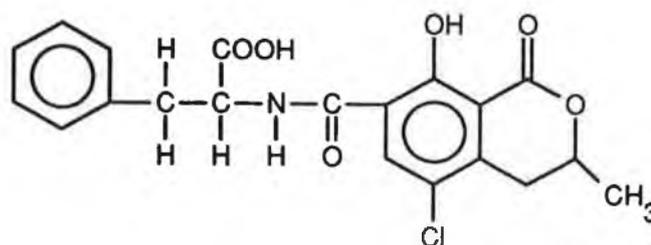


Fig. 5.5 Chemical structure of ochratoxin A.

5.1.2.3 Zearalenone

Zearalenone, also known as F-2, is produced by the *Fusarium* species, primarily *Fusarium roseum*, growing in grains stored at high moisture condition[11]. Zearalenone has been found in maize, corn screenings, wheat, sorghum, barley, oats, sesame seed, hay, silage and various mixed feeds[12]. Zearalenone is an oestrogenic substance and in the female pig zearalenone poisoning gives rise to hypertrophy and prolapse of the vulva as well as to infertility and reduction of litter size[13].

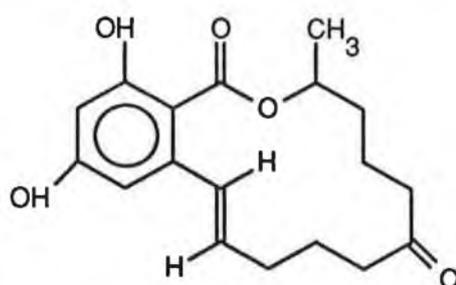


Fig. 5.6 Chemical structure of zearalenone.

5.1.3 Levels of tolerance

A tolerance level of aflatoxin B₁, in groundnut, copra, palm kernal, cotton seed, babassu, maize and products derived from the processing thereof is 20 µg Kg⁻¹ in the European Communities Regulations 1991. Levels have not been set for the other mycotoxins mentioned above.

5.2 EXTRACTION AND CLEAN-UP

In general, mycotoxins are soluble in slightly polar solvents and usually insoluble in completely non-polar solvents. Normally the extraction step is carried out using different organic solvents either alone or in combination with a small amount of aqueous solution which may contain salts or acids. Aqueous solvents more easily penetrate hydrophilic tissues and enhance toxin extraction. After this step the bulk of the sample has been discarded, and the mycotoxin of interest contained in the solution, free from particulate matter. The solution at this stage usually contains other components, such as fats and dyes, which may interfere with the separation and detection of the mycotoxin. This brings us to the next step which is the purification/clean-up process. This stage usually involves the use of a column which separates most interferents from the mycotoxin of interest. The separation may be based on; molecular weight, e.g. gel permeation chromatography (GPC), affinity e.g. immunoaffinity columns or polarity e.g. silica columns. Other purification/clean-up steps which do not use a column may use membranes for the separation of the compounds of interest. The separation using membranes is usually based on molecular weight differences.

5.2.1 Aflatoxins

Methanol/water are common solvent mixtures used for the initial extraction of aflatoxins. Thean et al.[14] used methanol/water 80/20 to extract aflatoxin from corn. After ammonium sulphate treatment (protein precipitation) the aflatoxins are partitioned into chloroform. A silica gel column was used for clean-up before HPLC analysis. Recoveries of added aflatoxin B₁, B₂, G₁ and G₂ were 84 - 118 % at levels of 1.5 - 125 µg Kg⁻¹. Park et al.[14] who carried out a collaborative study on aflatoxins B₁, B₂, G₁ and G₂ in raw peanuts, peanut butter and corn used methanol/0.1 M hydrochloric acid 4/1 for extraction. Following filtration it

was defatted with hexane and partitioned with methylene chloride before silica gel clean-up. Many of the fats and lipids, present in most extracts, can be partitioned into fat solvents such as hexane and discarded.

Holcomb and Thompson[6] used methanol/water 70/30 to extract aflatoxins from feeds. For the clean-up procedure an affinity column, containing antibodies designed to be specific for aflatoxins was used. Recoveries averaged 85 % for B₁ and G₁, 77 % for B₂ and 58 % for G₂. Affinity columns are very specific and usually result in chromatograms with essentially only the aflatoxin peaks present.

Kamimura et al.[16] extracted aflatoxins with chloroform/water 10/1 from cereal and nut samples. A florisil column was used for clean-up and recoveries were high. Florisil is a coprecipitate of magnesia and silica with the approximate composition of 16 % MgO and 84 % SiO₂. Florisil and silica have polar surfaces, however, florisil has higher activity than silica and can better separate different types of non-polar compounds. Paulsch et al.[17] used chloroform/water 10/1 to extract aflatoxins from feedstuffs and also used florisil for clean-up. A C₁₈ Sep Pak was also used for clean-up as Paulsch[17] had special interest in removing citrus pulp, a frequently used ingredient of compound feedstuffs, which contains a number of fluorescent components. Recoveries ranged from 81 to 87 %.

As already mentioned when using methanol/water as an extracting solvent partition into a non-polar solvent is required. To overcome this step and some other clean-up steps such as defatting, Tomlins et al.[18] have evaluated a non-polar bonded phase for the clean-up of maize extracts for aflatoxin determination. Tomlins used methanol/water to extract the aflatoxins and a variety of non-polar bonded phase cartridges, octadecyl(C₁₈), octyl(C₈), ethyl(C₂), cyclohexyl(CH) and phenyl(PH), were evaluated for the clean-up.

The aflatoxins were eluted with chloroform. The study showed that the process was efficient with the PH phase attaining the highest recovery, however, additional clean-up was necessary when quantifying low levels of aflatoxins. Hurst et al.[7] determined aflatoxins in peanut extracts using disposable bonded phase columns for sample clean-up with methanol/water 55/45 being used for extraction. Water was added to the extract and it was applied directly to an aminopropyl and C₁₈ silica column connected in series (pretreated with methanol followed by water before use). The aflatoxins were retained on the C₁₈ column and eluted with methanol. Recoveries ranged from 93 - 104 % for aflotoxins.

Gel permeation chromatography (GPC) has been mainly used for the clean-up of pesticide residues. GPC separates components based on molecular weight. Mycotoxins generally lie between 250 - 400 molecular weight units. Hetmanski and Scudamore[19] have used GPC as a clean-up procedure for aflatoxins. Water/dichloromethane 1/10 was used for the extraction of aflatoxins from cereal and animal feedstuffs followed by GPC for clean-up using dichloromethane/hexane 3/1 as eluent. Recoveries ranged from 70 - 80 %.

In general methanol/water or chloroform (or dichloromethane)/water has been used for the extraction of aflatoxins from feedstuffs. Early on silica gel was very popular for clean-up and is still used, but when extraction with methanol/water is carried out, partition into a non-polar solvent is required. This is time consuming and does not lend itself to automation. In this case it would seem, extraction with a non-polar solvent is the obvious choice. Florisil, although quite similar to silica gel, offers improved separation from interferences in most cases. The introduction of bonded phase clean-up helps get rid of more interferences when used in combination with silica gel and helps avoid the use of other time consuming extractions. This clean-up technique may be used on its own and therefore polar extraction is favoured. GPC uses differences in molecular weight to separate interferences and offers equal clean-up to the techniques already

mentioned and is readily adaptable to automatic procedures. In the past few years a lot of work has been carried on columns which consist of specific antibodies bound to a gel material and contained in a cartridge or column. These are found to be rapid and highly specific.

5.2.2 Ochratoxin A

Chloroform seems to be the most common solvent involved in the extraction of ochratoxin A. Frohlich et al.[20] extracted ochratoxin A from mouldy grain. using phosphoric acid 0.1 M and chloroform 1/12 for the initial extraction. After filtration through anhydrous sodium sulphate, the extract in chloroform was applied to reverse phase thin layer chromatography (RPTLC) for clean-up. The spot containing ochratoxin A, detected by UV light, was scraped and collected into a recovery device. Ochratoxin was eluted from the device with methanol and analysed by liquid chromatography with recoveries of 94 %.

Cohen and Lapointe[21] extracted ochratoxin A from animal feed and cereal grains using chloroform and ethanol 80/20 plus 20 ml 5 % acetic acid. After filtration through Celite 545 the extract in chloroform was purified using a silica gel cartridge followed by a cyano cartridge. Liquid chromatography was used for the final determination recoveries ranged between 82 - 99 %.

Nesham et al.[22] extracted ochratoxin from barley using 0.1 M phosphoric acid and chloroform 1/9. Clean-up was carried out on sodium bicarbonate-diatomaceous earth column. Formic acid and chloroform 1/99 was used to elute ochratoxin from the column. The extracts were quantified by thin layer chromatography (TLC) with recoveries of 81.2 %.

Roberts et al.[23] determined ochratoxin A in animal feedstuffs. Water and chloroform 1/10 was used for the extraction followed by clean-up using Sep-Pak

silica cartridges. The extract after filtration through anhydrous sodium sulphate was mixed with hexane which was then passed through a Sep-Pak cartridge. The Sep-Pak cartridge was washed with ethyl acetate and the ochratoxin was eluted with methanol/formic acid. The eluate was dried and dissolved in chloroform and analysed by TLC.

5.2.3 Zearalenone

Once again chloroform is the main solvent used for extracting zearalenone from feedstuffs. Ware and Thorpe[24] used chloroform and water 10/1 to extract zearalenone from corn. The extract was cleaned up by liquid-liquid extraction, first with 4 % sodium hydroxide, discarding the lower layer, secondly with citric acid plus benzene. Reverse phase HPLC with fluorescence detection was used for determination and the recoveries averaged greater than 89 %.

Moller and Josefsson[25] extracted zearalenone from cereals using chloroform and 0.1 M phosphoric acid 20/1. Silica gel was used for the clean-up. Benzene and hexane or cyclohexane-ethylene dichloride-ethyl ether was used for washing and the zearalenone was eluted with chloroform. The extract was further purified by extracting into alkaline solution and washing with water adjusted to pH 8 and back extracting into chloroform. HPLC was used for the determination with UV detection. Recovery was approximately 80 % with the cyclohexane-ethylene dichloride-ethyl ether mixture.

Cohen and Lapointe[26] extracted zearalenone with chloroform and ethanol from animal feeds. A Sep Pak silica cartridge was used for initial clean-up which removed most of the coloration and acted as a filter. A column containing Saphadex LH-20 in chloroform - isooctane was used for final clean-up which

removed most of the polar constituents. HPLC was used for the determination with fluorescence detection. Recoveries of greater than 90 % were achieved.

Malaiyandi and Barrette[27] extracted zearalenone from corn and mixed feed. Chloroform/water/methanol 10/1/1 was used for extraction which was then evaporated to almost dryness and redissolved in chloroform. Sodium hydroxide was used to extract acidic and phenolic components from the chloroform solution. The aqueous phase, i.e. sodium hydroxide extract, after acidification with hydrochloric acid was extracted with chloroform and then the chloroform layer was washed with sodium bicarbonate. A silica gel column was used for clean-up followed by HPLC for analysis using a UV detector. Recoveries averaged 72 % in corn and 67.3 % in pig starter.

Scott et al.[28] extracted zearalenone from corn based foods. Methanol was used for the extraction and water and hexane were added to the extract. The hexane layer was discarded and the pH of the aqueous layer was adjusted to 9.4 - 9.5 with sulphuric acid. Chloroform was added and the chloroform extract was evaporated to dryness and then dissolved in 0.5 ml of chloroform. Further clean-up which markedly reduces certain polar constituents was carried out by HPLC, with detection by fluorescence, or TLC using fast violet B salt as a spray reagent. Recoveries ranged from 84 - 104 %.

Bagneris et al.[29] extracted zearalenone from animal feeds and grains using chloroform and water. Sodium chloride and sodium hydroxide were added to the filtrate. Citric acid was added to the aqueous layer after the chloroform layer was discarded. The zearalenone was extracted with methylene chloride. Analysis was carried out by HPLC using fluorescence detection. Recoveries averaged 84 %.

5.2.4 Multi-toxin extraction and clean-up

Multi-toxin extraction and clean-up methods have become popular as they are more economical. Hunt et al.[30] extracted aflatoxins and ochratoxin A from a range of foods using aqueous acetonitrile. Lipid material was removed with 2,2,4-trimethylpentane before re-extraction into chloroform followed by clean-up by TLC. Bands containing mycotoxins, identified under UV 385, were removed and the mycotoxins eluted with chloroform/methanol 10/1. The clean extract was analysed by HPLC.

Howell and Taylor[31] determined aflatoxins, ochratoxin A and zearalenone in mixed feeds. Chloroform/water 10/1 was used for the extraction and the extracts were cleaned-up using disposable Sep-Pak silica cartridges. The different mycotoxins were eluted from the cartridges with different solvent mixtures. The mycotoxins were analysed by HPLC using different conditions for each analyte.

Langseth et al.[32] determined zearalenone and ochratoxin A in cereals and feed using chloroform/0.1 M phosphoric acid 10/1 for the extraction. Clean-up was carried out using silica Bond Elut columns. Zearalenone and ochratoxin A were eluted separately from the clean-up columns and both were determined by HPLC.

Chamkasen et al.[33] developed an on-line sample clean-up procedure for the determination of aflatoxins, ochratoxin and zearalenone in cereal grains, oilseeds and animal feeds. Acetonitrile/4 % potassium chloride in water/20 % phosphoric acid 178/20/2 was used for the extraction. Water was added to the filtrate to increase the polarity of the extract and after filtration this was loaded onto an Adsorbosphere C₁₈ precolumn with phosphate buffer/methanol/acetonitrile 9/0.5/0.5. The mycotoxins were eluted from the precolumn onto an analytical column by gradient elution and detected by fluorescence detection.

Scudamore and Hetmanski[34] developed a method for the clean-up of extracts from cereals and animal feeds containing a range of mycotoxins. Dichloromethane/1 M hydrochloric acid 10/1 was used for the extraction. GPC (Bio-beads S-X3) was used for the clean-up. The mycotoxins were determined by HPLC, under separate conditions, using fluorescence and UV detection.

Many mixtures of solvents are used in extracting mycotoxins, however, only one step is generally required for the extraction. In the clean-up procedure several steps may be required in order to get an almost interference free mycotoxin solution to allow for its quantitative determination. Individual mycotoxins have a wide range of properties and there is usually a large number of other constituents present with the mycotoxins which may be co-extracted. It is difficult to have a multitoxin clean-up which can get rid of the interfering constituents and at the same time leave the mycotoxins for quantitative determination.

5.3 SEPARATION AND DETECTION OF MYCOTOXINS

A number of analytical techniques have been developed for the determination of mycotoxins[2, 6, 29, 31 - 33, 35 - 53]. TLC and HPLC are by far the most popular[2, 6, 29, 31 - 33, 35 - 41, 46 - 53]. These two methods are applicable to the analysis of nearly all known mycotoxins. Gas chromatography (GC) has been used mostly for the analysis of zearalenone[52, 53]

5.3.1 Thin-layer chromatography

Up till recently TLC was by far the most commonly used method for mycotoxin determination[35 - 41]. TLC has been widely used for multimycotoxin screening methods as well as for individual mycotoxin analysis. The TLC technique involves applying a concentrated sample to a baseline of a TLC plate (a glass or foil plate coated with silica gel), separation by solvent migration, drying and characterisation of the resultant spots. With different combinations of solvent each mycotoxin will have a characteristic migration and separation pattern known as the R_f value. A vast number of solvent mixtures has been investigated[35 - 41].

Gimeno[35] determined zearalenone in corn, sorghum and wheat using TLC. Various developing solvents were used with UV detection. Aluminium chloride and fast violet B salt were used as spray reagents to enhance the sensitivity. Detection limits were 140 - 160 $\mu\text{g Kg}^{-1}$ when aluminium chloride was used and 85 - 110 $\mu\text{g Kg}^{-1}$ when fast violet B salt was used.

Two dimensional TLC is also used in which the sample is developed in one direction with a given solvent, dried and then developed in a second direction, perpendicular to the first, with a second solvent. Two dimensional

chromatography is particularly suitable for sample extracts containing large amounts of co-extracted substances. Thus, the development in the first direction serves as a clean-up step while the second direction is for the actual detection/quantification.

Shotwell et al.[36] determined aflatoxins in corn dust using TLC. The use of both one or two dimensional TLC was reported. For one dimensional TLC the optimum solvent was found to be chloroform/acetone/water 91/9/1. Optimum solvents for two dimensional TLC were, first direction, ether/methanol/water 91/9/1 and, second direction, toluene/ethyl acetate/formic acid 60/30/10. A detection limit for aflatoxin B₁ of 9 ng g⁻¹ was achieved using densitometry.

In many cases the mycotoxins present are not known and therefore screening methods for their simultaneous detection is required. Egon et al.[37] developed a TLC screening method for aflatoxins, ochratoxin, patulin, sterigmatocystin and zearalenone in cereals. Benzene/hexane 3/1 was used as the first developing solvent to separate the lipids from the mycotoxins. Benzene/ethyl acetate/formic acid 80/20/0.5, which was the second solvent, was used to separate the mycotoxins. Detection was by UV both short and longwave and the addition of aluminium chloride solution was used to enhance the response for zearalenone and sterigmatocystin. The limit of detection was 5 µg for aflatoxins, 10 µg for ochratoxin, 50 µg for patulin, 10 µg for sterigmatocystin and 35 µg for zearalenone per Kg.

Roberts et al.[38] analysed twelve mycotoxins in mixed animal feedstuffs using TLC. The chromatogram was developed in toluene/ethyl acetate/90 % formic acid 60/30/10 and examined under UV light. The detection limits for aflatoxin B₁, ochratoxin A and zearalenone were 3, 80 and 1000 ppb respectively. Takeda et al.[39] determined fourteen mycotoxins in grains using a range of developing

solvents. Detection was by fluorodensitometry and detection limits ranged from 10 to 800 $\mu\text{g Kg}^{-1}$ depending on the mycotoxin.

In the previous examples the mycotoxins were determined on one plate using in some cases one or more solvent mixtures. Detection limits have been found to be lower when the a developing solvent is optimised for each individual mycotoxin, although this is more time consuming and does not act as a screening method. Howell and Taylor[31] determined aflatoxins, ochratoxin and zearalenone in mixed feeds but the mycotoxins were determined singly on separate TLC plates. Zearalenone was developed in chloroform/methanol 97/1, aflatoxins in chloroform/acetone 9/1 and ochratoxin in chloroform/methanol 97/3. Detection was by UV limits in mixed feeds of 3, 0.9, 3, 0.9, 200 and 10 $\mu\text{g Kg}^{-1}$ for aflatoxin B₁, B₂, G₁, G₂, zearalenone and ochratoxin respectively.

Soares and Rodriguez-Amaya[40] analysed aflatoxins, ochratoxin, zearalenone and sterigmatocystin in Brazilian foods using TLC. For screening toluene/ethyl acetate/formic acid 60/40/0.5 was used as a developing solvent with UV detection. For quantification each mycotoxin had a different solvent:- acetone/chloroform 1/9 for aflatoxins, toluene/ethyl acetate/formic acid 5/4/1 for ochratoxin and toluene/ethyl acetate/formic acid 60/40/0.5 for zearalenone. The detection limits were 2, 5, 15 and 55 $\mu\text{g Kg}^{-1}$ for aflatoxins, ochratoxin, sterigmatocystin and zearalenone respectively.

Silica gel is the usual adsorbent used in TLC, however within the last few years reverse phase chemically bonded adsorbents have become popular. Thus changing the choice of mobile phase from a more non-polar to a more polar state. Reverse phase chemically bonded adsorbents include C₂, C₈, C₁₂, C₁₈ and diphenyl. Abramson et al.[41] studied mycotoxins using C₁₈ and diphenyl bonded phases and found that they performed well and could serve as a

convenient confirmation for mycotoxins appearing in normal phase (silica) TLC screening procedures.

5.3.2 High performance thin-layer chromatography (HPTLC)

HPTLC has been found to have better sensitivity and efficiency as compared to TLC. It also has the advantages of using less solvent and can run a large number of samples per plate, it does however require an expensive densitometer[42]. HPTLC obtains better resolution due to the uniform particle plates, new sample application apparatus and multi-optic scanning devices[42]. Lee et al.[43] used HPTLC to simultaneously determine thirteen mycotoxins. Detection limits in the low nanogram range were obtained using UV/visible absorption and in the low picogram range using fluorescence.

Tosch et al.[44] determined aflotoxins in peanut products using HPTLC and compared the technique to HPLC. HPTLC appeared to be equivalent to LC with respect to precision, accuracy and sensitivity. The disposable nature of the HPTLC stationary phase eliminates the problem of residual contamination that effect the life and performance of microparticulate silica gel LC columns. The amount of solvent used was found to be much less with HPTLC. Dell et al.[45] analysed aflotoxins in peanut butter by HPTLC, HPLC and a commercially available enzyme-linked immunosorbent assay (ELISA). The HPTLC method gave more consistent results but the ELISA kit had the advantage of being rapid, cheap and sensitive, however, this method was less precise. The HPLC method was found to be precise but biased i.e. the HPTLC-ELISA methods gave better agreement than HPTLC-HPLC methods.

HPTLC, although it offers several advantages over TLC and it is in most cases equivalent to HPLC. HPLC still remains a more popular method than HPTLC probably due to HPLC being a more versatile instrument.

5.3.3 High performance liquid chromatography

High performance liquid chromatography (HPLC) is applicable to the analysis of nearly all known mycotoxins. Both normal and reverse phase HPLC have been used[6, 21, 29, 31 - 33, 46 - 51]. Pons and Franz (1976)[46] determined aflatoxins in cottonseed and used a silica gel column with a water saturated chloroform- cyclohexane acetonitrile elution solvent. Hunt et al. (1978)[47] determined aflatoxins and ochratoxin in food. Silica gel was used as the stationary phase and chloroform saturated with water and acetic acid as the mobile phase. Detection limits ranged from 0.3 to 12.5 $\mu\text{g Kg}^{-1}$ depending on the mycotoxin determined.

Due to the difficulties in reproducing the mobile phase, reverse phase HPLC is the more common method employed. A C_{18} column is generally used with several solvent mixtures such as water, acetonitrile and methanol. UV and fluorescence detection are by far the most common means of detection. Photodiode array detection (PDA) has also been used for mycotoxin detection[51].

Bagners et al.[29] determined zearalenone in animal feeds and grains. An ODS column was used with a mobile phase of methanol/water 70/30 and a fluorescence detector at 236/418 nm. The limit of detection was 10 ng g^{-1} for zearalenone. Hetmanski and Scudamore[48] analysed zearalenone in cereal extract using an ODS-1 column and a mobile phase of ethanol/water 80/20. Detection was by fluorescence at 285/440 nm after post-column derivatisation.

Aluminium chloride is known to enhance the fluorescence of zearalenone on TLC plates and this reaction has been applied to the determination of zearalenone on-line. The reaction was found to enhance the zearalenone fluorescence by a factor of five. The nature of the chemical reaction involved in the derivatisation is not clearly understood but aluminium chloride is a powerful Lewis acid and it may form a conjugate with zearalenone under derivatisation conditions.

Osborne[49] determined ochratoxin in flour and bakery products. A C₁₈ column was used with a mobile phase of acetonitrile/0.1 % orthophosphoric acid 55/45. An acidic mobile phase is necessary because ochratoxin A is a carboxylic acid and must be chromatographed in the unionised form or peak tailing will occur. Fluorescence detection at 343/430 nm was employed. Detection limits varied between 0.5-1.0 µg Kg⁻¹ for ochratoxin. Cohen and LaPointe[21] determined ochratoxin in animal feed and cereal grains. A C₁₈ column was used with a mobile phase of acetonitrile/water 55/45 plus 1 % acetic acid. Fluorescence detection was used at 330/460 nm. The limit of detection was 0.005 ppm.

Holcomb and Thompson[6] determined aflatoxins B₁, B₂, G₁, and G₂ in rodent feed. A C₁₈ column was used and a mobile phase of water/methanol/acetonitrile 50/40/10. Fluorescence detection of 365/440 nm was used. Post-column derivatisation with iodine was carried out to enhance the fluorescence of B₁ and G₁. Limits of detection were 0.25 ppb for B₁, B₂ and G₁ and 0.12 ppb for G₂. Kok et al.[50] determined aflatoxins in cattle feed using a C₁₈ column with a mobile phase of water/ methanol/acetonitrile 13/7/4 for the separation. Post-column derivatisation was also used with electrically generated bromine. Bromide and nitric acid were included in the mobile phase. This post column derivatisation has the advantage over derivatisation with iodine because iodine reagent is not stable and must be prepared fresh each day. A thermostating oven is also required. Fluorescence was used for detection at 360/420nm. The limit of detection was down to 1 µg Kg⁻¹.

Langseth et al.[32] determined ochratoxin and zearalenone in cereals and feed. A C₁₈ column was used and methanol/0.01 M phosphoric acid 58/42 as the mobile phase. Fluorescence detection set at 270/465 nm for zearalenone and 340/465 nm for ochratoxin. Separate injections were made of each mycotoxin due to the different wavelengths of detection required for each. Limits of detection were 2 - 5 µg Kg⁻¹ for zearalenone and 0.1 - 0.3 µg Kg⁻¹ for ochratoxin. Howell and Taylor[31] determined aflatoxins, ochratoxin and zearalenone in mixed feeds. An ODS column was used. Different mobile phases were used for the determination of each. Fluorescence detection at 274/440 nm for zearalenone, 365/425 nm for aflatoxins and 333/470 nm for ochratoxin. The limit of detection for all mycotoxins was 1 µg Kg⁻¹.

Chamkasem et al.[33] determined aflatoxins, ochratoxin and zearalenone in grains, oilseeds and animal feeds using on-line sample clean-up. A C₁₈ column was used for the separation and gradient elution was carried out with phosphate buffer, methanol and acetonitrile mixtures. Post-column derivatisation with iodine was also included with two fluorescence detectors were used:- one before the derivatisation to detect the zearalenone which is affected by derivatisation and one after derivatisation. Limits of detection were 5 ppb for aflatoxin and ochratoxin and 30 ppb for zearalenone.

Frisvad and Thrane[51] developed a HPLC method for the determination of 182 mycotoxins based on retention indices and photo diode array (PDA) detection. A C₁₈ column was used and gradient elution with water and 0.05 % trifluoroacetic acid in acetonitrile. The advantage of PDA is that it provides both multiwavelength and spectral information in a single chromatographic run. This method was not applied to real samples, however, it did provide the base for the development of a multitoxin detection method. Kuronen[2] developed a method for mycotoxins using retention indexes and diode array detection (DAD). Gradient elution using acetonitrile and water mixtures and a C₁₈ column were

used and the detection of aflatoxins from spiked almond paste was demonstrated. Many interfering compounds were found to co-elute despite a clean-up of the sample. Background peaks interfered with the HPLC-DAD determination, and although the aflatoxins could not be determined in a one step HPLC procedure they were easily identified by retention index monitoring (RIM) and DAD after collection, concentration and re-injection of the separate aflatoxin fractions

The use of RIM-DAD does need further investigation in its use for real samples where interferences may be present. However the efficiency and detection do depend on the extraction and clean-up method employed especially when dealing with multimycotoxin determinations.

Reverse phase HPLC is by far more popular for the analysis of mycotoxins. It is a more suitable method for the analysis of mycotoxins when the toxins are sensitive to environmental factors such as oxygen and light. HPLC has a wide range of applications and is therefore readily available.

5.3.4 Gas chromatography

GC is mainly employed for the analysis of zearalenone. HPLC and TLC are more popular techniques as they may be used for a wide range of mycotoxins. Scott et al.[28] determined zearalenone in cornflakes and other corn based foods by HPLC, TLC and GC-high resolution mass spectrometry. GC was found to be the most sensitive and selective method but its use is limited by the availability of the instrumentation. Bata et al.[52] determined zearalenone in cereal samples by capillary GC which allowed shorter columns to be used. A flame ionisation detector (FID) was used and the limit of detection was 100 ppb. Thouvenot and Morfin[53] determined zearalenone in corn by GC on a capillary glass column. An FID was used and the limit of detection was 100 ppb zearalenone in corn.

Rosen et al.[54] used GC/MS/select ion monitoring for confirmation of aflatoxin B₁ and B₂. Analysis of aflatoxins by GC/MS had been impossible until recently because aflatoxins could not be chromatographed on packed or open tubular capillary columns probably because of binding and/or decomposition by trace metals in the glass columns. With the advent of fused silica capillary columns (containing < 1 ppm metals) coupled with medium resolution selected ion monitoring confirmation of aflatoxins B₁ and B₂ was possible. Peanuts were analysed by TLC and those found to be negative i.e. < 1 ppb were then analysed by GC/MS. The limit of detection for aflatoxins B₁ and B₂ in peanut samples was 0.1 ppb.

Because GC may only be used for a few mycotoxins it is not a popular technique for this analysis. The availability of equipment i.e. MS, is another drawback.

5.4 CONCLUSION

Due to the increasing awareness of the hazards posed to both animal and human health by mycotoxins in feeds and foodstuffs the development of methods for extraction/clean-up, separation and detection of these has been well documented. There is however room for development in the area of multitoxin analysis which would speed up the analysis time over individual assays. Ideally with one extraction, clean-up, separation and detection method, the analysis of a range of mycotoxins could be carried out. TLC has been the most successful in this area, but more so in relation to screening, however, as already mentioned mycotoxins have a wide range of properties and to find a single method to include all mycotoxins would seem impossible. Some recent publications are trying to overcome this barrier. Scudamore and Hetmanski[34] have developed a multitoxin clean-up method for a wide range of mycotoxins. Kuronen[2] used HPLC DAD to separate and detect many mycotoxins. Further work needs to be

carried out to use these methods together and to develop the HPLC separation beyond just screening.

The work presented here describes a method which allows the determination of aflatoxins B₁, B₂, G₁ and G₂, ochratoxin A and zearalenone in animal feed using a multitoxin extraction and clean-up method. Gradient elution in conjunction with HPLC was used for the determination. Maize, palm and wheat were used for recovery, reproducibility and repeatability studies.

5.5 EXPERIMENTAL

5.5.1 Reagents

Aflatoxin B₁ and ochratoxin A were obtained from Calbiochem (San Diego Calif., USA). Aflatoxins B₂, G₁ and G₂ were obtained from Makor Chemicals Ltd. (Jerusalem, Israel). Zearalenone was obtained from Carl Roth (KG 1975 Karlsruhe 21, Germany). All solvents were of analytical reagent grade and were purchased from Merck (Darmstadt, Germany), Water was obtained from a Milli-Q system (Millipore, Bedford, M.A. USA).

The following stock solutions were prepared: a) aflatoxin B₁, 1 µg ml⁻¹ in chloroform, b) aflatoxin B₂, 1 µg ml⁻¹ in chloroform, c) aflatoxin G₁, 1 µg ml⁻¹ in chloroform, d) aflatoxin G₂, 1 µg ml⁻¹ in chloroform, e) ochratoxin A, 1 µg ml⁻¹ in methanol, f) zearalenone, 100 µg ml⁻¹ in methanol.

5.5.2 Equipment

The gel permeation chromatography (GPC) equipment consisted of a 60 mm x 6 mm i.d. glass column (Spectrum Medical Industries Inc., Los Angeles, California, USA) fitted with a 40 - 60 µm porous bed support and adjustable plunger packed with Bio-Beads SX-3 gel (Bio-Rad Ltd., Watford, U.K.). The gel was suspended in a mixture of dichloromethane, ethylacetate and formic acid (49.9/49.9/0.2) for one day before loading onto the glass column. The height of the column was 55 mm. A Waters (Waters Associate Inc., Made St. Milford MA, USA) M-45 pump was used and a Waters WISP 710B automatic injector. A Gilson (Villiers, le Bel, France) 202 fraction collector and 201 - 202 fraction controller were used for collecting fractions.

The HPLC equipment included a Gilson 305 and 302 pump, a Gilson 805

manometric module, Gilson 811B dynamic mixer, chromosphere RP-C₁₈ column (Chrompack, Middelburg, Netherlands), KOBRA device (Lamers and Pleuger, Den Bosch, Netherlands) for generating bromine for the post-column derivatisation and a Perkin Elmer LS4 fluorescence detector (Perkin Elmer, Norwalk, CT, USA). An automatic Gilson 231 sample injector with a Gilson 401, dilutor was also used.

Other equipment included a Desaga flask shaker (Heidelberg, Germany) and a Büchi rotary evaporator (Switzerland).

5.5.3 Procedure

5.5.3.1 Extraction

A 25 g portion of well mixed, finely ground sample was weighed into a 250 ml erlenmeyer flask. 12.5 g Celite (Johns-Manville, Denver, CO, USA), 12.5 ml 1M hydrochloric acid and 125 ml dichloromethane were added. The flask was stoppered and shaken for 30 minutes before filtering the sample through a Whatman No. 1 filter paper into a 250 ml round bottom flask. The residue in the filter paper was rinsed with 3 x 25 ml portions of dichloromethane. The combined filtrate and washings were evaporated to near dryness (approx. 0.5 ml) by rotary evaporation at 30 °C. The residue in the flask was transferred to a 10 ml volumetric flask with at least four rinses of dichloromethane, approximately 1 ml each time, 5 ml of ethylacetate and 0.02 ml formic acid were added and the solution was made up to the mark with dichloromethane.

5.5.3.2 Clean-up

Approximately 1 ml of the sample extract was filtered through a disposable 0.45

μm organic filter (Acrodisc CR PTFE, Gelman Science 600 S Wagner Rd. Ann Arbor, MI 45106-1445, USA). 200 μl of the filtrate was injected onto the GPC column using a WISP 710 B automatic injector. Dichloromethane, ethyl acetate and formic acid (49.9/49.9/0.20) were passed through the column at 0.3 ml min.⁻¹ One fraction from 25 - 45 minutes was collected. 2 ml of water were added to the fraction which was stoppered and well shaken. The lower organic layer was passed through anhydrous sodium sulphate. The sodium sulphate was rinsed with 5 ml dichloromethane. The combined filtrate and washings were evaporated to near dryness (approximately 0.5 ml). The residue was taken up in water/acetone 85/15. This was well shaken and sonicated for 5 minutes and then filtered through a disposable organic filter before HPLC determination.

5.5.3.3 High performance liquid chromatography

A schematic diagram of the HPLC set-up is shown in figure 5.7. A gradient solvent system was used with a mobile phase A of water/methanol/acetonitrile (180/70/40) plus 1 mM nitric acid plus 1 mM potassium bromide. A mobile phase B of 0.01 M phosphoric acid/acetonitrile (50/50). The initial percentage of A was 100 %. This was maintained for 8 minutes after injection. Over the next 5 minutes the percentage A was reduced to 30 % and the percentage of B increased from 0 % to 70 % linearly. These were maintained at these levels for the following 14 minutes. The percentage A was then increased to 100 % and the percentage B decreased to 0 % linearly over the next 5 minutes and maintained for 8 minutes at which point the next injection could be made.

Post-column derivatisation with bromine was used to enhance the sensitivity of aflatoxin B₁ and G₁ in conjunction with fluorescence detection with a pre-programmed wavelength change. The derivatisation with bromine decreased the sensitivity of zearalenone. It was therefore necessary to inject the extract again

without derivatisation in order to determine zearalenone.

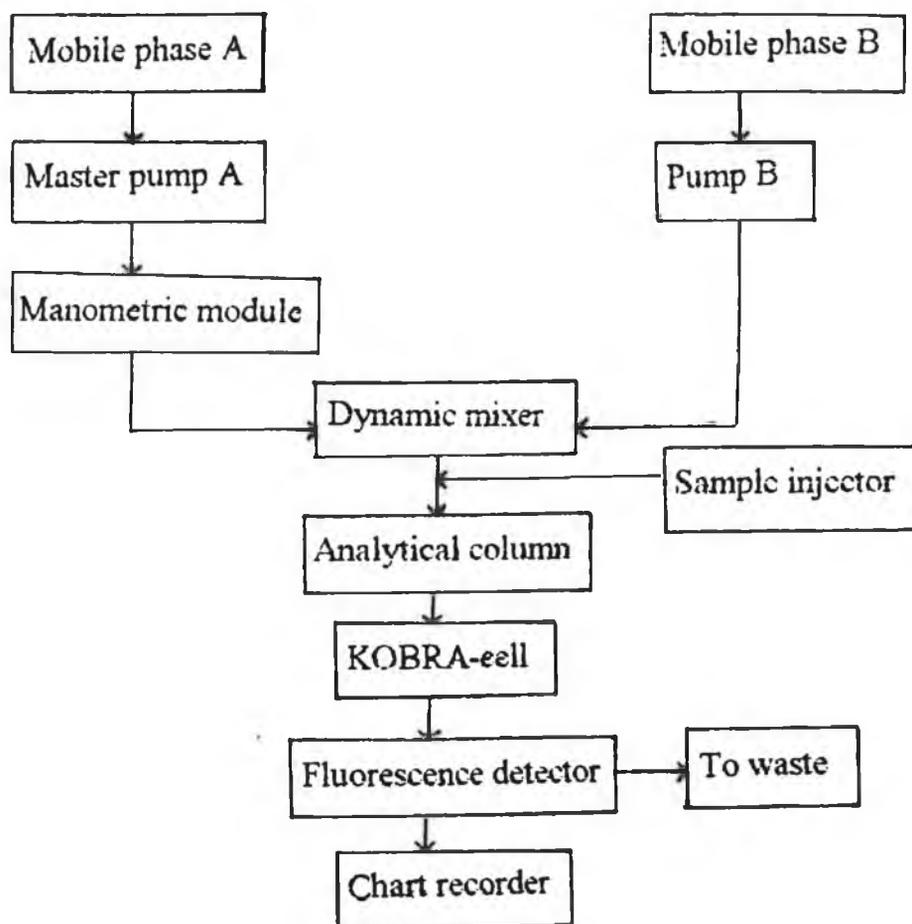


Fig. 5.7 Schematic diagram of HPLC gradient elution setup.

The wavelength of excitation and emission were changed as follows:

0 - 20.0 min.	= 369/422 nm
20.0 - 24.0 min.	= 335/500 nm
24.0 - 26.2 min.	= 310/470 nm
26.2 - 38.9 min.	= 335/500 nm
38.9 - 40.0 min.	= 369/422 nm

5.6 RESULTS AND DISCUSSION

5.6.1 Extraction and clean-up

The extraction and clean-up method used was developed by Scudamore and Hetmanski[34]. Some minor modifications were made. Before the GPC injection, extracts were filtered through a 0.45 µm disposable filter in order to remove any suspended particles. The GPC column and injection volume were smaller than that used by Scudamore and Hetmanski[34] however, these were reduced proportionally in our work. The flow-rate was also reduced proportionally. The solvent consumption is reduced due to the smaller injection volume and lower flow rate, thus making the analysis more economical.

The fraction in which the mycotoxins eluted from the GPC, was determined by monitoring the output, using fluorescence detection at an appropriate wavelength of excitation and emission, for each mycotoxin. Results are shown in table 5.1. Mycotoxins with a higher molecular weight eluted earlier, as expected. In table 5.1 all mycotoxins are eluted between 32 and 50 minutes. The elution time of these mycotoxins may change with time possibly due to compression of the column. The top of the column may need to be repacked from time to time due to the build up of impurities. These factors lead to a change in elution time of the mycotoxins and therefore before a batch of samples are collected the elution time of the first and last eluting compound should be checked.

Mycotoxin	Wavelength (Ex/Em)	Elution time (min.)	Concentration (ng)
Aflatoxin B ₁	369/425	35.0 - 46.0	0.100
Aflatoxin B ₂	369/425	36.5 - 49.5	25.000
Aflatoxin G ₁	369/425	34.0 - 48.5	0.010
Aflatoxin G ₂	369/425	34.0 - 45.5	0.002
Ochratoxin A	335/500	32.5 - 40.0	0.200
Zearalenone	310/510	35.5 - 46.0	20.000

Table 5.1 Elution of mycotoxins from the GPC column.

5.6.2 High performance liquid chromatography separation

Water/acetone was used to dissolve the mycotoxins for HPLC analysis as it had been used previously by Kok et al.[50] to determine aflatoxins in cattle feed. The dissolved mycotoxins were filtered through a 0.45 µm disposable filter in order to remove residue drops which did not dissolve. Residue drops were also present if acetonitrile/water (1/1) was used. This was the solvent used by Scudamore and Hetmanski[34]. The filtration did not affect recovery. It also ensured that a clean sample was injected onto the HPLC column.

Kok et al.[50] used a mobile phase of water/methanol/acetonitrile 130/70/40 plus 1 mM nitric acid and 1 mM potassium bromide to separate aflatoxins B₁, B₂, G₁ and G₂. By using a water ratio of 180, better separation of aflatoxins was

achieved. In order to elute ochratoxin A and zearalenone the polarity of the mobile phase had to be decreased. The elution of ochratoxin A containing a carboxylic acid group also requires an acidic mobile phase[32]. The second mobile phase in the gradient elution system was 0.01 M phosphoric acid/acetonitrile (50/50). A similar mobile phase had been used by Howell and Taylor[31] for the determination of zearalenone and ochratoxin A.

Calibration graphs of aflatoxin B₁, B₂, G₁ and G₂, ochratoxin and zearalenone are shown in figures 5.8.1 to 5.8.6. Standard solutions containing known amounts of aflatoxins B₁, B₂, G₁ and G₂, ochratoxin A and zearalenone, made up in the GPC mobile phase, were injected onto the GPC column, collected and determined by the HPLC method. Table 5.2 shows the recoveries obtained.

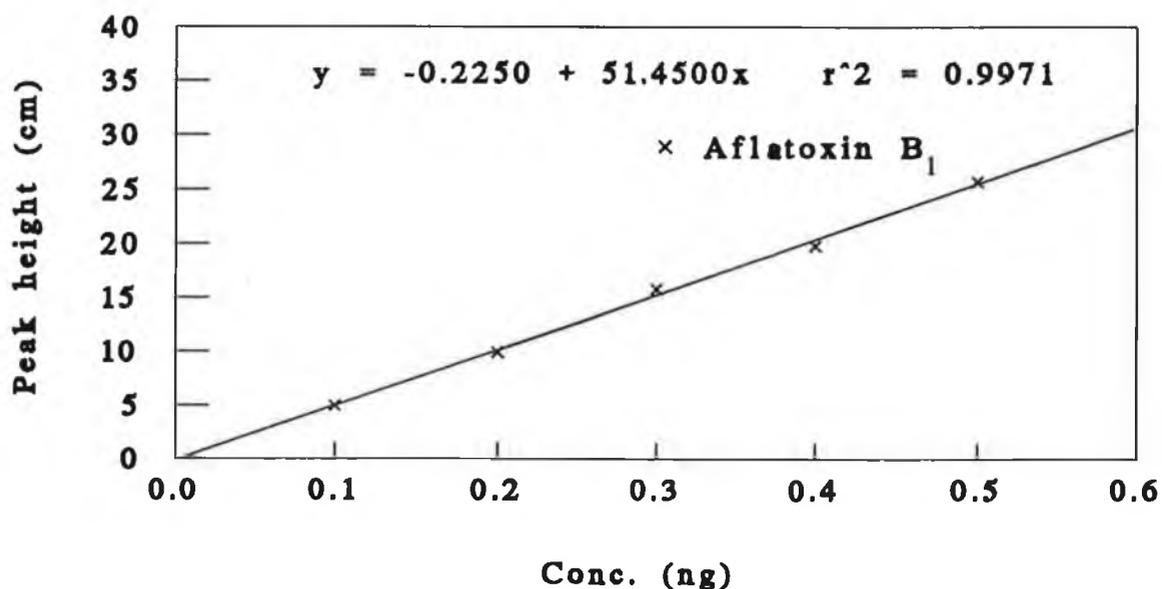


Fig. 5.8.1 Calibration graph for aflatoxin B₁.

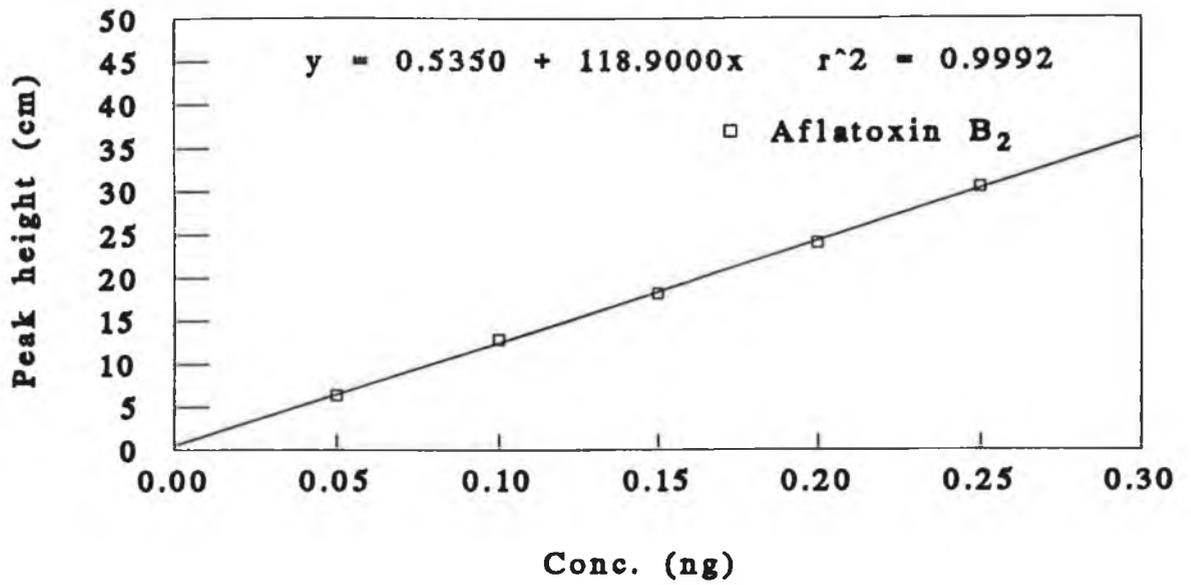


Fig. 5.8.2 Calibration graph for aflatoxin B₂.

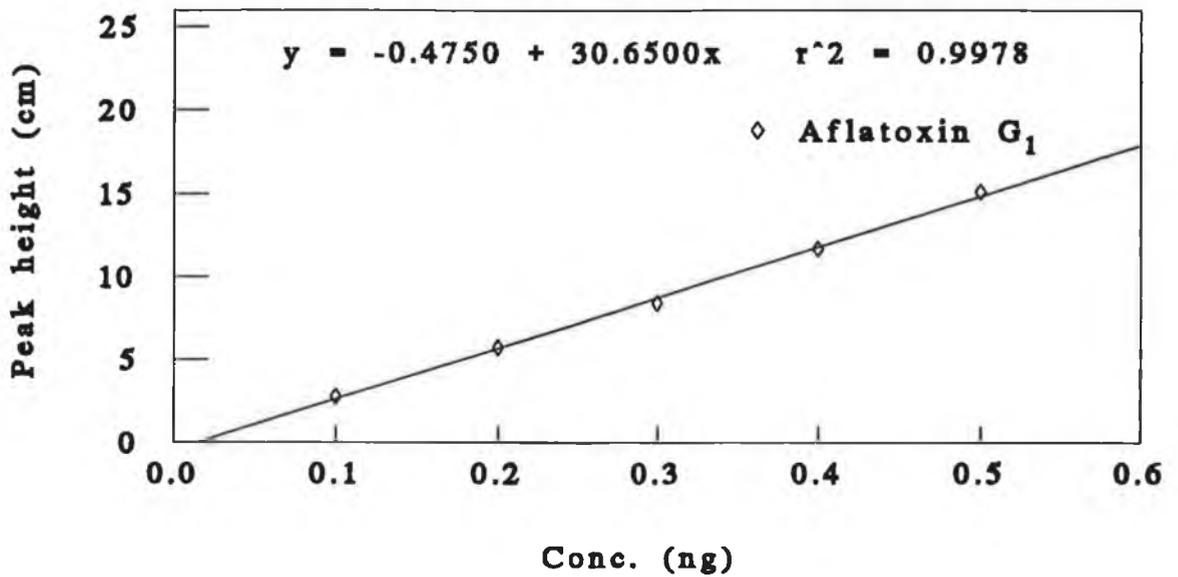


Fig. 5.8.3 Calibration graph for aflatoxin G₁.

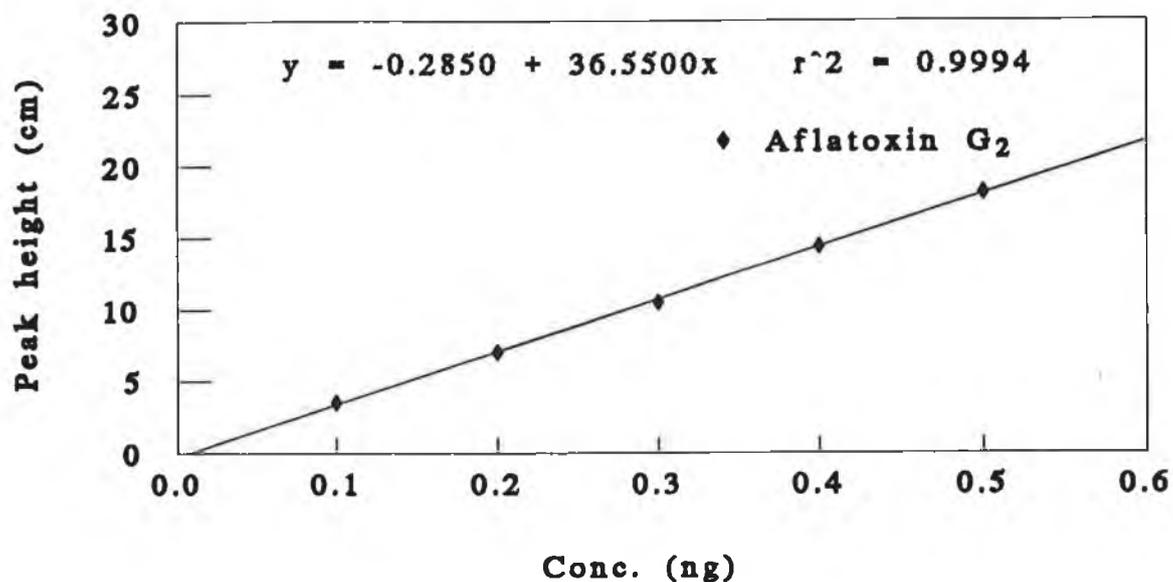


Fig. 5.8.4 Calibration graph for aflatoxin G₂.

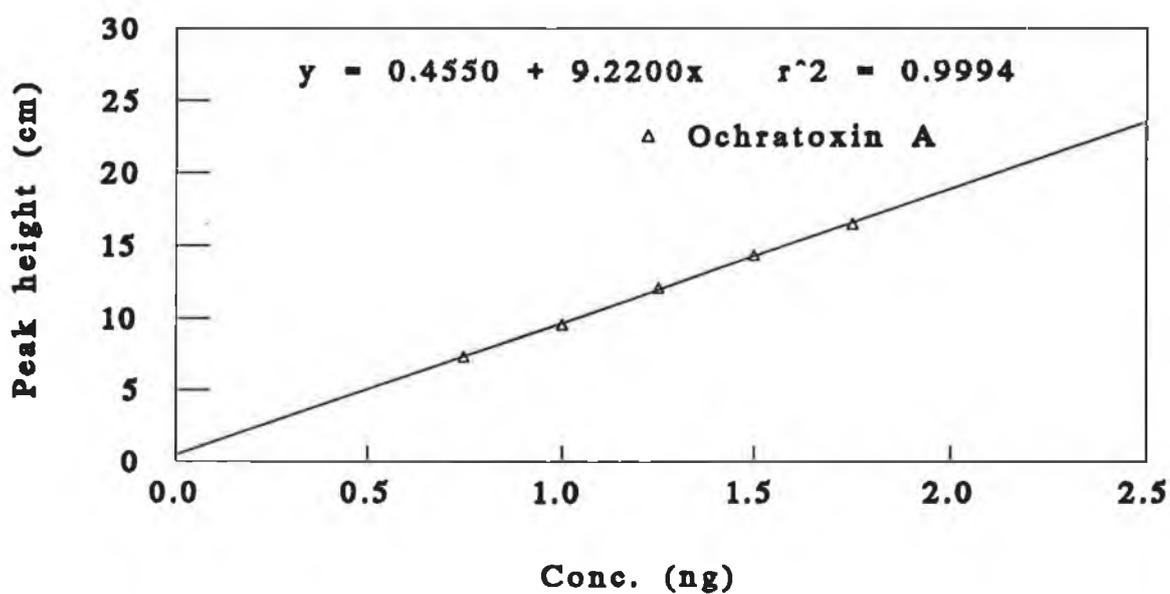


Fig. 5.8.5 Calibration graph for ochratoxin A.

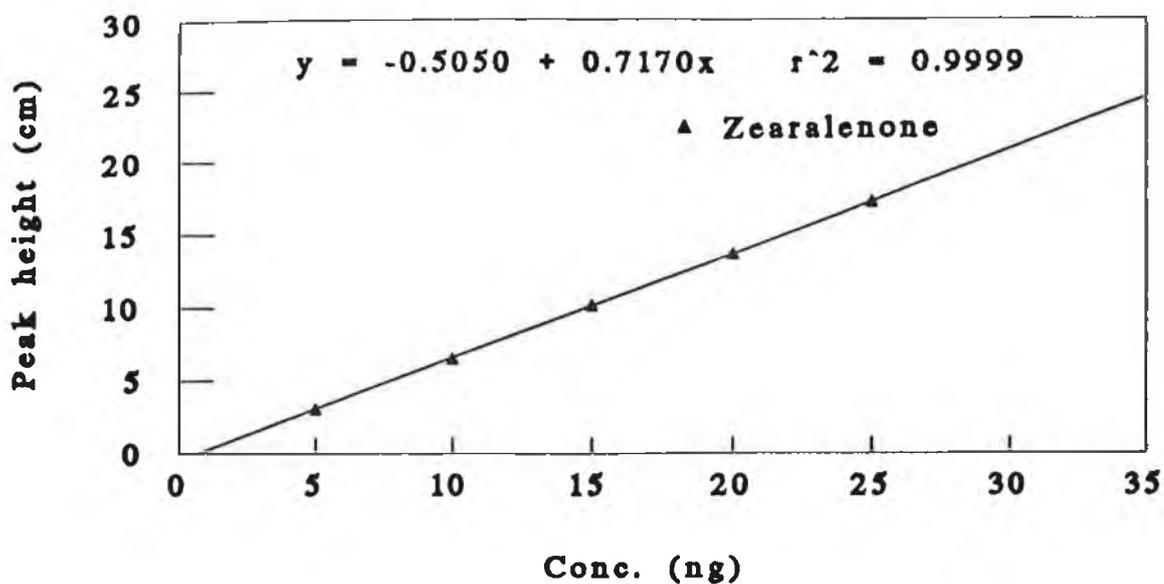


Fig. 5.8.6 Calibration graph for zearalenone.

% Recovery	B ₁	B ₂	G ₁	G ₂	Ochratoxin A	Zearalenone
Mean	99.1	96.5	100.5	98.5	74.0	87.3
S.D.	5.8	4.0	13.7	11.2	10.4	5.9
% CV	5.8	4.1	13.6	11.4	14.1	6.7

Table 5.2 Recovery of mycotoxin standards. Levels of mycotoxins used were 0.2 ng aflatoxins B₁, G₁ and G₂, 0.1 ng aflatoxin B₂, 1 ng ochratoxin A and 20 ng zearalenone. The analysis was repeated three times.

5.6.3 Post-column derivatisation

Post-column derivatisation with bromine was used to enhance the sensitivity of aflatoxin B₁ and G₁ in conjunction with fluorescence detection with pre-programmed wavelength change. Kok et al.[50] showed that the fluorescence intensity of aflatoxin B₁ and G₁ increased after the addition of bromine solution. The reaction is believed to be the bromination of the 8,9 double bond. Aflatoxin B₂ and G₂ do not react with bromine owing to the absence of the double bond. The fluorescent signal enhancement of the aflatoxin is carried out by derivatisation on-line, with electrochemically generated bromine. Bromine is produced from bromide present in the mobile phase in an electrochemical cell after the column. The derivatisation with bromine decreases the sensitivity of zearalenone. It was therefore necessary to inject the extract again without derivatisation in order to determine zearalenone.

Typical chromatograms of samples spiked to contain aflatoxins B₁, B₂, G₁ and G₂, ochratoxin A and zearalenone are shown in figures 5.9 and 5.10. The peak identified as zearalenone in the blank sample in figure 5.10 has the same retention time as that of the spiked zearalenone sample. The zearalenone peak also disappears when post-column derivatisation is used as indicated in figure 5.9.1 thus confirming the peak is zearalenone. Any change in baseline at 20, 24 and 26.2 minutes is due to a wavelength change. Although the post-column derivatisation with bromine enhances aflatoxin B₁ and G₁, the zearalenone peak disappears under these conditions. This however, can be used as a useful confirmation test for zearalenone in particular with samples that contain interferences that coelute with zearalenone and give false positive results[33]. In order to determine zearalenone the extract must be re-injected without derivatisation. To avoid this re-injection a second detector, set for the detection of zearalenone, may be placed after the column and before derivatisation. The KOBRA-cell also continues to influence the chromatogram for some time i.e. a

few hours, after it is switched off. In the set-up used it is therefore not possible to determine zearalenone immediately. The zearalenone should be determined first, before derivatisation is carried out for the determination of the other mycotoxins, on the other hand the KOBRA-cell may be by-passed. Post-column derivatisation with bromine not only enhances the fluorescence intensity of aflatoxins B₁ and G₁ but also reduces and in some cases completely diminishes, the fluorescence intensity of many interfering components.

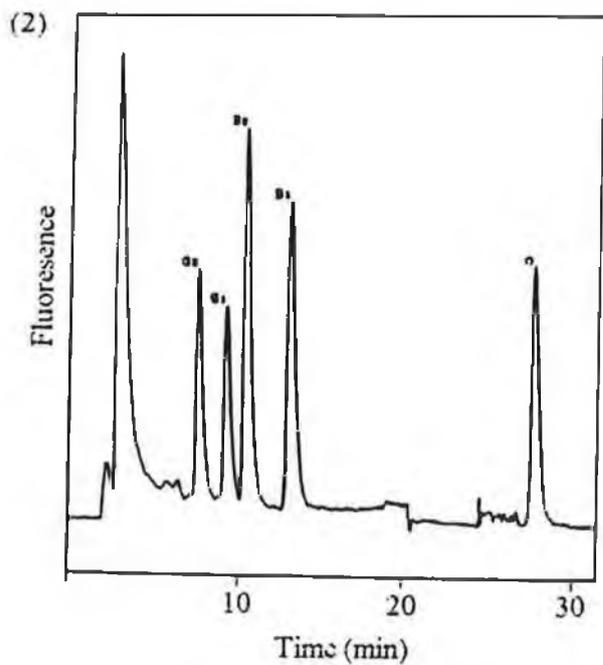
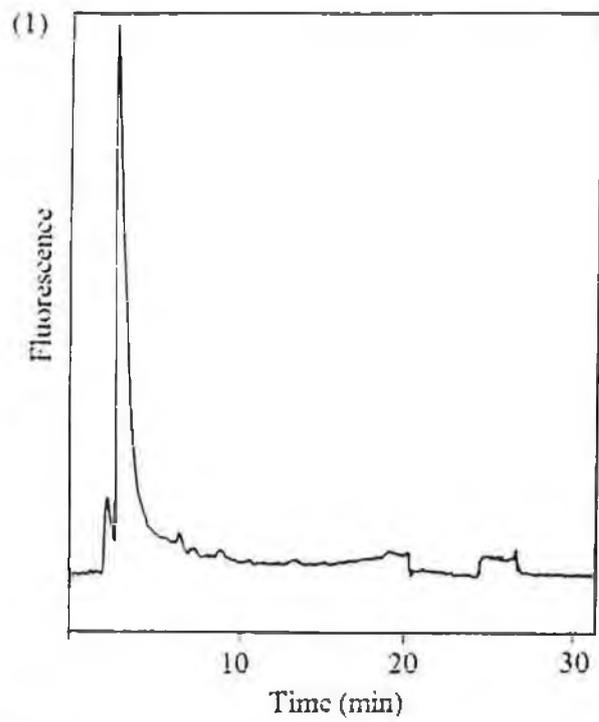


Fig. 5.9 Chromatograms of (1) maize samples and (2) maize sample spiked to contain $3.2 \mu\text{g Kg}^{-1}$ aflatoxin B_1 , G_1 , and G_2 , $1.6 \mu\text{g Kg}^{-1}$ aflatoxin B_2 , $16 \mu\text{g Kg}^{-1}$ ochratoxin A and $320 \mu\text{g Kg}^{-1}$ zearalenone with post-column derivatisation. No zearalenone detected due to post-column derivatisation.

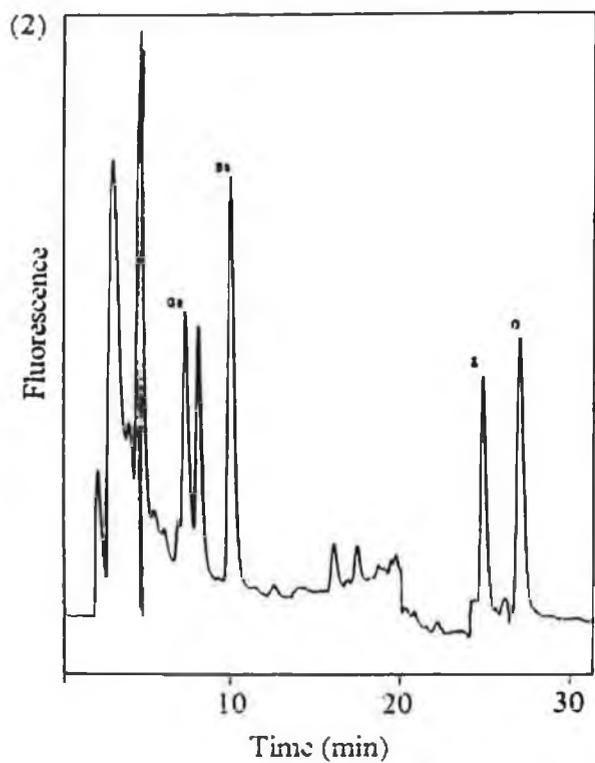
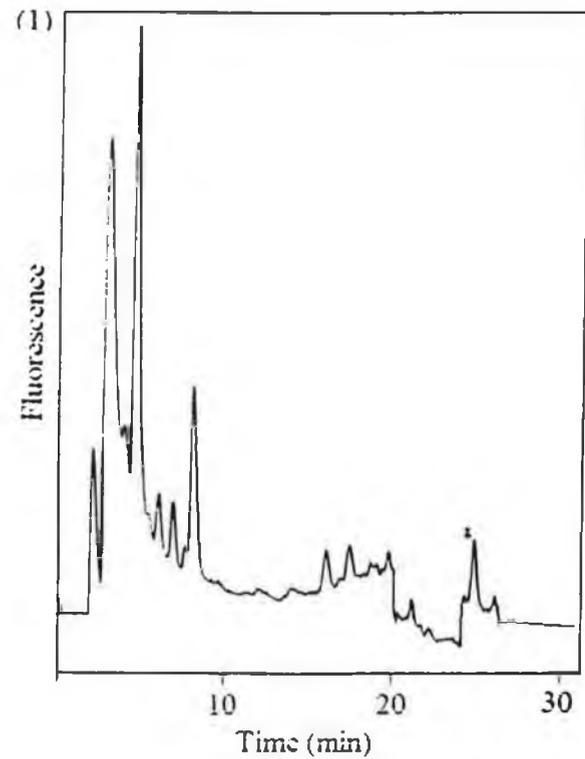


Fig. 5.10 Chromatograms of (1) maize samples and (2) maize sample spiked to contain $3.2 \mu\text{g Kg}^{-1}$ aflatoxin B_1 , G_1 , and G_2 , $1.6 \mu\text{g Kg}^{-1}$ aflatoxin B_2 , $16 \mu\text{g Kg}^{-1}$ ochratoxin A and $320 \mu\text{g Kg}^{-1}$ zearalenone without post-column derivatisation.

5.6.4 Analysis

5.6.4.1 Recoveries

Recoveries for spiked extracts of maize, palm and wheat are shown in table 5.3. Known amounts of standard mycotoxin solutions were added to extracts of maize, palm and wheat. Recoveries for zearalenone in maize and palm were low.

% Recovery	B ₁	B ₂	G ₁	G ₂	Ochratoxin A	Zearalenone
Maize	93.4	102.4	97.3	97.9	N.D.	49.7
Palm	81.4	99.6	93.1	90.2	N.D.	17.2
Wheat	90.9	102.2	103.9	103.0	N.D.	71.6

N.D. = not determined

Table 5.3 Recovery of mycotoxins from spiked extract. Levels of mycotoxins used the same as in table 5.1.

5.6.4.2 Reproducibility

The reproducibility of the method was checked using different types of feed. Three feed ingredients were analysed three times each for aflatoxins B₁, B₂, G₁ and G₂, ochratoxin A and zearalenone, which were spiked onto the feed ingredient. The results are shown in table 5.4. The aflatoxin recoveries are greater than 73 % for all feeds. The aflatoxin recoveries obtained for wheat had a tendency to be higher than those obtained by Scudamore and Hetmanski[34], the ochratoxin A recoveries compare favourably and the zearalenone recoveries are much less than those obtained by Scudamore and Hetmanski[34]. The recoveries for palm are lower than other feed ingredients analysed, but this is due to the higher background interference especially in the case of aflatoxin B₂, G₁ and G₂. Refer to figure 5.11.

% Recovery	B ₁	B ₂	G ₁	G ₂	Ochratoxin A	Zearalenone
Maize Mean	96.3	101.0	102.6	102.6	77.6	24.6
S.D.	5.1	7.3	9.6	9.5	2.4	1.7
Palm Mean	73.1	82.0	87.5	76.7	12.5	12.9
S.D.	7.3	5.5	10.5	10.3	3.7	0.9
Wheat Mean	75.9	84.6	96.7	88.7	59.4	38.8
S.D.	5.6	2.6	7.3	12.2	12.6	9.0

Table 5.4 Reproducibility test on three different spiked feeds. Levels of mycotoxins used were 3.2 µg Kg⁻¹ aflatoxins B₁, G₁, and G₂, 1.6 µg Kg⁻¹ aflatoxin B₂, 16 µg Kg⁻¹ ochratoxin A and 320 µg kg⁻¹ zearalenone.

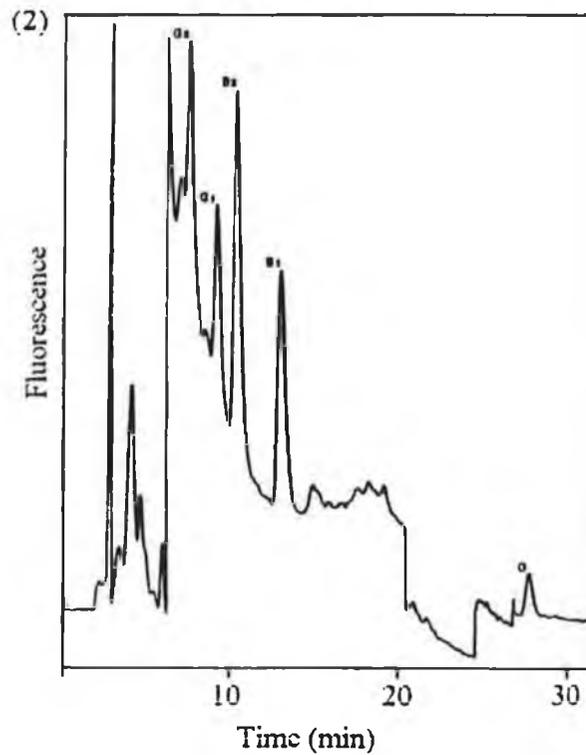
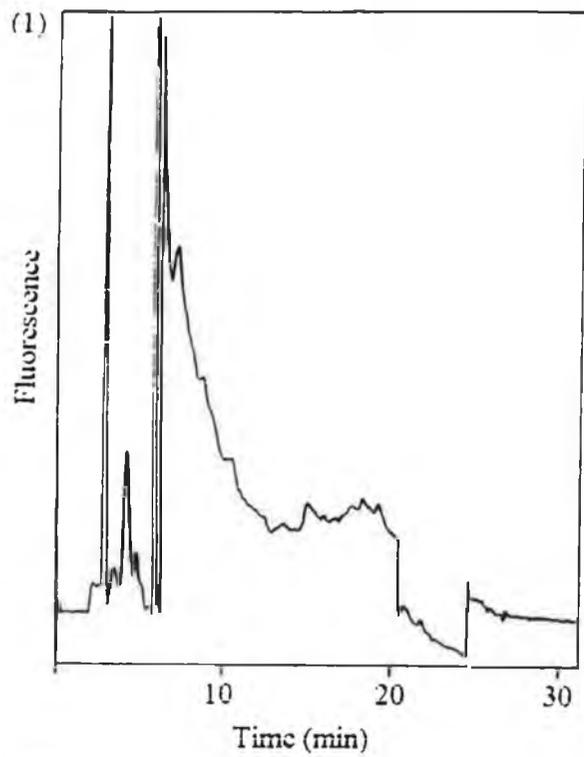


Fig. 5.11 Chromatogram of (1) palm sample and (2) palm sample spiked to contain $3.2 \mu\text{g Kg}^{-1}$ aflatoxin B_1 , G_1 , and G_2 , $1.6 \mu\text{g Kg}^{-1}$ aflatoxin B_2 , $16 \mu\text{g Kg}^{-1}$ ochratoxin A without post-column derivatisation.

5.6.4.3 Repeatability

Repeatability of the method was checked using maize. Ten portions of feed ingredient from the same batch were spiked with aflatoxins, ochratoxin A and zearalenone. The results are shown in table 5.5.

% Recovery	B ₁	B ₂	G ₁	G ₂	Ochratoxin A	Zearalenone
Mean	102.7	105.6	108.2	106.5	73.1	N.D.
S.D.	4.1	4.9	5.8	4.6	3.8	N.D.
% CV	4.0	4.6	5.4	4.3	5.2	N.D.

N.D. = not determined

Table 5.5 Repeatability test on maize (n = 10). Levels of mycotoxins used the same as in table 5.4.

5.6.4.4 Detection limits

Detection limits for each mycotoxin are shown in table 5.6. These are based on the noise x 2 and are in ng levels. Taking the original feed ingredient and % recovery into account the detection limits are quoted in $\mu\text{g kg}^{-1}$. This detection limit depends on the type of feed being analysed as the % recovery varies from feed to feed. Therefore a range of values are included which take into account the different types of feed ingredient being analysed.

The detection limits are good, despite the poor recoveries in some cases for ochratoxin and zearalenone.

Mycotoxin	Detection limit	
	ng	$\mu\text{g Kg}^{-1}$
B ₁	0.006	0.096 - 0.131
B ₂	0.002	0.032 - 0.039
G ₁	0.011	0.171 - 0.223
G ₂	0.009	0.139 - 0.181
Ochratoxin A	0.033	0.687 - 4.233
Zearalenone	0.413	17.017 - 51.183

Table 5.6 Limits of detection.

5.7 CONCLUSION

The HPLC method developed is fast, sensitive and economical. It allows the determination of six mycotoxins using one HPLC set up with gradient elution. The clean-up procedure involving GPC lends itself to partial automation. The six mycotoxins investigated eluted within 50 minutes from the GPC. The HPLC method developed allows good separation and quantification of the mycotoxins. Recoveries, reproducibility and repeatability were excellent for the aflatoxins and ochratoxin. The zearalenone results were poor but none the less were detectable and quantifiable. The detection limits were good and compare favourably with individual clean-up and detection assays..

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CHAPTER 6

Conclusions

6.0 CONCLUSIONS

Mycotoxins are hazardous materials and great care and handling precautions were needed when in contact with them even when using the smallest of concentrations. The organic solvents used in the extraction procedure are also toxic and care here must also be taken. In the analysis of arsenic similar safety precautions must also be obeyed. Arsenic species are toxic but the materials used in their determinations are also dangerous such as perchloric acid. Perchloric acid can explode if left to dry out, the borohydride solution produces excessive amounts of hydrogen when mixed with acid. Care must be taken at all times when using these chemicals.

The investigation of analytical techniques for the determination of arsenic and arsenic species was very successful. The flow injection system described here allows a rapid and economical analysis to be carried out. It is easily assembled and requires minimum operator manipulation and expertise. It offers an alternative, precise and sensitive approach for the trace determination of arsenic species if present in a single form. A sampling rate of 90 injections per hour can be achieved using this system. This is a significant improvement over a direct method of analysis and allows for rapid analysis of liquid samples. The analysis time is reduced even in the analysis of coal, where the majority of time is taken up by the digestion procedure (4 hours).

This flow injection technique is suitable for the determination of total arsenic present in complex matrices but is not suitable for the analysis of individual species in the presence of each other.

The coupling of HPLC to hydride generation AAS achieved the desired separation and detection of arsenite, DMA, MMA and arsenate. The

reproducibility and accuracy of the system developed was demonstrated by participation in the European inter-laboratory comparison. The detection limits obtained for the species are acceptable for many applications, however with the increasing demand for lower detection limits especially in the analysis of environmental samples a reduction in these limits is desirable

Column switching coupled with hydride generation AAS was developed for the preconcentration and analysis of two arsenic species. A precolumn is used to achieve pre-concentration and the species are separated by ion exchange HPLC before being detected by hydride generation AAS. The two species studied were arsenate and MMA and using this on-line pre-concentration system detection limits of 5 and 10 ppb respectively could be achieved. This is an improvement of approximately 50 - fold on the detection limits achievable without the incorporation of the on-line pre-concentration step.

Matrix solid phase dispersion was developed for the isolation of arsenic species from fish tissue. This new technique had only been applied to organic extractions ie drug residues. Matrix solid phase dispersion offers a new approach for the isolation of analytes from complex matrices not only for organic but from this work it has been shown to be suitable for inorganic extractions also. In this work this extraction procedure has been applied successfully to the extraction of arsenic species from fish samples. The matrix solid phase dispersion isolation of arsenic species from fish uses small sample size and low volume of washing and extracting solvents. The results obtained are consistent, with inter and intra-assay variabilities achievable being very low. Extraction efficiencies of between 50 % and 70 % were achievable for the species studied. Even though the efficiency could be improved the extractions have been shown to be very reproducible with coefficient of variations up to 5.0 %. This method offers the major advantage of being able to extract individual species. As the technique does not involve the use of oxidative or reducing reagents the information obtainable should be very

representative of the speciation within the sample in the case of fish. Matrix solid phase dispersion is an attractive alternative method to the classical approaches which are labour and material intensive, may require multiple manipulations and can result in inconsistent assays. It is sufficiently rapid compared to other extracting techniques which result in accurate information.

The method developed for the determination of six mycotoxins using one HPLC set up with gradient elution is fast, sensitive and economical. The clean-up procedure involving GPC lends itself to partial automation. The six mycotoxins investigated eluted within 50 minutes from the GPC. The HPLC method developed allows good separation and quantification of the mycotoxins. Recoveries, reproducibility and repeatability were excellent for the aflatoxins and ochratoxin. The zearalenone results were poor but none the less were detectable and quantifiable. The detection limits were good and compare favourably with individual clean-up and detection assays.