Trace Metal Speciation Studies

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy 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 work

Signed : Leonie Byrne ID No: 93700326

Date: 19th September 1997

To my parents and Eilis

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Abstract

The development of methods capable of determining individual metal species at trace levels is a widely researched topic. The majority of the techniques developed for this purpose involve the use of "coupled" instrumental techniques to separate and detect metal species at low concentrations, also additional derivatisation steps are often required prior to detection. This approach requires lengthy analysis times and high manual input to construct and maintain the interface between the two instruments. Complicated sample preparation procedures are also necessary for tissue samples. These factors discourage the implementation of routine environmental monitoring of trace metal species. In the course of this study, alternative techniques for trace metal speciation are examined. The criteria for these techniques is that each should be straightforward in design and operation requiring a minimal level of manual input while remaining cost effective.

The use of a solid phase extraction technique known as Matrix Solid Phase Dispersion (MSPD) is evaluated for extracting arsenic species from fish tissues. MSPD involves physically grinding up tissue samples with a quantity of chromatographic packing material and packing the resulting mixture into a column followed by selective elution of the analytes with mobile phase. The technique is developed and validated for five arsenic species. The effectiveness of this technique is then assessed by comparison with an existing liquid-liquid extraction protocol.

The feasibility of capillary electrophoretic methods (CE) for routine trace metal speciation is examined for arsenic and methylated tin species respectively. This technique involves the separation of charged species in narrow bore capillary tubing under the influence of an electric field. CE offers the benefits of highly efficient separations, short analysis times, minimal sample preparation requirements and small reagent consumption. Problems with low sensitivity can be overcome with electrostacking and indirect detection methods which allows the technique to be extended to the area of trace metal analysis.

Chapter one

Instrumental techniques for Trace Metal Speciation Studies.

1.1. The implications of heavy metal pollution in the environment.

Metals are prevalent and immutable constituents of the natural environment which exhibit great diversity in their interaction with living organisms. Pier[1] has demonstrated that the metallic elements can be classified according to the influence they exert on a biological system as follows:

1) The essential elements which are required by the organism in relatively large quantities and consequently for which there is a large tolerance. This group mainly comprises the lighter metals such as sodium or potassium but certain transition metals such as iron are also included

2) Metals such as selenium and copper, also essential for life but only at trace levels. Above a certain threshold, these metals will have an adverse effect on biological functions.

3) Metals which have no function in the life process of an organism and are toxic at very low levels, these are mostly the heavy metals such as arsenic, lead and mercury.

The onset of the industrial revolution in the 19th century brought with it the associated problems of pollution. Activities such as mining, smelting and fossil fuel combustion resulted in the release of certain heavy metals into the atmosphere. This ultimately led to an increase in the levels of these metals in ground water, rivers, in farm and garden soils and subsequently in vegetation and drinking water. Developments in chemical technology in the early part of this century further supplemented this environmental contamination by heavy metals as organometallic derivatives of elements such as arsenic, tin and lead found application in agriculture, medicine and the petroleum industry respectively[2].

Inevitably, the elevated levels of heavy metals in the environment were manifested by an increase in the incidences of human exposure through the ingestion of contaminated food. One of the earliest cases of accidental poisoning by a heavy metal was recorded

by Reynolds[3] at the turn of the century. In this instance, 70 people died as a result of drinking beer contaminated by arsenic contained in glucose. Similar cases have subsequently been reported involving arsenic contaminated milk powder[4], rice and river water poisoned by cadmium[5] and high levels of lead in certain acidic foods as a result of leaching from the storage containers[5].

Incidents such as these, highlighted the necessity for stringent controls on the acceptable levels of toxic metals in foodstuffs and drinking water. These requirements in turn, have strongly influenced the development of modern analytical chemistry. A number of highly specific and sensitive instrumental techniques such as atomic absorption spectroscopy, anodic stripping voltammetry and neutron activation analysis have evolved which were capable of determining these metals in the parts per million range and below.

In the early stages of analytical research, each metal was analysed as a single entity without distinction between redox states or physicochemical forms. Organometallic compounds were not considered to occur naturally in the environment and therefore except for isolated industrial accidents, not widely regarded as a health risk for the general population. This assumption was however, ultimately proven to be invalid.

As early as 1935, Challenger[6] demonstrated that inorganic arsenic could be converted to a volatile methylated analogue through the action of the mould *Scopulariopsis brevicaulis*. This compound identified as trimethylarsine [$(CH_3)_3As$] had been previously known as "Gosio Gas" and was responsible for a number of deaths where it had been formed as a result of the action of the mould on wallpaper coloured with arsenic containing pigments[7].

In spite of this evidence, the theory that metal-carbon bonds could be formed via a biological process received little credence until an actual outbreak of organometallic poisoning occurred in the 1950s at Minamata Bay, Japan. In this case, poisoning was linked to the ingestion of methylmercury from contaminated fish tissue[8]. The effluent from a local chemical plant was identified as being the principal source of methylmercury pollution, however subsequent research also revealed that bacteria

present in the sludge were capable of methylating inorganic mercury also present in the industrial discharge[9-10]. Furthermore, this methylated form of mercury was appreciably more toxic than the corresponding inorganic species exerting a greater effect on the brain and central nervous system[5].

Ensuing research has since revealed that the organometallic derivatives of most heavy metals also follow this trend of increased toxicity[2]. Arsenic however, is an exception and in this instance increased biomethylation is considered a detoxification mechanism. In addition to this many metals such as arsenic, tin and chromium can exist in more than one oxidation state, this has also been found to have significance in terms of toxicity and bioavailablity[11].

These findings not only revealed the enormous diversity of metallic species existing in the environment but also emphasised the inadequacies of total element determinations as an estimation of their toxic hazard. Techniques which facilitate the quantification of individual physico-chemical forms of an element has thus been the focus of much research particularly over the past two decades. These methods have collectively become known as speciation analysis and have led to improvements in the understanding of the environmental behaviour, fate and impact of these trace metals such that large scale poisonings such as the Minamata incident have been avoided.

1.2. Early developments in elemental speciation; Electrochemical techniques.

Electrochemical methods, polarography and stripping voltammetry in particular, were among the first instrumental techniques to find application in the area of trace metal speciation. The approach is limited by the fact that individual chemical species cannot be determined, however it is possible to discriminate between labile (chemically active) and inert (chemically inactive) fractions of the total metal concentration[12]. The labile fraction is composed of the free metal ion and the metal weakly bound in complexes or adsorbed onto colloid particles of iron or manganese oxides. Young et al.[13] reported a strong correlation between lability and bioavailability of copper in sea water using a larval shrimp as a test species. This correlation was further verified by Florence and

co. workers[14] who demonstrated that the fraction of electrolabile copper in sea water closely matched its toxicity toward the marine diatom *Nitszschia closterium.* The presence of synthetic ligands such as 8-hydroxyquinoline or nitrolotriactetic acid however had an adverse effect on this correlation.

In the majority of cases it is also possible to differentiate between oxidation states due to the fact one half of the redox pair is not electrochemically active under certain conditions. Some of these electrochemically inactive valance states include, arsenic (V), chromium(III), manganese(IV), antimony(V) and tin(IV)[12]. The exact concentration of the electroactive state can thus be determined in the presence of its inert analogue. A simple chemical treatment of the sample can subsequently convert all the metal into a reactive form, allowing a total metal determination to occur[15].

Anodic stripping voltammetry (ASV) has proved the most popular electrochemical technique for labile metal determinations and has been applied to the determination of the majority of the environmentally important metals[12]. The electrodeposition step provides an inherent preconcentration effect, resulting in high sensitivity and limits of detection in the sub ppb range, this has made ASV attractive for analysis of metals such as arsenite and chromium (VI) which are highly toxic at very low levels. The technique is hampered however by a number of interference problems. Adsorbance of organic matter onto the surface of the mercury electrode can have a detrimental effect on the diffusion of metal ions at the surface. Bhat et al.[16] reported that this adsorption resulted in a non linear relationship between stripping current and deposition time. The appearance of "tensammetric" peaks have also been observed[17]. These are caused by the process of adsorption and desorption of organic dipoles at the mercury surface. The experiments of Bately and Florence[18] revealed that these peaks though non-faradaic in nature, will appear at potentials similar to the stripping potentials of cadmium, lead and copper. Interfering organic matter can be destroyed by UV irradiation, but this in turn will alter the metal speciation by releasing organically bound metals into the labile fraction. Dissolved oxygen has also proved to be problematic in ASV techniques, it has been linked with an apparent increase in the stripping peaks of copper[19] and lead [20], this increase is often manifested as peak broadening.

A distinct form of stripping voltammetry known as adsorptive cathodic stripping voltammetry has also been employed in the determination of total dissolved and labile metals in environmental samples. The technique can be considered a three step process in which an organic (analytical) ligand is first adsorbed onto the surface of a hanging mercury drop electrode. This subsequently complexes with metal ions from the sample solution. The adsorbed ligand complex will strip back into solution, when the potential is scanned in the cathodic direction, with the formation of a metal mercury amalgam. Wang et al[21-23]. were among the first exponents of this technique and explored the use of the dihydroxyazo dye, Solochrome violet RS (SVRS) as the analytical ligand for the determination of titanium[21], gallium[22] and iron[23]. Due to the complexation step, the adsorptive stripping technique was found to be less prone to interferences which ultimately resulted in better sensitivity than the corresponding anodic stripping approach. The presence of surfactants such as Triton X and strong complexing agents such as EDTA led to an overall reduction in peak size. Boussemart and co. workers[24,25] have also successfully adapted this approach to the speciation of chromium in sea water. In this case, diethyenetriaminepentaacetic acid (DTPA) was used as the complexing ligand. Determinations were carried out at pH 5.20 with a DTPA concentration of 2.5mM. The cathodic scan produced a single peak at -1.275V this was due to the fact that chromium (VI) is reduced electrochemically to chromium (III) at potentials below -0.05V. However, the contribution to the signal from the dissolved chromium(III) complex, diminished with time, due to the formation of a second chromium(III)-DTPA complex which is electrically inert. This made it possible to determine the exact concentrations of each redox state of chromium in the sample.

The inability to directly quantify the individual chemical species present in the sample remains a serious limitation for electrochemical speciation techniques. Furthermore, the very nature of monitoring current while scanning the potential of the solution resulted in a disturbance of the equilibrium between redox states and complexed forms which in turn leads to the destruction of the "natural" speciation in the original sample. These restrictions combined with the problems of interferences from naturally occurring ligands, prompted researchers to explore alternative schemes for trace metal speciation.

1.3. Hydride generation atomic absorption spectroscopy

In order to accurately discriminate between redox states and organometallic forms of a metal ,the individual species need to be physically separated prior to detection. This requirement is the dominant feature of all speciation studies and has brought about the development of hyphenated techniques whereby two or more instrumental methods are used in tandem with each other.

Atomic absorption spectroscopy has been almost exclusively employed for detection in these speciation schemes. The technique affords a high degree of selectivity though the use of element specific hollow cathode lamps as a light source. In addition to this, detection sensitivity is in the part per billion range (μg/L) making the technique highly suited for determination of toxic metals in environmental samples.

A select group of elements can be converted into volatile covalent hydride derivatives, upon reduction with sodium borohydride in an acidic medium. These hydride analogues are generally more easily atomised than the corresponding solvated metal ions, subsequently bringing about an increase in the population of atoms in the light path and hence an improvement in sensitivity. Among this group of "hydride active" metals are a number of environmentally important elements such as arsenic, antimony, lead, selenium and tin. Consequently, early development of hyphenated speciation schemes was accompanied by a great deal of research promoting the generation and collection of hydrides of these compounds for further analysis.

Initial work concentrated on batch hydride generation systems where sample and reducing agents are injected into a reaction vessel and any hydrides produced are purged or swept by a stream of carrier gas either directly to the detector or into a cold trap. In 1969, Holak[26] reported a straightforward design for the generation of arsine which was composed of a reaction vessel and liquid nitrogen trap, consisting of a U-tube filled with glass beads and immersed in liquid nitrogen. This was connected between the aspirator of a Perkin Elmer model 303 AAS and the reaction vessel fitted with a calcium chloride drying tube (fig. 1.1).

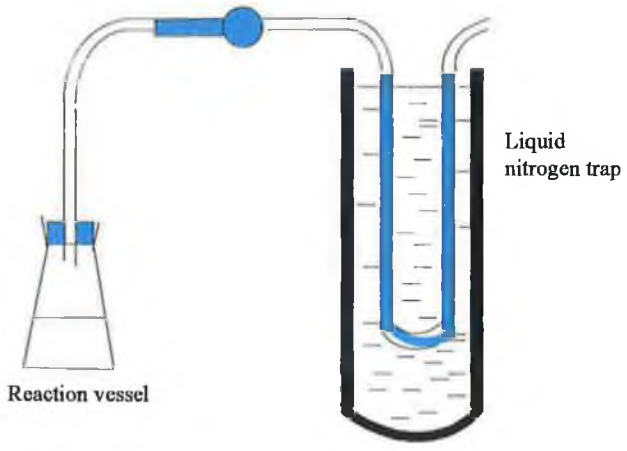


Fig.1.1; Prototype for batch hydride generation as designed by Holak[26]

Reagents were added to the flask and after a suitable time period the trap was closed and allowed to come to room temperature. Nitrogen was then allowed to flow through the U-tube sweeping the converted arsines into the optical beam of the AAS. This method gave an acceptable linear response with detection limits at 0.04mg arsenic.

The considerable diffusion of sample which occurred upon passage of the hydrides from the collection vessel into the atomisation flame, somewhat limited the sensitivity of detection. This was further impaired by the high background absorbance due to the presence of liberated hydrogen. An additional disadvantage of this approach was the long time duration required to ensure complete evolution and collection of hydrides. A more advanced prototype for batch hydride generation was proposed by Thompson and Thomerson[27]. With this model, the reaction vessel was connected directly to a silica tube, 17cm long, 0.8cm i.d., which in turn, was mounted just above the burner head of an air/acetylene flame. Atomisation of the hydride was now confined to the narrow dimensions of the tube, which served to improve sensitivity to a considerable extent. The design also dispensed with the use of a collection vessel, instead, reaction products were continuously swept into the heated silica tube, by a constant flow of nitrogen carrier gas through the reaction chamber, this allowed the combined processes of generation and detection to occur within 40 seconds. An auxiliary nitrogen stream was also introduced via two transverse tubes positioned at either end of the

atomisation tube, this prevented ignition of hydrogen, which had previously contributed to background absorbance.

This apparatus was successfully used in the generation and subsequent determination of hydrides of arsenic, antimony, bismuth, germanium, lead, selenium, tin and tellurium, using a 1% sodium borohydride solution and 0.2M hydrochloric acid. Detection limits ranged from 0.002μg/ml for bismuth to 0.5μg/ml for lead.

Following the development of straightforward, efficient systems for the generation of hydrides, the possibility of separating individual metal species by exploiting differences in their susceptibility to the reaction or the volatilities of the corresponding hydrides was explored. The simplicity of interfacing a hydride generation vessel with an atomisation cell led to the development of a primitive but effective analytical technique for speciation which served as a precursor for future development of more intricate chromatographic - atomic absorption spectroscopy interfaces.

Hodge et al.[28] were among the first exponents of a technique termed "selective volatilisation" and succeeded in separating a number of inorganic and organometallic tin compounds on the basis of differences in the boiling points of their hydride derivatives. In this case, two U tube assemblies, the first filled with water and immersed in dry ice and the second packed with glass wool and immersed in liquid nitrogen were connected in series between the reaction chamber and atomisation cell. The water trap served to remove the hydrogen and other volatile interferants with generated hydrides being retained by the glass wool in the second tube. The hydride reaction was initiated by injecting a 4% solution of $NaBH_4$ via a rubber septum into the reaction vessel. This solution was added at regular intervals, until the evolution of hydrogen ceased. At this point the hydride trap was removed from the liquid nitrogen where upon the collected hydrides volatilised in order of increasing boiling points. Inorganic tin(IV), mono, di and tri methyl tin and butyl tin, all evolved from the trap within 2 minutes, with the resulting detector output closely resembling a chromatogram.

For compounds such as tributyltin hydride, with boiling points in excess of 100°C it was necessary to heat the hydride trap in boiling water before evolution could occur. Limits of detection ranged from 0.4ng for tin(IV) to 2ng for tributyltin chloride. This protocol was successfully applied to the determination of tin species in sea water, lakewater and human urine.

A modification of this approach was employed by Braman and Tompkins[29] for the determination of methyltin compounds in natural waters. In this case the hydride trap was packed with OV-3 Chromasorb -W support material. This allowed the tin compounds to be separated with an efficiency of approximately 1300 theoretical plates. Resolution of the components was found to be dependant on the rate at which the trap was heated. Higher warming rates resulted in a loss in resolution but an increase in the sharpness of response. For these purposes, a heating rate of 15°C a min was chosen as optimum. Determination of the eluting hydrides was carried out using a hydrogen-rich, hydrogen air flame emission detector, with limits of detection in the parts per trillion range.

Shaikh and Tallman[30] extended this application to the area of arsenic speciation. In addition to volatility differences, it was also possible to differentiate between arsenic compounds by varying the pH at which the reduction reaction occurred. Total inorganic arsenic, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) could be determined at pH 1 whereas determination of arsenite (arsenic (III)) was possible at pH 5.

Howard and Arbab-Zavar[31] improved upon the design of the arsine generator by replacing manual mixing by a pumping mechanism whereby reactant was continuously added to the flowing sample stream and thorough mixing was achieved by passing reactants through a 14 turn mixing coil. A carrier gas stream transported the resultant gas/liquid mixture through a separator from where gas passes through a lead acetate scrubber (to remove H_2S) and a drying agent into a liquid nitrogen trap. This trap was subsequently heated to room temperature where arsines volatilise in order of increasing boiling points into the atomisation cell. Differentiation between the two inorganic

arsenic species was achieved as before, by performing the reduction at two different pH levels. At pH 5 arsenite alone will undergo reduction whereas in the presence of 1M HCl both redox states will react. Therefore the arsenate concentration can be calculated by difference.

This approach was rather restricted in its ability to separate compounds of similar boiling points and was ineffective for the quantitative determination of the less volatile hydrides where substantial peak tailing and loss in sensitivity occurred. Furthermore, the method was unsuitable for trace metal speciation in complex matrices such as tissue samples, where organic constituents could potentially interfere with the reduction reaction.

1.4. Coupled gas chromatography - atomic absorption spectroscopy techniques.

1.4.1 Gas chromatography coupled to flame atomic absorption spectroscopy

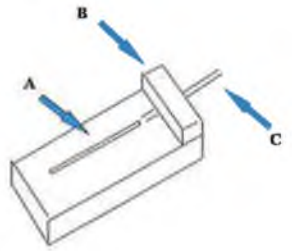
The superior resolving power of various chromatographic techniques were ultimately required for assurance of unequivocal separation of individual metal compounds and matrix interferants. Gas chromatography was the first of these techniques to be examined for its suitability to this purpose. The interface between the gas chromatograph and atomic absorbance spectrometer was pioneered by Kolb et al.[32]. This simply consisted of a short glass lined heated transfer tube connecting the tip of the column with the nebulisation chamber of a flame atomiser. The rapid flow of fuel and oxidant gases towards the burner head functioned in carrying column effluent into the air acetylene flame. This system was successfully applied to the separation and determination of tetramethyllead and tetraethyl lead compounds in a petrol sample and has subsequently been used to measure a number of alkylead compounds in gasoline[33] and environmental samples[34].

Coker[35] improved on this design by dispensing with this transfer tube, instead, the GC was interfaced directly to the burner head via a 1/16 inch gas union, threaded into a hole at the side of the burner. Column effluent was introduced into a short manifold positioned just under the burner slot which contained four outlet holes. This allowed

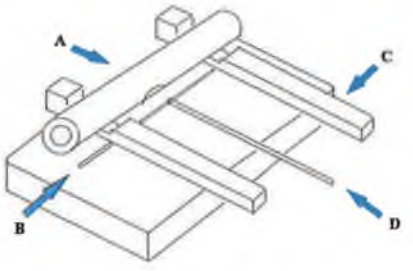
the gas to be evenly distributed along the entire length of the flame. In bypassing the nebulisation chamber, peak broadening due to the diluting effect of the large gas volume and loss of analyte due to condensation at the cold walls of the mixing chamber could be avoided. This system was used to analyse the alkylead composition in leaded and unleaded gasoline samples. Separation was carried out on a column packed with 10% PEG 20M (Carbowax) on 100/120 mesh Poracil, which allowed complete resolution of the seven main alkyllead compounds commonly found in petrol samples. Detection limits for all of the compounds were as low as 0.2ppm.

The sensitivity of flame atomic absorption detection is restricted due to the short residence time experienced by the atoms in the flame. This was the principal drawback of using this system as a means of detecting eluting metal species in gas chromatography. Ebdon and co. workers[36] addressed this problem and designed an atomisation cell consisting of a ceramic tube, suspended over the burner head. Preliminary investigations showed that by confining the atomisation process to this tube, a five fold increase in the lead absorbance signal could be achieved. A number of configurations of ceramic tube, burner head and column interface were evaluated in terms of their effect on the sensitivity of detection. The first of these, consisted of a ceramic tube suspended over the flame via aluminium rods, there was a hole on the underside of this tube (fig. 1.2a). Column effluent passed via a heated glass lined transfer tube, so that it impinged perpendicularly on the analytical flame, just below the hole. This arrangement was modified for the second atomisation cell, so that the interface tube from the GC column passed through the burner head, emerging just below the hole in the ceramic tube (fig. 1.2b). For the last design, this hole was placed at the front of the ceramic tube (fig. 1.2c). The interface tube was also altered so that column effluent passed into a T-piece, into which an auxiliary flow of hydrogen was introduced. A hydrogen diffusion flame was then ignited at the tip of this T piece, so that it coincided with the hole in the ceramic tube. The purpose of this flame, was to atomise the alkylead compounds before entry into the cell, leaving the air acetylene flame with the sole function of heating the tube. This further served to increase the length of time the atoms resided in the flame.

a; Atom cell I



b; Atom cell II



c; Atom cell III

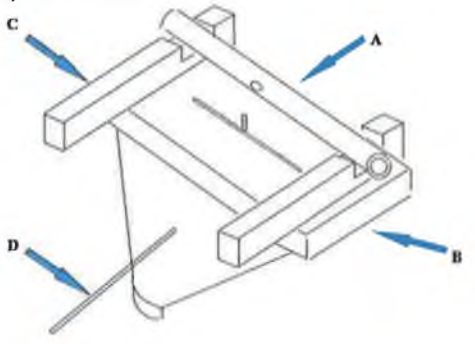


Fig. 1.2; Flame atomisation cells for coupling with gas chromatography; a; Atom cell I consisting of an air-propane burner head(A), steel clip(B)and a glass lined interface tube (C). b; Atom cell II consisting of a ceramic tube(A), an air acetylene burner head(B), aluminium supports(C) and a glass lined interface tube(D), c; Atom cell III, abbreviations as for atom cell II[36].

In all cases, the introduction of the ceramic tube above the flame was found to have a profound impact on the detector response. With at least twenty-fold improvement in the limit of detection over the direct flame atomisation approach, for alkyllead compounds. The greatest improvement in response was achieved with the separation of atomisation and tube heating processes. The linear range obtained using the third atomisation cell was 0.1-15ng alkyllead with the limit of detection calculated to be 17pg.

1.4.2. Gas chromatography coupled to furnace atomic absorption spectroscopy.

The feasibility of coupling electrothermally heated furnaces with gas chromatography systems has also been examined. Chau et al.[37] initially studied this combination for the separation and determination of dimethylselenide and dimethyldiselenide species. Separation of the compounds was carried out on a glass column packed with 3% OV1 on Chromasorb W, with a nitrogen carrier gas flowing at 70ml per minute. The column was interfaced to a silica furnace via a stainless steel transfer line, 2mm o.d. Column effluent was introduced though a side arm in the silica furnace, with hydrogen being added at a second point. This temperature of the furnace was maintained at 1000°C. A glass U tube, 2mm in diameter and 26cm long, packed with 3% OV-1 on Chromasorb and immersed in dry ice was used to cryogenically trap the selenide compounds. A known volume of an atmospheric sample was sucked through this trap by a peristaltic pump. The trap was then mounted between the carrier gas inlet and the injection port of the GC and heated to 100°C. This caused the selenides to be desorbed onto the column. With this arrangement, it was possible to separate the two selenide compounds within 6 minutes, with limits of detection in the nanogram region. This approach has since been adapted to the analysis of organolead in the atmosphere[38]

Van Loon and Radziuk[39], developed a simple, inexpensive method of coupling a GC column with a silica furnace tube. In this system, the GC column was contained in the long arm of a heated quartz T piece, with effluent passing into the cross piece atomiser which in turn was purged with nitrogen and hydrogen gases. This was successfully applied to the determination of a number of dialkylselenium compounds absorbed by the organism, *Astragalus racemosus*[40]. A sample trap consisting of DC-550 on Chromasorb W immersed in dry ice, was used to adsorb these compounds from the samples. Separation was then carried out on a glass column packed with 20% polymetaphenylether on 60-80 mesh Chromasorb W at 82°C and a nitrogen carrier gas flowing at 23ml per minute. Detection limits of 10, 20 and 20ng were achieved for dimethylselenide, dimethyldiselenide and diethyldiselenide respectively.

1.4.3. Derivatisation techniques for GC-AAS applications.

The direct use of gas chromatography-atomic absorption spectroscopy for speciation of metals is limited to compounds such as the alkyleads, which are relatively volatile. In order to expand the range of application of the technique, an additional derivatisation step is required to convert inorganic and organometallic species into more volatile derivatives.

Hydride generation has proven popular for this purpose. Burns and co. workers[41] reported a five-fold improvement in sensitivity in the determination of alkyl tins by GC-AAS when a hydride conversion step was introduced prior to atomisation. By placing the hydride generation manifold before the GC column, Heitkemper et al[42]. succeeded in converting a number of organometallic compounds of arsenic, antimony, bismuth, tin and selenium into forms more amenable to gas chromatographic separations.

A novel on-line method of hydride formation followed by GC-AAS was reported by Sullivan et al.[43] for the determination of butyltin compounds. In this method derivatisation was accomplished in a pre column reactor, which consisted of a glass injection port liner packed with sodium borohydride, placed inside the injector. Separation of dibutlytin, tributyltin and triphenyl tin hydrides was carried out on a wide bore capillary column coated with 5% phenylmethylsilicone, using a temperature gradient of 70°-200°C at 10°C a minute. Detection was carried out using a flame photometric system. The conversion of these tin compounds to their hydride analogues, served to reduce peak tailing and improve separation efficiency, and was successfully applied to the analysis of organotin compounds in salmon tissue.

The hydride generation-cryogenic tapping scheme reported by Howard and Arbab-Zavar[31] was modified by Ebdon et al.[44] so that the liquid nitrogen trap could be incorporated into a GC-FAAS instrument. By placing this trap between the GC injection port and gas inlet valve and applying a gradual heat, the generated arsines were swept onto the analytical column. Separation was carried out isothermally at 30°C on 10% OV-101 on Chromasorb W, with a carrier gas flow rate of 30ml per

minute. Detection limits ranged from 0.22ng for monomethylarsonic acid to 0.56ng for
arsenite.

Alternative methods of derivatisation have also been explored. Hewitt et al.[45] reported the separation of ionic alkyllead species in atmospheric samples by GC-FAAS, following their propylation with a grignard reagent. The analytes were collected from air by bubbling through distilled deionised water, and subsequently extracted into n-hexane, where conversion was carried out by the addition of propylmagnesium chloride. The high sensitivity of this method made it possible to determine very low levels of these compounds in ambient air samples.

A similar procedure was adopted by Gomez-Ariza et al.[46] for the speciation of butyl and phenyl tin compounds in river and sea water samples. The analytes were first extracted into tropolone and subsequently refluxed at 40°C for an hour with an ether solution of pentylmagnesium bromide grignard reagent. The alkyltin derivatives were then extracted, concentrated by rotary evaporation and analysed on a capillary gas chromatograph fitted with a flame photometric detector. Separation of mono, di and tri butyl and phenyl tins could be achieved using a temperature gradient, from 50°-250°C at 10°C per minute. with very low variation in retention times between replicate injections. The method was highly sensitive with limits of detection ranging from 19ppt for tributyltin to 52ppt for phenyltin.

This process of grignard derivatisation prior to gas chromatographic separation has also found successful application in the speciation of butyltin compounds in sewage, sludge[47] and shellfish[48] samples.

1.4.4. Gas chromatography coupled to plasma based atomisation

Plasma based excitation sources were among the early techniques to be investigated for their suitability for coupling with gas chromatography for metal speciation. The experiments of Bache and Lisk[49] utilised a microwave powered, low pressure helium

plasma connected to the outlet of a GC column for determination of methylmercury compounds in fish tissues. Separation was carried out on 20% OV-17 and QF -1 (1:1 w/w) on 80.100 mesh Gas Chrom Q, with detection of eluting compounds at the mercury atomic emission wavelength of 253.7nm. Separation of four environmentally important organomercurials was achieved within 7 minutes and limits of detection were in the ppb range. This sensitivity was improved upon by Quimby and co-workers[50], due to the inclusion of a heated interface between the GC and plasma source. This led to a ten fold decrease in the limit of detection for diphenylmercury.

The high compatibility of microwave induced plasma systems (MIP) with gas chromatography effluents lies in the fact that both techniques use nitrogen as a carrier gas at very similar flow rates. Furthermore, the low gas operating temperature of the MIP allows for the introduction of small volumes of sample, such as the effluent from a capillary gas column, without the risk of extinguishing the plasma. Problems associated with plasma stability and solvent interfaces coupled with advances in lower cost gas chromatography - flame atomic absorption spectroscopy interfaces have however, hampered the development of microwave induced plasma for use in the area of speciation. Recently, Hill[51] has reported the use of an improved commercial GC-microwave induced plasma instrument for the speciation of organotin compounds in soil samples. With this system it has been possible to monitor both metallic and non-metallic compounds simultaneously, which has important implications for the analysis of environmental samples.

In spite of developments in on-line derivatisation and volatilisation techniques, the application of gas chromatography is still confined to thermally labile compounds which possess favourable gas-solution partition coefficients. Inorganic redox species and a number of their metabolic intermediates do not belong to this category of compounds and are thus excluded from the speciation data obtained from GC-AAS analytical techniques. In addition to this, derivatisation procedures involving grignard reagents involve complicated, lengthy processes which would discourage their use for routine speciation analysis in commercial laboratories.

1.5. Liquid chromatography coupled to atomic absorption spectroscopy techniques.

1.5.1. Liquid chromatography coupled to flame atomic absorption spectroscopy.

Liquid chromatography is a more versatile approach for the purpose of metal speciation. The array of stationary and mobile phases available allows for the separation of a diverse range of cations, anions and high molecular weight species without the need of extensive sample preparation.

The coupling of atomic absorption spectroscopy with the LC column provides a highly sensitive element specific detection scheme for the eluting metal containing compounds. The heated transfer tube which served as a simple but effective interface for gas chromatography-atomic absorption spectroscopy was incompatible with LC systems. This was primarily due to the fact that the rate of nebulisation needed to be closely matched with the rate of mobile phase flow through the column and the low nebulisation efficiency of aqueous systems and the large background interference generated by organic solvents in the flame severely hampered the detection sensitivity. The design of a suitable interface between LC column and AA spectrometer which maximised sensitivity was therefore the focal point of initial research in this technique.

Early work explored the use of direct nebulisation type couplings. Messman and Rains[52] connected the LC column directly with the aspiration uptake capillary of a nebuliser using a short piece of polyethylene tubing. This system was successfully applied for the determination of the five major tetraalkyllead compounds found in gasoline. The separation was carried out on a C18 column with a 70% acetonitrile in water mobile phases pumping at 3ml. per minute. Maximum compatibility between rate of sample nebulisation and the flow rate of the mobile phases was achieved when the aspiration uptake rate was adjusted to be slightly lower than the flow rate of the eluting compounds.

Under these conditions, all five compounds were completely resolved within four minutes, without interference from co.-eluting hydrocarbon compounds and with retention time reproducibility in the 0.3-1.0% range. This was a significant

improvement on UV detection schemes, where the determination of alklyleads was not viable in the presence of high levels of organic constituents in the background matrix. The interface, did suffer from a lack of durability, with the acetonitrile constantly causing corrosion of the epoxy seal on the connections. This inevitably led to leakage and the resin had to be reapplied on a regular basis. The relatively high flow rates required to match the nebulisation rate of the detector, further limited the applications of this system to compounds which were easily resolved from each other.

This problem can be surmounted through the use of a discrete volume nebuliser in which a micropipette tip, attached to the end of the sample uptake tube, serves as a funnel to collect effluent dripping from the end of the HPLC column (fig. 1.3A). The effluent was thus introduced to the detector as a series of pulses. This interface originally reported by Bernt and Slavin[53] for the determination of amino acids after metal labelling, was refined by Hill et al.[54] for the determination of alkyltin compounds in harbour water. Replacement of the funnel arrangement by a vented capillary tube prevented the accumulation of dust and the associated problems of blockage and contamination (fig. 1.3B). The inclusion of a slotted tube atom trap just above the flame, maximised sensitivity for those elements readily dissociated to their ground state atoms.

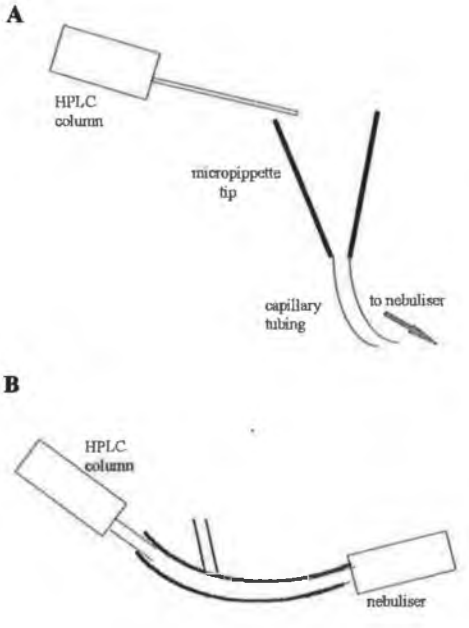


Fig 1.3; A; Discrete volume nebuliser for coupled LC-FAAS
B; Vented capillary tube arrangement[54].

Ebdon et al.[55] constructed an elaborate interface in which the nebuliser was replaced by a quartz tube in the flame acting as an atom trap. This increased the residence time of atoms in the flame and thus improved the sensitivity of the technique. Column eluant was collected as discrete aliquots on a series of platinum wire spirals. These spirals were mounted on a motor driven rotating disk controlled by a microprocessor. Each spiral was passed in turn over a desolvating flame before being positioned in turn in the analytical flame just below a small inlet hole in the atom trap. The entire assembly is illustrated in fig. 1.4.

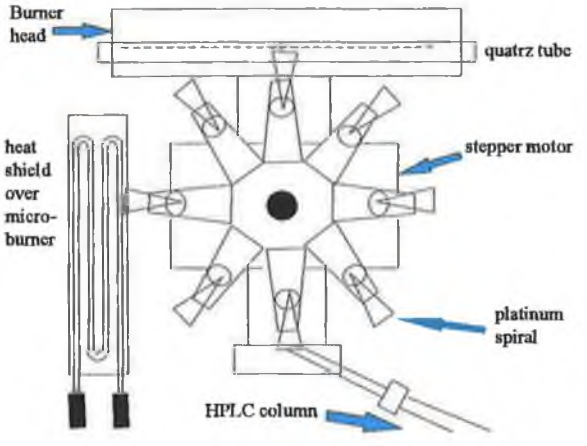


Fig. 1.4; Rotating Spiral interface for coupled LC-FAAS[55]

The superior limits of detection and reproducibility obtained using this system was demonstrated for the determination of di and tri alkylead compounds in the picogram range, which previously could only be analysed following derivatisation.

Gustavsson and Nygren[56] proposed a nebulisation interface in which the flow of oxidant was split, with a portion being used for the purposes of aspirating sample (fig. 1.5). This nebulising gas was pumped at right angles to the column outflow, causing the effluent to be introduced as an aerosol spray into a heating zone. The flow of auxiliary oxidant and fuel gases subsequently carried the analyte towards the flame. A four fold improvement in the limit of detection of tributyltin was achieved using this interface.

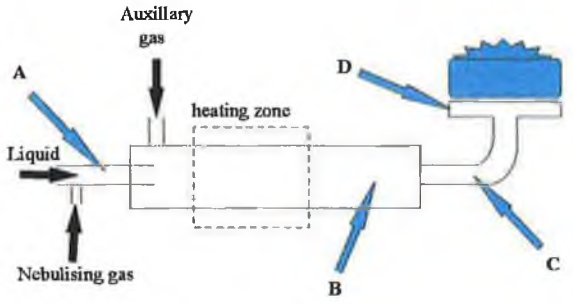


Fig. 1.5; HPLC-FAAS interface designed by Gustavsson and Nygren, A; concentric nebuliser, B; aerosol chamber, C; adapter & D; burner head[56]

1.5.2. Liquid chromatography coupled to Electrothermal Atomisation - AAS

Direct nebulisation flame atomic absorption spectroscopy is generally considered an inefficient process with only 10% of the sample actually reaching the flame and the remainder going to waste. This limits the sensitivity of detection rendering the approach unsuitable for many environmental applications. A substantial improvement in the limit of detection can be achieved if atomisation is controlled by an electrothermal process, mainly through the use of a graphite furnace. The discontinuous nature of the furnace operation, makes interfacing with a flowing system an extremely complicated procedure. An indirect approach to coupling is thus required to transfer eluting compounds to the detector.

Initial work employed specially adapted autosampler systems for this purposes. Brinkman et al.[57] , constructed a system in which a Teflon well sampler positioned in the autosampler tray of a graphite furnace atomic absorption spectrometer, acted as the interface between column and atomisation tube. Eluent from the column was transferred via medium walled Teflon tubing into the bottom of the sampler through a zero-dead volume screw fitting. This was then sampled at specific intervals by an automatic pipette for introduction to the graphite furnace. This arrangement was used for the detection of organometallic derivatives of arsenic, mercury, lead and tin after separation on a C18 column using a methanolic mobile phase. The system gave a very linear response, operating in a range suitable for most environmental applications.

Detection limits for triphenylarsine, triphenyllead, triphenyltin and methyl, ethyl and butyl mercury compounds, ranged from 4.8ng to 111ng.

Subsequent research has demonstrated the versatility of this system for the analysis of arsenic species in environmental samples. A range of chromatographic techniques have been investigated. In a related study, Brinkman and co.-workers[58] examined the efficiency of ion exchange and ion pair chromatography for speciation of arsenic in water. Employing a 0.04M acetic acid / ammonium acetate buffer as the mobile phase, arsenite, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) could be successfully separated on a SAX anion exchange column. Arsenate, being too strongly retained on the anion exchange was eluted from a SCX cation exchange resin along with DMA and arsenite with a 0.03M acetic acid/ 0.045M ammonium acetate mobile phase, however resolution was poor. A more successful separation of all four compounds was achieved on a C18 column when a lipophilic counter ion was incorporated into the mobile phase. The solvent used in this case was water/methanol 75/25 v/v which was completely saturated with tetraheptyl ammonium nitrate (THAN) as the ion pairing agent. For each of these experiments, volumes of 25µl were injected automatically into graphite cuvettes every 25 seconds, analyte solutions were then dried in the furnace and atomised for 10 sec at 2700°C. Sensitivity of the instrument was dependant on the characteristics of the graphite cuvettes which deteriorate with use, leading to a decrease in the overall performance. Variation in response between different cuvettes was also problematic.

The sensitivity of the graphite furnace atomisation approach can be impeded by various interferences of both spectral and non spectral nature. Organic solvents from the mobile phase can introduce background molecular absorbance which requires the use of Zeeman effect correction systems. This was particularly important for the determination of volatile organometallic compounds such as the alkyl leads, where separate drying and ashing stages were not possible without incurring considerable loss of sample and therefore atomisation occurred in presence of the matrix constituents.

The use of such Zeeman correction systems for the determination of tetraalkyllead compounds in the presence of benzene was demonstrated by Koizumi et al.[59].

Furthermore, a specially adapted high gas temperature furnace was employed in which volatilisation and atomisation were carried out in separate compartments. Sample vapour passed through a thermal converter consisting of porous graphite, before reaching the absorption cell. The temperature of the thermal reactor was raised sufficiently to allow decomposition and atomisation to occur. Using this system the signals obtained from all inorganic and tetraalkyl lead compounds were equal in magnitude indicating that complete atomisation of the organometallic compounds had occurred. This was not observed with conventional graphite tubes, where the signals obtained from tetramethyl, tetraethyl and tetraphenyl lead compounds differed considerably. This had important implications as it was possible to obtain accurate quantitative information about organolead compounds without the exact structure being known.

Vickery et al.[60] discussed the use of certain matrix modifiers to eliminate interferences prior to graphite furnace AAS determination of organotin and organolead compounds after separation by HPLC. Separation of tetraaalkyllead species was carried out on a C18 ODS column with gradient elution using an 80-100% methanolic mobile phase. A fraction collection scheme was employed with manual transfer to the graphite furnace tube at regular intervals. The addition of iodine as a matrix modifier prior to the dry-char-atomisation analysis cycle gave rise to a three fold increase in the observed signal for each tetra alkyl lead compound. This arose due to the reduction in volatility of the lead compounds by the action of iodine, thus minimising losses at the ashing stages. An improvement in the precision of the analysis was also apparent. This publication also discussed the use of graphite cuvettes which had been chemically modified with zirconium for the analysis of organometallic tin compounds. As with the previous experiment, a C18 ODS column was used for the separation with 97.5% methanol in water as the eluting solvent. In this case an on-line pulsed mode of sampling was employed, in which a timing device controlled a sampling valve which facilitated the introduction of column eluant into the graphite tube at regular intervals. The use of these treated cuvettes yielded a three fold improvement in the signal/noise response for all tin compounds, with an equal response being obtained for each species.

One of the principal reasons which would discourage the use of graphite furnace atomic absorption spectroscopy as a detector for routine speciation is that the discontinuous nature of graphite furnace atomisation and the lengthy analysis time periods required, are incompatible with continuous elution of mobile phase from the HPLC column. In addition to this, the necessity for Zeeman background correction systems increases the overall cost of the instrumentation, further limiting the application of the technique.

1.5.3. Liquid chromatography coupled with hydride generation atomic absorption spectroscopy

The ideal situation for a HPLC-Atomic absorption hybrid technique is one which combines the high sensitivity of graphite furnace methods with the continuous sample introduction of flame atomisation methods.

One approach to improving the sensitivity of flame atomic absorption spectroscopy is to replace the inefficient nebulisation process with a more efficient method of sample introduction. The introduction of a hydride generator between chromatographic column and atomisation cell has become one the most popular methods of eliminating direct nebulisation for the HPLC-flame AAS determination of arsenic and selenium species.

The feasibility of coupled liquid chromatography - hydride generation for the purposes of arsenic speciation was evaluated by Ricci and co-workers[61]. The separation of arsenite, arsenate, MMA, DMA and p-aminophenylarsonate (pAPA) was investigated using a Dionex 3x500mm anion exchanger column with on-line continuous arsine generation. In this instance a graphite furnace mode of atomisation was used for detection. In order to achieve adequate resolution, two eluent systems were employed; (1) a buffer solution of 0.002M $NaHCO_3$/0.0019M Na_2CO_3/0.001M $Na_2B_4O_7$ allowed the separation of MMA, p-APA, and arsenate. DMA and arsenite however co.-eluted requiring a lower ionic strength mobile phase of 0.005M $Na_2B_4O_7$ (2) for resolution . Gradient elution was found to be a very lengthy process and so an isocratic approach was employed in which eluent 1 separated MMA, p-APA and arsenate and

subsequently the column was re-equilibrated with eluent 2 for the resolution of arsenite and DMA. Detection limits of less than 10ppb were obtained for each species.

An analogous arrangement was utilised by Tye and co-workers[62] except in this case the atomisation of hydrides was carried out in a silica tube mounted in an air/acetylene flame. Separation of arsenate, arsenite, MMA and DMA was achieved on a BAX-10 anion exchange column with a single mobile phase of 0.1M ammonium acetate. Solutions of 5M HCl and 4% NaBH4 were pumped separately at 1.6mlmin^{-1} into the column effluent stream and mixed thoroughly in a glass coil where the reduction took place. A gas/liquid separator with nitrogen continuously flowing through it provided a means of isolating the volatile arsines and sweeping them towards the heated atomisation tube. The passage of analytes through a zippax™ anion exchange column prior to separation allowed for the removal of some major interferants and the preconcentration of individual compounds. This gave rise to lower limits of detection of 2ppb for arsenite, arsenate and MMA and 1ppb for DMA.

HPLC followed by continuous hydride generation/AAS detection has therefore found wide application in the area of arsenic speciation. Additional refinements to the original design have served to enhance the response of these systems and led to lower limits of detection. Principally these improvements have dealt with the hydride generation manifold. Electrical heating blocks are in many ways preferable to flame atomisation techniques due to the fact that temperatures can be regulated to suit a particular analyte and a high degree of reproducibility can be attained. Commercially available heating systems consist of a closed block equipped with a quartz T shaped tube, however, this design has one fundamental drawback; as reported by Welz[63]. Namely that the atomisation of arsine and related hydrides is mediated by hydrogen radicals generated at high temperatures inside the tube. Residual contaminants which are burnt onto the surface inside the cell with time form a catalytic film promoting radical recombination thus severely hampering the sensitivity of the analysis. Moreover, heating quartz to high temperatures leads to the formation of high temperature cristobalite[64] which also impedes atomisation. Both these problems can be remedied by regular conditioning of the quartz cell with HF. With classical electrical heating systems, the quartz T-piece cannot be removed without dismantling the entire

assembly, a time consuming and difficult process. To combat this problem, Mayer et al[64]. have modified an electrical heating block which is open at both ends and is easily opened and closed. This allows for rapid and convenient change of the quartz tube. In this paper, it was reported that when the treatment was repeated after every 100 measurements the same T piece could be used for up to two years without a decrease in sensitivity for the determination of arsenic and selenium.

Le et al.[65] eliminated the requirement for a separate mixing coil and gas/liquid separator by combining both processes in a novel arsine generator which improved the efficiency of separation and enhanced the signal to noise ratio. In this design, a Buchner funnel enclosed in a glass cylinder served as both reaction chamber and gas liquid separator. Nitrogen gas introduced from the bottom of the funnel through a fritted disk ensured efficient solution mixing and transportation of arsine to the atomisation tube, which was mounted in the flame for AAS measurement. Liquid waste overflowed into the outer cylinder, draining out through a side arm into an open reservoir. This maintained constant pressure within the apparatus.

The dimensions of the funnel were optimised with respect to signal/noise ratio for arsenic, antimony and tin determinations. The performance of this new generator/separator was compared to that of a more classical hydride system and a noteworthy increase in signal to noise ratio was achieved. This is probably due to the fact that fine air bubbles of carrier gas travelling through the mixture improved the efficiency of the reaction and separation procedure. Radioactive tracer studies carried out using ^{3}H labelled MMA demonstrated that over 95% of MMA was converted to the corresponding hydride in the generator when it was operating in continuous mode.

When straightforward hydride generation is interfaced with HPLC systems, arsenate shows significantly lower selectivity towards the hydride reduction reaction than its trivalent counterpart. In total arsenic determinations, potassium iodide has been used to pre-reduce arsenate to arsenite. However, strong acid media is required and the process may take up to 4 hours for complete conversion at room temperature. Using a gas flowing batch system, Chen & co-workers[66] demonstrated the use of L-cysteine as a highly effective pre-reducing agent for arsenate at lower acid concentrations. At

room temperature in 0.02M acetic acid, complete reduction was accomplished with 1mg/ml l-cysteine in 35 minutes. Reaction time was reduced to five minutes when the vessel was heated in boiling water. The arsine signal was also increased by 75% in the presence of L-cysteine and the effects of interfering ions were substantially reduced. These factors combined with the innocuous nature of L-cysteine and the fact that the lower acid concentrations required means that corrosion to the tubing is reduced makes it attractive as a pre-reductant.

Low acid cysteine systems are however incompatible with conventional continuous hydride generators due to the fact that gas liquid separators are designed for use with concentrated acids and therefore operate under the assumption that copious amounts of hydrogen gas is produced in the reaction. This serves to strip hydrides from solution. The absence of high levels of gas when more dilute acids are used for the reaction, consequently causes a reduction in the efficiency of the gas-liquid separation process.

A system which overcomes these problems was designed by Brindle et al.[67]. Due to the rapid nature of the reaction in the presence of L-cysteine the need for a reaction coil can be eliminated, sample and reagents are continuously pumped into the generator/separator via two glass tubes, reactants meet at the tips of the tubing and drop into the bottom of the vessel. Argon flowing at a rate of 400ml per minute was introduced through a glass frit at the bottom of the vessel producing fine bubbles which violently mixed the solution and also provided a large gas liquid interface area which promoted evolution of the hydrides. A second flow of argon at 2l per minute flowed into the vessel from the top. The combined gas flow swept arsines into the plasma through an outlet port via a U tube partly filled with water, which functioned in moderating any surges in flow. Splitting the gas flow eliminated problems associated with foaming in the generator while keeping required total gas flow constant. Waste solution was continuously pumped to a drain via a tube at the base of the generator. A prereduction coil placed before the hydride generator allowed for thorough mixing of sample, acid and L-cysteine solutions. This was heated to 980°C to ensure complete reaction, a 0.5m x 0.5mm water cooled coil reduced the temperature of the solution resulting in reduced foaming within the reaction chamber. Hydrides were analysed by

direct current plasma atomic emission spectroscopy which gave a limit of detection of 4ppb for arsenate.

Hydride generation atomic absorption spectroscopy thus combines the sensitivity and compatibility required for on-line detection of environmental metal species after separation by liquid chromatography. The essential drawback of the technique is that not all metal species are susceptible to reaction with sodium borohydride. These include certain organometallic arsenic compounds and a number of butyltin compounds. Therefore in order to determine the concentrations of all species present in a sample by the HPLC-HGAAS process, additional steps need to be inserted between the separation and detection stages which will chemically convert these compounds into hydride active forms. These include hot acid[68] or base[69] digestions or photo-oxidation[70] procedures. The incorporation of these steps into the analysis may render the analysis a cumbersome and lengthy process. In addition to this, the increased distance which the analyte will have to travel between the column and final detection may lead to substantial peak broadening and a complete loss in resolution for closely eluting peaks. These limitations may discourage the use of this technique for routine speciation studies. The development of alternative detection schemes which maintain the high sensitivity and LC compatibility of hydride systems but with a wider range of application has thus been the principal objective of the most recent research into metal speciation techniques.

1.5.4. Liquid chromatography coupled to plasma emission sources

The use of high temperature plasma excitation with atomic emission spectroscopy for detection of eluting metal species was investigated at an early stage of research in this area.

Fraley et al[71]. were among the first to evaluate inductively coupled plasma atomic emission spectroscopy (ICP-AES) as a HPLC detector. The HPLC outlet was connected to the nebuliser of the ICP instrument via narrow bore teflon capillary tubing fastened to the column exit by a stainless steel swagelock fitting. This ensured a

flexible connection while minimising dead volume. A non-retentive dummy column was employed throughout the experiment. This eliminated peak spreading due to mass transfer and other retention effects. The limits of detection for a number of elements were subsequently evaluated and compared to those obtained in the absence of a HPLC column. For elements such as chromium, nickel, magnesium and lead, the presence of a HPLC column made little difference to the response obtained, however there was a marked disparity in the limits of detection obtained for calcium, copper, cadmium and selenium after HPLC. The determination of arsenic was problematic with this system due to high background interference from carbon emission at 193.6nm. These results were due to inherent problems of the ICP-AES system rather than that of the interface. This was illustrated by the fact that signals obtained for copper(II)-aminocarboxyllic acid chelates with this ICP-AES arrangement after HPLC correlated well with those obtained when a more conventional flame atomisation process was employed.

Gast and co.-workers[72] investigated the feasibility of a similar interface for the HPLC-ICP-AES determination of iron and molybdenum carbonyl complexes, alkylmercury and organoarsenic compounds, however, in this case a fixed cross flow nebuliser was employed. In order to simulate the situation in the presence of a HPLC column, small volumes of sample where injected into the nebuliser. The rate of sample delivery which corresponded to the flow rate of the mobile phase, was observed to have a profound effect on the sensitivity of the response with peak areas dropping rapidly with increasing delivery rates. Considerable dilution of the sample also observed in the tubing prior to the nebuliser and in the spray chamber.

In a related study, Whaley et al.[73] examined the optimum position for the ICP-spray chamber, in terms of its effect on peak broadening and overall sensitivity of detection. Two possible configurations were examined, one in which the spray chamber remained in its conventional position inside the plasma coupling box, and one in which it was placed externally close to the column exit. With this latter arrangement, the analytes were transported to the plasma as an aerosol rather than in a flowing liquid, thus minimising transfer time and interaction with solvent molecules which ultimately

resulted in improved peak symmetry. A second advantage of this system was that peak height remained invariant to changes in the flow rate of the mobile phase.
This initial research was primarily concerned with demonstrating the interface between a plasma excitation system and HPLC and little emphasis was placed on the applications of this approach.

The most attractive feature of ICP-AES is its capacity for multielement determinations with a single sample aspiration, thus reducing analysis time and sample clean up. This was demonstrated by Robbins and Caruso[74] with the simultaneous determination of arsenic, selenium, tin and antimony by microwave induced plasma atomic emission spectroscopy after derivatisation with sodium borohydride. A Chromasorb 102 column was placed between the hydride generator and plasma tube with the function of isolating the analytes from matrix constituents which may cause spectral background interference. A sequential volatilisation approach was used for the partial separation of the individual hydrides prior to passage onto this column. Derivatisation was carried out in a separate reaction vessel where a flowing stream of helium served to carry the hydrides into a liquid nitrogen cooled condensation chamber. A three-way valve system was used to remove the excess hydrogen and other gaseous contaminants. These valves were then adjusted and the condensation tube heated so that the hydride compounds could volatilise and be swept onto the column by the flow of helium. The maximum permissible column length for the separation of the four hydrides from the matrix within the integration period of the AES instrument was five inches. This caused a slight overlap of the peaks though they were sufficiently resolved from the background. The limits of detection reported for arsenic, selenium, tin and antimony were 0.07μg, 0.6μg, 0.13μg, and 0.02μg respectively with the relative precision values for each element all below 10%.

Morita et al.[75] employed an ICP-AES system for the on-line detection of arsenic species after separation by anion and cation exchange chromatography with a phosphate buffer. As with previous HPLC-ICP couplings, teflon tubing provided the interface between the two instruments with the nebuliser uptake rate set at 1ml per minute to match that of the eluant flow rate. Arsenic was monitored at the 193.7 emission line. Calibration curves for arsenite, arsenate, MMA, DMA and

arsenobetaine were linear in the range of 50-1000ng with a high level of reproducibility in peak heights and retention times for three replicate injections. In this publication both cation and anion exchange separation mechanisms were investigated. While it was possible to separate all five species on an anion exchange column, peaks due to arsenite and DMA were very broad due to the high affinity of these species for the anion exchange stationary phase. Improved separation efficiency was observed with cation exchange chromatography, however, with this approach arsenite and arsenate remained unresolved.

The problem of improving efficiency while maintaining resolution was addressed by Low and co-workers[76] who availed of a column switching method coupled to ICP-AES. Anion exchange and C18 columns were connected via a 6 port automated switching valve. Ammonium buffer was employed as the mobile phase, this was suited to both stationary phases and therefore allowed for the smooth transition between columns without the need for reconditioning. Setting the switching valve to position 2 the unresolved arsenite and Asbet components were allowed to flow onto the C18 column, the valve was then returned to position 1 for separation of MMA, DMA, and arsenate by anion exchange. Returning the valve to the original position allowed the separation of arsenite and arsenobetaine.

Direct current plasma atomisation methods for atomic emission spectroscopy were explored by Ahmad et al.[77] for analysis of chromium (III) and chromium(VI) after separation on activated alumina. The separation exploited the fact that alumina will preferentially adsorb anionic chromium(VI) species with the cationic chromium(III) redox state, eluting in the void volume. The retained species can be eluted with a high pH solvent such as ammonia. Calibration graphs were reported to be linear in the range 50-250ng with relative standard deviations in peak heights of 8.04% and 7.44% for chromium (III) and chromium (VI) respectively.

The essential drawback of plasma atomisation coupled to atomic emission spectroscopy is that inefficient sample nebulisation into the plasma, results in considerable losses and hence a reduced sensitivity. The high atomisation efficiencies and increased residence times counterbalanced the effects of poor nebulisation to a

certain extent, such that the sensitivity of the technique generally exceeds that of flame atomic absorption, though still remains inferior to graphite furnace or hydride generation techniques. These sensitivity problems coupled with the high cost of the instrumentation, would discourage the widescale use of coupled HPLC-ICP-AES for the purposes of routine speciation studies in commercial laboratories.

1.6. Inductively coupled plasma-mass spectrometry

The true potential of chromatography combined with inductively coupled plasma excitation was realised with the development of ICP-mass spectrometry instrumentation. The efficiency of ICP for producing singly charged ions makes it an ideal ionisation source for mass spectroscopy. With this system, ions are extracted from the atmospheric pressure plasma into the low pressure mass spectrometer via a two or three stage differentially pumped interface. They then pass through a cooled sampling cone, with an orifice 1mm in diameter. The gas expands behind this orifice and a portion passes through a second orifice in the skimmer cone. A series of ion lenses, maintained at appropriate voltages are used to focus the ions into the quadropole mass analyser, where they are separated on the basis of their charge to mass ratio and detected by an electronmultiplier tube[78].

Plasma mass spectroscopy offers a substantial increase in sensitivity with improvements in the limits of detection of up to three orders of magnitude over atomic absorption or emission techniques. Inductively coupled plasma-mass spectroscopy is also possibly the most versatile detection system examined to date in the area of metal speciation with the capability of determining many metal species without the need for additional chemical derivatisation steps. These factors combined with the facility for multi element analysis and a wide linear dynamic range makes the technique highly attractive as a detection system for environmental speciation studies.

Using a HPLC-ICP-MS arrangement, Shiomi et al.[79] identified arsenobetaine as the major arsenic compound in tissue of the clam Merotrix Lusoria and characterised tetramethyl arsonium salt as being the chief component in the gill region. Beauchemin

et al.[80] also used a similar system for the quantification of arsenobetaine in the dogfish muscle Dorm 1 reference material. Separation of seven main arsenic species was performed on a C18 column with a mobile phase of 10mM sodium dodecyl sulphate solution containing 5% methanol and 25% glacial acetic acid. This column was directly interfaced via teflon tubing to the nebuliser of the ICP instrument which was linked to the mass spectrometer with detection limits ranging from 50-300pg.

In the case of arsenic speciation, interference due to the formation of $^{40}Ar^{35}Cl^{+}$ was problematic as it coincides with the arsenic peak at m/z = 75. This was remedied by Sheppard and co-workers[81] who succeed in isolating chloride from arsenite, arsenate, MMA and DMA by ion exchange chromatography. Improved sensitivity was possible through the use of a mixed gas Helium-Argon plasma. This was due to the fact that the high ionisation energy and metastable state energies of helium, caused an increase in the energy of the plasma. Limits of detection for arsenite, arsenate, MMA and DMA were 0.063ng, 0.037ng, 0.080ng and 0.032ng respectively, this was an improvement of one order of magnitude on those obtained when a single argon plasma source was employed. The method was successfully applied to the determination of arsenic species in matrices such as urine, club soda and wine where the chloride content was exceedingly high.

An alternative approach for the removal of this $^{40}Ar^{35}Cl^{+}$ spectral interference was proposed by Goossens and co-workers[82]. In this case, the addition of 4% ethanol to the matrix coupled with careful optimisation of the nebuliser gas flow rate had a diminishing effect on the magnitude of this interference. Observations indicated that this was due to the fact that the $ArCl^{+}$ signal was shifted to a lower frequency at lower gas flow rates. The method was applied to the determination of arsenic and selenium species in human serum and urine reference materials with excellent agreement between the certified and experimentally obtained values.

Al-Rashdan and co.-workers[83-84] explored the use of ICP-MS for the detection of triorganolead species after separation as their ion pairs with sodium pentane sulfonate on a reverse phase C18 column. A gradient elution system whereby the methanol content was increased from 40-90% over 10 minutes ensured the separation of triethyl

lead, triphenyllead, tetramethyllead and inorganic lead within a single run. Detection limits ranging from 0.2-3900pg were reported. This approach has also been adapted by Shum, Pang and Houk[85] for the determination of methyl, ethyl and phenyl mercury compounds with limits of detection as low as 7pg achieved.

In a more recent publication, Kallio and Manninen[86] have reported a coupled ion chromatography system with ICP-MS, for the purposes of chromium speciation. An anion exchange guard column was connected in series with cation exchange column. Chromium species were pumped onto the anion exchange column with 5mM nitric acid. Chromium (VI) species were retained by this column and chromium (III) eluted with the solvent onto the cation exchange column. A gradient elution protocol was then initiated for elution of each of the retained chromium species into the nebuliser of the plasma MS instrument. Elution of chromium(III) from the cation exchange column was possible with 5mM HNO_3 whereas a more concentrated acid solution (40mM) was required for the removal of chromium(VI). With the ICP-MS detection, the lowest concentration for quantification was 0.3μg/l for chromium(III) and 0.5μg/l for chromium(VI). This was a tenfold improvement on corresponding graphite furnace modes of detection.

1.7. Conclusions

The evolution of analytical instrumentation for the purposes of measuring individual chemical forms of an element has served the dual purpose of demonstrating the great diversity of metal species which exist in the environment while providing a highly specific and sensitive means of analysing them.

As many of these metals are toxic at very low levels, the main emphasis of analytical research has been development of methods capable of detecting species in the sub ppb level. Early electrochemical experiments met these sensitivity requirements but were ineffective in discriminating between all physicochemical species and for the most part were destructive to the indigenous speciation in the sample.

Coupled chromatography - atomic absorption spectroscopy in a variety of forms has become the accepted approach for studying metal speciation in an environmental sample. Both gas and liquid chromatographic separations have been investigated. Coupled GC-FAAS has proven successful for the speciation of a limited group of the volatile compounds such as alkylleads or dialkylseleniums. To extend the superior resolving power of GC to other metallic species additional derivatisation such as hydride generation or grignard propylation is required.

The liquid chromatographic approach is more universally applicable. The use of flame atomisation AAS for detecting eluting species suffers from a lack of sensitivity due to inefficient nebulisation and spectral interferences from the mobile phase solvent. Electrothermal methods of atomisation, while providing the required sensitivity and enabling the matrix to be removed prior to the absorption measurement being recorded, are generally time consuming due to the fact that a fraction collection and manual transfer system needs to be incorporated between column and furnace tube. This combined with the high cost of graphite furnace atomic absorption instrumentation would discourage the use of this technique in the context of routine speciation.

The development of hydride generation atomic absorption spectroscopy has been an important breakthrough for the purposes arsenic, selenium and tin speciation. The original batch generation techniques were quickly replaced with continuous flow systems, which could be easily coupled with HPLC. Hydride generation AAS has now become the main form of detection in speciation work for these elements and much research has been carried out to introduce improvements to the design to reduce the amount of manual input required..

The coupling of plasma atomisation with liquid chromatography has also significant advancement of the area of trace metal speciation. The original combination of plasma atomisation and atomic emission spectroscopy still possessed certain problems associated with flame and furnace methods. However, in recent years the use of these plasma sources with mass spectrometry has provided an innovative detection system capable not only of measuring metal species in the sub ppb range, but also of providing

structural information on these species. These systems have also made it possible to determine a number of metal species simultaneously.
HPLC-ICP-MS has found application in all areas of metal speciation. The high cost of the instrumentation is still a major restriction for the use of this technique and therefore the development of a more economically viable alternative is becoming the chief focus of research in this field.

In recent times a technique known as capillary electrophoresis has emerged as a complementary technique to HPLC. With this technique analytes are separated in a fused silica capillary column due to differences in their migration under the influence of an electric field. The chief merit of capillary electrophoresis lies in the fact that an extremely high separation efficiency, analogous to that obtained by capillary gas chromatography techniques however the range of compounds which can be analysed by capillary electrophoresis extends to those compounds conventionally analysed by HPLC methods. Furthermore, the low reagent consumption and low cost of the capillary tubing compared to that of a HPLC column makes capillary electrophoresis a suitable candidate for use as an analytical technique for routine environmental monitoring studies. Problems with sensitivity due to low injection volumes and narrow dimensions of the UV detection system have hampered the development of capillary electrophoresis for the purposes of trace metal speciation although the technique has been successfully applied to the determination of the total concentration of various metals in a sample. Improved understanding of the processes involved in capillary electrophoretic separations have led to the development of on-line pre-concentration and other sensitivity enhancing methodology. This has further broadened the range of applications of this technique to the analysis of small inorganic and organometallic ions which do not absorb strongly in the UV region. These techniques are discussed at length in chapter 3 and the feasibility of capillary electrophoresis as an analytical technique for the determination of arsenic and methylated tin species is evaluated in chapters 3 and 4 respectively.

An important aspect of trace metal speciation which has been neglected by previous studies is the actual preparation of the sample prior to analysis. In the case of water samples a simple filtration step is sufficient however for more complex matrices such

as tissue samples, a complicated series of liquid-liquid extraction protocols and subsequent chromatographic clean up steps may be required to render the sample suitable for injection into an analytical system. These steps result in lengthy analysis times and require a considerable amount of manual input which inevitably lead to losses and irreproducible results. In the following chapter, a solid phase extraction technique known as matrix solid phase dispersion (MSPD) will be evaluated for use in the extraction of arsenic species from fish tissue samples. This technique has previously been successfully employed in the extraction of a variety of drug residues and pesticides from tissue matrices. The primary objective for this particular study is the adaptation of the MSPD approach so that it provides a more rapid and straightforward method than the existing liquid-liquid extraction approach for the isolation of the arsenic species from fish tissue. This liquid-liquid extraction procedure in addition to being time consuming and labour intensive involves the use of harmful and corrosive solvents such as phenol. These combined factors would discourage the use of this technique for routine arsenic speciation analysis in tissue samples. The MSPD technique however, with its low requirement for organic solvents and simplicity of design would be ideal for use for routine purposes.

In the course of this research, the techniques of matrix solid phase dispersion and capillary electrophoresis are examined as viable alternatives to existing methods in the area of trace metal speciation. Each of the methods researched are more straightforward in design, cost effective and simpler to operate than the established speciation techniques. These criteria are vital considerations if individual metal species present in environmental samples are to be analysed on a routine basis.

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Chapter Two.

Extraction of Arsenic Species from fish tissue by Matrix Solid-phase Dispersion.

2.1. Introduction

2.1.1. The occurrence of arsenic in the environment

Arsenic is widespread in the environment. The 20th most abundant element in the earth's crust, it is associated in nature mostly with igneous and sedimentary rocks, being particularly prevalent in areas rich in mining deposits of gold, silver and tin[1]

Arsenic exists in a variety of chemical forms including inorganic arsenic III (arsenite) and arsenicV (arsenate) as a number of mono-, di- and tri-methylated organometallic compounds. These exhibit diverse chemical and toxic properties ranging from those which are completely harmless to those which have damaging effects on the nervous, respiratory, cardiovascular and immune systems. Inorganic arsenic is classed as a group 1 carcinogen[2], this means that there is sufficient evidence from epidemiological studies to support a casual association between exposure and cancer. Case studies of a number of chronic arsenic poisoning incidents[3] indicate that inhaled arsenic usually leads to respiratory cancers whereas colon cancer and leukaemia tend to arise when arsenic is ingested. Liver sarcoma and carcinoma, myeloma and oral cancer have also been linked to arsenic exposure[2].

Inorganic arsenic compounds are the most toxic with the trivalent redox form more harmful than its' pentavalent analogue. Alkylated organometallic arsenic compounds exhibit a gradually diminishing toxicity with increasing degree of alkylation to the extent that large complex molecules such as arsenobetaine and arsenocholine are completely non-toxic.

It is estimated that anthropogenic activity such as smelting and mining operations and fossil fuel combustion accounts for over 40% of the overall cycle of arsenic in the environment, the remainder arising from various weathering and biologically mediated processes, as well as volcanic activity. These combined routes give rise to arsenic being present in sediments from stream beds, in farm and garden soils and subsequently

in vegetation and drinking water. In addition to this, high levels of exposure to arsenic can occur in the glass making industry and in the manufacture of certain chemicals such as wood preservatives and herbicides where it is used as one of the starting products[4]

An area which has been extensively studied is that regarding the nature of arsenic species in the marine environment. The concentration of arsenic in sea water is relatively low, typically about 1-2 μgdm^{-3} globally, the majority of oceanic arsenic occurring in sediments. However, in spite of this, the element is found in appreciable quantities in fish and other marine biota. As early as 1926, A.C. Chapman[5] reported the occurrence of high levels of arsenic in crustaceans and shellfish. His investigations showed that this arsenic was in the form of a complex organic substance which appeared to be non-toxic and which was excreted by humans within a day of seafood ingestion. In 1977 Edmonds et al.[6] identified the organoarsenical trimethylarsonioacetate [$Me_3As^+CH_2COO^-$] (arsenobetaine) as being the principal arsenical constituent of the Western rock lobster *Panulrius Longpipes Cygnus George*. Arsenobetaine has subsequently been reported in a plethora of marine organisms including halibut, haddock, cod, herring and mackerel[7]. A number of related compounds such as arsenocholine [Me_3AsCH_2OH][8] and trimethylarsineoxide [Me_3AsO][9] have also been isolated from these species. Arsenosugars[10,11,12] identified in a number of lower tropic organisms are believed to be precursors to arsenobetaine and arsenocholine which apparently are assimilated by fish via the food chain.

2.1.2. Analysis of arsenic species in fish tissue

The World Health Organisation[13] recommend that the daily intake of arsenic should not exceed $2mgkg^{-1}$ body weight. However, due to the high concentration of these non toxic arsenic species in seafood samples, determination of the total arsenic content in the tissues will give an exaggerated assessment of potential exposure to individuals. The development of analytical techniques for the specific determination of these

complex organoarsenicals has thus become one of the most popular research topics in the area of trace metal speciation.

As outlined in chapter one, the majority of metal speciation techniques are characterised by a chromatographic separation coupled to atomic absorption spectrometric detection. Gas chromatography has found limited application in the area of arsenic speciation as additional derivatisation is required to convert the compounds into more volatile forms. The polar nature of most inorganic and organometallic arsenic species makes them more amenable to ion exchange or reverse phase ion-pair chromatographic techniques. All modes of atomic absorption spectroscopy have been investigated for the detection of eluting arsenic species however, hydride generation atomic absorption spectroscopy has proved to be the most popular in terms of selectivity, sensitivity, relatively low cost and compatibility with an on-line flowing system such as eluant from a HPLC column. The essential drawback of hydride generation for the purposes of routine arsenic speciation in fish tissue is that compounds such as arsenobetaine and arsenocholine are not susceptible to reaction with $NaBH_4$.

2.1.2.1. Wet digestion techniques for the conversion of arsenobetaine into a hydride active form

A number of workers have concentrated on methods of conversion of arsenobetaine and related compounds into hydride active forms. Kaise et al.[14]. proposed a reaction whereby arsenobetaine was converted to trimethylarsine oxide which is susceptible to reduction to trimethylarsine. This conversion was brought about by reacting the arsenobetaine with hot sodium hydroxide. In the initial stages of the reaction, the negatively charged hydroxide group becomes associated with the positively charged arsenic atom of the arsenobetaine molecule and an intermediate compound results. The negatively charged oxygen molecule in the acetate part of the molecule then reacts with the hydrogen atom of the newly attached hydroxide group. This part of the original molecule is then released as a molecule of formic acid and the remaining

Attached negatively charged oxygen atom forms a second bond with the arsenic to form trimethylarsine oxide. The reaction is summarised in fig 2.1.

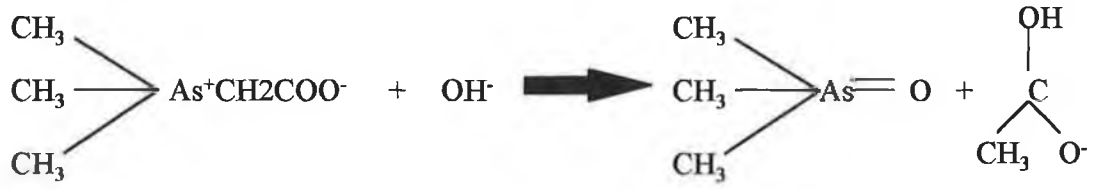


fig. 2.1; Hot base digestion of arsenobetaine as proposed by Kaise et al.[14]

In this particular example, the digestion was carried out by heating purified methanolic tissue extracts along with standards of arsenite, arsenate, MMA, DMA arsenobetaine and arsenocholine with 2.0 M NaOH in a water bath at 85°C for 3 hours. These digests were then neutralised with Hcl and made up to 20cm3 with distilled deionised water before being introduced into the hydride generator where 0.6M HCl and NaBH4 (2% in 0.2M NaOH) were pumped through a mixing coil at 6.0cm3min-1. Arsines were collected in a liquid nitrogen cooled trap for a period of 30 seconds after which the trap was slowly heated to 200°C causing the trapped arsines to be swept in order of increasing volatility onto a gas chromatography column for analysis. Separation was carried out on a glass column packed with 3% silicone OV-17 on 80/100 mesh Chromasorb W. Electron impact mass spectroscopy was used to monitor the eluting arsenic species, this was operated in selected ion mode at 70eV, an ion accelerating voltage of 3.0kV and an oven temperature of 180°C.

The EI mass spectra obtained indicated that under the digestion conditions studied arsenobetaine was quantitatively converted to trimethylarsine oxide and that this was then reduced to trimethylarsine by the action of the borohydride. Results also showed that arsenocholine did not undergo any transformation to a hydride active species in the course of the reaction and that arsenite, arsenate, MMA and DMA were unaffected by hot base digestion and formed arsines as normal.

Analysis of the digests of the tissue extracts revealed the presence of trimethylarsine and to a much lesser extent, dimethylarsine. The fate of the arsenobetaine standard solution following the hot base digestion indicates that arsenobetaine was the principal arsenical originally present in the tissue extracts.

The effectiveness of this digestion was questioned by Sturgeon[15] et al. In a controlled experiment 800mg of arsenobetaine was heated with 25cm^3 of 4M NaOH at 90°C for 72 hours. However only 35% conversion of arsenobetaine to trimethylarsine oxide was achieved. The remaining unconverted arsenobetaine was recovered by anion exchange column chromatography with ammonium buffer as the eluant.

An alternative digestion procedure reported in this paper yielded 86% conversion of arsenobetaine to a hydrolysable form[15]. This involved heating samples in a mixture of 5cm^3 H_2SO_4, 5cm^3 HNO_3 and 5cm^3 $HClO_4$ to yield yellow fumes of SO_2.

Ideally, conversion of arsenobetaine to a hydride active form should be included on-line with the analytical procedure in order to minimise losses and experimental error due to manual input. While these hot base digestion procedures were successful in converting arsenobetaine into a hydride active form they were not amenable to automation. Furthermore, the use of high concentrations of corrosive acids and bases may discourage their use for routine arsenic speciation analysis.

2.1.2.2 Thermochemical Hydride Generation

A significant advancement with respect to determining arsonium cations has been the invention of the thermochemical hydride generation interface. This design was first presented by Blais and co-workers.[16] and is illustrated in fig 2.2 overleaf. The all quartz main body consisted of an optical tube situated in the AAS optical beam, an analytical flame tube, a combustion chamber positioned within the side arm of the lower T tube, a thermospray tube and O_2/H_2 inlets the former being upstream from the latter. The combustion chamber and thermospray tube assembly met the analytical

flame tube at an angle of 45°. The assembly was composed of the main body, a capillary transfer line which was connected to the HPLC column outlet and was positioned inside the thermospray tube by means of a quartz guide tube. The heating element consisted of a coil of resistance wire which was heavily insulated by refracted wool and surrounded by shaped fire brick casing held together by a screw clip. Two stainless steel swagelock assemblies functioned in fixing the guide tube within the thermospray tube and the oxygen inlet within the analytical flame tube.

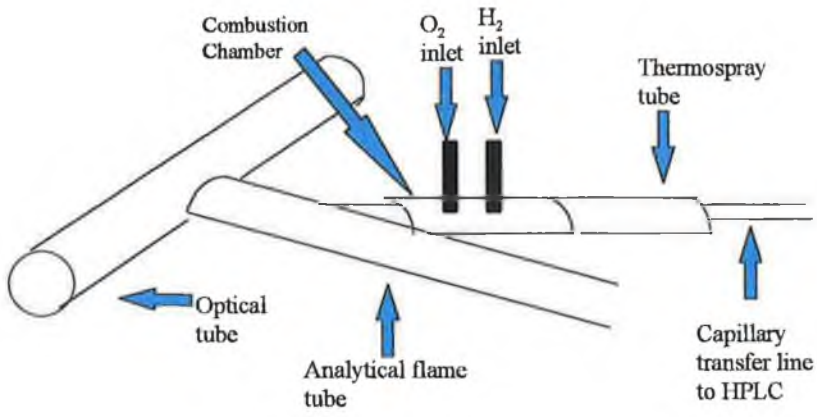


Fig 2.2 The thermochemical hydride generation interface designed by Blais et al.[16].

HPLC eluent was nebulised by the thermospray effect followed by pyrolysis in an oxygen mediated kinetic flame. Each analyte was then thermochemically derivitised to the corresponding hydride in the presence of excess hydrogen. These volatile derivatives were then transported to a cool diffusion H_2/O_2 flame atomiser.

The high compatibility of the diffusion flame atomisation process and the hydride generation reaction was due to the fact that a high hydrogen flow rate was required for both processes. Hydrogen radicals formed in the diffusion flame react with hydrides resulting in the atomisation of the latter. This reaction zone was formed in a spatially limited cloud of free radicals which does not reach the AAS optical beam. This effectively limits spectral background noise.

The proposed mechanism for thermochemical hydride generation in this interface was corroborated by the following observations

1. In the absence of the analytical diffusion flame no AAS signal was generated thus indicating that the species emerging from the interface were molecular and volatile.

2. The signal disappeared when the post thermospray hydrogen was replaced by helium .

3. Inorganic arsenic was also derivitised which can only be explained by thermochemical derivitisation to arsine.

Arsonium standards were separated isocratically on a cyanopropyl bonded phase column using a methanolic eluent containing 30% diethyl ether, 1% acetic acid and 0.05% triethylamine. This gave rise to well-resolved symmetrical peaks. Spectral interferences were negligible due to the highly selective nature of the diffusion flame atomiser. Calibration curves were linear in the concentration range studied (50ng-1mg) with limits of detection of 13.3ng for Asbet, 14.5ng for Aschol, and 7.6ng for tetramethylarsonium cation.

The suitability of this thermochemical hydride generation interface towards routine analysis of arsenicals in biological matrices was assessed by Momplaissir et al..[17]. Levels of arsenobetaine and arsenocholine and tetramethylarsonium cations in spiked lobster, shrimp and cod fillet samples were determined using a similar cyanopropyl stationary phase. The mobile phase composition was as follows; 80% v/v methanol, 1% v/v diethyl ether, 1% glacial acetic acid containing 0.129% v/v triethylamine and 20mg/100cm^3 picrylsulfonic acid. This was delivered at 0.65cm^3/min. A spiking range of 10% the anticipated value was employed with recovery from each sample at least 83%.

HPLC thermochemical hydride generation AAS was thus deemed to be an inexpensive alternative to the existing HPLC GFAA techniques for arsenic speciation in fish tissue. The main limitation of this design is the requirement for an organic rich (in excess of 80% methanol) mobile phase which acts as a fuel to support the combustion. This would render this approach incompatible with anion exchange chromatographic methods

2.1.2.3. Photo-Oxidation

A more innovative and universally applicable approach is the conversion of organoarsenicals to hydride active compounds by a simple photo-oxidation step. First described by Cullen and Dodd[18] it provides a useful substitute for more complex digestion procedures. Decomposition of a number of aromatic and aliphatic organoarsenic compounds was carried out by irradiating aqueous standard solutions for 1 hour with a 1200V medium pressure lamp. Up to 24 samples could be simultaneously exposed. These were contained in sealed tubes and arranged around the lamp in a fan cooled carousel.

Irradiated solutions were analysed by ion-pair chromatography on a reverse phase column using a tetrabutylammonium nitrate in a 95/5 water/methanol mobile phase and detection by hydride generation graphite furnace atomic absorption spectroscopy. Resulting chromatograms identified arsenate as the principle degradation product of these organoarsenic compounds. The variation in hydride generation atomic absorption response following conversion of arsenobetaine to arsenate in a number of acid media and in the presence of methanol, ethanol and acetonitrile was also examined. Findings indicated that at high acid concentrations a longer exposure time was necessary for complete photo-oxidation, this increase in required irradiation time was attributed to the quenching effect of the other species introduced by the acid. A similar effect was observed for organoarsenic solutions irradiated in the presence of organic solvents although the extent of this interference was less than that experienced in acidic solutions. In order to establish the viability of the photo-oxidation method for

the determination of organoarsenicals in "real" samples, a number of methanolic extracts of clam tissues were spiked with arsenobetaine and subjected to the irradiation. Analysis of the arsenate concentration in the solutions following irradiation revealed that the recoveries were in the range 89.6-103.8%. The irradiation method was also highly reproducible with the percentage relative standard deviation reported in the range 0.9-1.4%.

This technique was further developed by Atallah and Kalman[19] who reported the on-line photo-oxidation of organoarsenicals to inorganic arsenate. Initially, the most suitable conditions for this photo-oxidation were established by carrying out the reaction in batch mode, using a range of acidic, neutral or basic media. Results showed that optimum conversion of MMA, DMA, phenylarsonic acid (p-AsA) and ortho arsanilic acid (o-AsA) to arsenate occurred when these organoarsenicals were irradiated in the presence of a 20mg/cm^3 potassium persulfate solution in 50mg/cm^3 NaOH. The authors proposed that photo-oxidation in the presence of persulfate is mediated by highly reactive hydroxyl radicals which were produced by the photo-decomposition of persulfate. Using these conditions, on-line photo-oxidation was then investigated using a system composed of a flow injection hydride generation manifold, photo reactor, gas/liquid separator and AAS detector. This system is described schematically in fig. 2.3. The photoreactor unit was constructed using a mercury lamp emitting short wavelength light with a maximum at 254nm. This was wrapped in 5m of PTFE tubing and enclosed in aluminium foil which increased the light intensity and prevented exposure to the operator. Eluent from the photoreactor was acidified with 8M HCl flowing at 0.8cm^3/min before merging with 4% $NaBH_4$ solution. A high concentration of borohydride was necessary to reduce the excess persulfate as well as the arsenate. The reduction reaction took place in a mixing coil and arsine vapour was stripped with a 350cm^3/min flow of helium to the atomisation tube of the AAS. Efficiency of the photo-conversion was found to be dependant on residence time in the reactor. For the experimental conditions studied, the optimal photoreactor residence time was found to be 20-25 seconds with coil lengths of 2m or more and pumping speeds of 1cm^3/min.

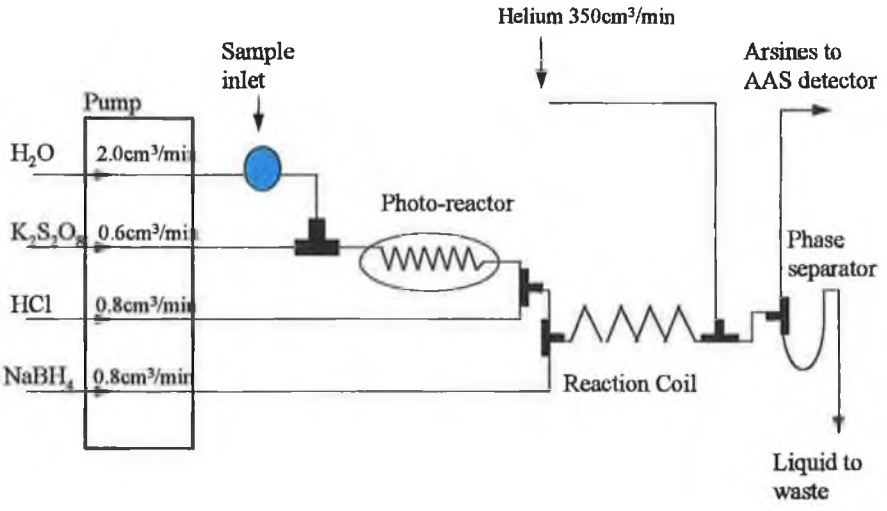


Fig 2.3; On line photo-oxidation hydride generation system reported by Atallah and Kalman[19].

Under the experimental conditions a conversion efficiency of above 95% was achieved for all organoarsenicals investigated. It was observed that the presence of ammonium, methanol or urea in the sample cause depression of the analytical signal, which would be an important factor to consider when analysing biological samples.

Rubio et al.[20] have subsequently published optimum coupling conditions for introducing photo-oxidation on-line between chromatographic elution and hydride generation. Separation of arsenite, arsenate, MMA, DMA, Asbet and Aschol was carried out on a Hamilton PRP-X-100 polystyrenedivinylbenzene trimethylammonium ion exchange column employing a $NaHPO_4$-Na_2PO_4 20mM mobile phase at pH 5.75. The photoreactor was designed to be compatible with the exit from the LC column and the entrance to the reduction chamber while keeping band broadening to a minimum. With column effluent flow rate at 1cm^3/min, persulfate was introduced at 0.2cm^3/min into PTFE tubing. A 0.5% persulfate solution in alkaline medium was used which required a 40 second reaction time for complete conversion this corresponded to a capillary tube length of 9m. A 1% solution of $NaBH_4$ in 1% NaOH and 8M HCl was

used for the hydride reaction. Arsines were determined by ICP - AES although all forms of AAS are applicable for detection purposes.

Compounds eluted in the order Aschol, Asbet and arsenite, DMA, MMA and arsenate however arsenite and Asbet co.-eluted under these conditions. The limits of detection were reported to be 6.1, 7.9, 2.6, 9.8, 13.0, and 9.6 μg As l^{-1} for Aschol, Asbet, arsenite, DMA, MMA, arsenate respectively when the photo-oxidation process was included in the analysis.

The introduction of on-line photo-oxidation did not lead to an appreciable loss in precision or an increase in analysis time. This hyphenated technique of LC-UV-HGAAS thus marks an important development in coupled arsenic speciation systems as it facilitated the analysis of all forms of the element within a single run therefore rendering it highly suitable for the purposes of routing analysis.

2.1.3. Sample preparation for arsenic speciation analysis

2.1.3.1. Digestion procedures for total arsenic determinations

In order to facilitate the majority of analytical determinations it is necessary to first remove the analyte from its sample environment. This often involves extensive extraction and sample preconcentration steps.

For total arsenic determinations destruction of the matrix can be carried out by digestion with a $HNO_3/HClO_4/H_2SO_4$ mixture for about three hours[21]. Using microwave technology [18] the digestion time can be reduced to a matter of minutes. Compounds such as arsenobetaine and arsenocholine however are stable to the action of some acids and therefore digestion may be incomplete. Alternatively a dry ashing procedure[22] may be employed whereby sample and an ashing aid [20% $Mg(NO_3)_2$/2% MgO] are subjected to a high temperature program in a muffle furnace for about 24

hours. Resulting ash can subsequently be re-dissolved in dilute HNO_3 for analysis by AAS.

For the purposes of speciation however, these digestion processes are too severe causing breakdown of metal-carbon bonds in some compounds and inter-conversion between redox states. Techniques therefore need to concentrate more on separation of organoarsenicals from their environment while maintaining their integrity rather than complete destruction of the matrix.

2.1.3.1. Liquid-Liquid extraction for speciation analysis.

The majority of arsenic speciation studies have dealt with water or biological tissue samples, particularly fish tissue. Water requires very straightforward sample handling procedures consisting of filtration and possibly the use of solid-phase extraction cartridges. In the case of biological tissues, isolation of organometallic compounds demands a complicated series of liquid-liquid extraction steps combined with various preparative chromatography techniques.

Edmonds and Francesconi initially developed a protocol for isolating arsenobetaine from the tail muscle of the western rock lobster[6]. Homogenised tail muscle was first shaken a number of times with methanol. The extracts were then combined, evaporated to dryness and the residue dissolved in water. Fat soluble impurities were removed by extraction with ether and the remaining solution was acidified and shaken with phenol. The arsenic containing compounds were recovered by dilution with ether, followed by extraction with water. The extraction solution was then passed through a Dowex 2x8(OH^-) and an Amberlite IRC 50 (H^+) column. Preconcentration of the arsenic containing fraction was carried out by application to a Zeokarb 255 (H^+) (SRC 8 2% DVB 3.5WR < 200 mesh) column followed by elution with dilute ammonia.

Variations of this methanolic extraction have since been used for a large number of arsenic studies[6,7,23,24].

Lawrence et al.[7] extracted homogenised fish tissue for 24 hours with ethanol-free chloroform and subsequently extracted the residue with 500cm^3 methanol for 24 hours which extracted the compounds of interest. This methanolic phase was then rotary flash evaporated at 300°C and the residue was then taken up in 100cm^3 chloroform. A 10cm^3 aliquot of this solution was applied to a column containing 25g of basic alumina and eluted with 100cm^3 of chloroform which was then discarded. The column was then subjected to a sequential elution procedure using 10cm^3 aliquots of solutions comprising of 1, 3, 5, 7, 10 and 15% methanol in chloroform. These were also discarded. Finally, 20cm^3 each of 20, 40, 60, 80, 100% methanol in chloroform solutions were applied to the column. These last five fractions contained the arsenic species. These were combined and flash evaporated to 0.2cm^3 at 30°C. The residue was then taken up in 10cm^3 of chloroform and applied to a second alumina column and the clean up procedure was repeated twice more. The final collected fractions were then combined and flash evaporated and the residue was dissolved in 50cm^3 methanol for HPLC.

These liquid-liquid extraction protocols as outlined however have some obvious drawbacks. They are highly time consuming and labour intensive which is a considerable limiting factor for routine analysis. The large number of steps involved makes the technique highly susceptible to errors making optimisation of extraction conditions a difficult task. The conditions may be still rigorous enough to cause breakdown of organoarsenicals and oxidation/reduction of various species thus leading to erroneous results. In addition to this, the process necessitates the use of large volumes of organic solvents which must be evaporated. These may contribute background interferences and furthermore cause detrimental effects to both the analyst and the environment.

2.1.4. Matrix Solid-phase Dispersion Extraction Techniques

In recent years, Long and co-workers[25,26,27,28,29] have developed a technique known as matrix solid-phase dispersion (MSPD) which may be used as an alternative to existing

liquid - liquid extraction methods for biological matrices. MSPD involves the blending of fortified tissue sample with a specific amount of bulk chromatographic packing (usually C18) to form a homogenous mixture which has the appearance of a fine powder. This C18/sample homogenate is then quantitatively transferred to a plastic syringe barrel plugged with filter paper, compressed and washed with two column volumes of a non-polar solvent such as n-hexane. This will remove any lipid soluble components. Residual hexane can then be removed by evaporation in an oven and the analyte in question can subsequently be eluted with a small volume of appropriate mobile phase for further analysis.

Even though the elution volume is at maximum about $8cm^3$ the process may be considered an exhaustive extraction as a relatively large volume of solvent is in effect passed over a very thin layer of sample. Dispersion of sample onto the C18 is a combination of mechanical and hydrophobic forces. Non-polar materials such as membrane lipids associate with the C18 polymer facilitating the disruption and unfolding of structural components. Conversely hydrophilic moieties preferentially arrange themselves away from the non-polar regions thus extending outwards, water soluble compounds affiliating with these hydrophilic ends. Scanning electron micrographs of uncoated and tissue coated C18 beads support this theory.

This technique has found successful application in the extraction of various classes of antibiotics including benzimidazoles[26], β-lactams[27], tetracyclines[28-29] and sulphonamides[30], from a range of animal tissues from chicken liver to oysters. Pesticides such as lindane, dieldrin, α BHC and β BHC have also been successfully isolated from tissue[31]. The technique has also been used to extract oxolinic acid from shellfish[32] and clenbuterol from beef tissue[33].

For all compounds, recoveries of greater than 60% were achieved over the range of concentrations examined and in all cases little or no sample clean up steps were required post extraction. This led to a substantial improved analysis and a reduction in the analysis time.

The matrix solid-phase dispersion method therefore eliminates many of the problems associated with classical isolation techniques. The small sample sizes required, minimal number of steps and lack of chemical manipulations such as pH and polarity adjustments makes MSPD highly favourable for routine work.

2.1.5. Conclusions

The development of matrix solid-phase dispersion techniques has been primarily concerned with the isolation of drug residues and other organic molecules from biological matrices. In order to apply the technique to the extraction of inorganic and organometallic arsenic species a number of modifications are required. These include the investigation of alternative chromatographic packing materials such as ion exchange resins and selection of appropriate solvents for the selective elution of analyte and matrix constituents. Once the suitability of the technique for the extraction of arsenicals has been established, statistical validation is necessary in terms of reproducibility and linear dynamic range.

2.2. Experimental.

2.2.1. Materials.

The following arsenic compounds were investigated; Sodium arsenite (NaAsO2); Dimethlyarsinic acid (DMA) ($(CH_3)_2AsOOH$), both from Aldrich; Disodium hydrogen arsenate (Na_2HAsO_4) - BDH; Monomethylarsonic acid (MMA) $CH_3AsO(OH)_2$ and Arsenobetaine (Asbet) $(CH_3)_3AsCH_2COOH$, both obtained from the European BCR program as 1000ppm stock solutions in distilled-deionised water. C18 30-70 micron 608, cyanopropyl, anion (SAX) and cation (SCX) exchange packings were all supplied by Alltech Associates/Applied Science. Solid-phase extraction columns were prepared using $1cm^3$ syringe barrels obtained from Bond Elut, Analchem International. Sodium dihydrogen phosphate (Merck) and Disodium hydrogen phosphate (Riedel de Haen) were employed as the eluting buffer. Sodium Borohydride powder (Aldrich Chem. CO,) and Sulphuric acid (Riedel de Haen) were used for the generation of hydrides.

Unless otherwise stated, all solutions were prepared in distilled-deionised water obtained from a Millipore water purification system.

2.2.2. Apparatus

Arsenic determinations were carried out by flow injection hydride generation atomic absorption spectroscopy.

This flow injection system consisted of a peristaltic pump, a four way rotary valve with external sample loop, a T-piece mixer and a gas liquid separator and is described schematically in fig 2.4. The analysis process was divided into two sampling cycles, a 2% sodium borohydride solution ($NaBH_4$) in 1% sodium hydroxide (NaOH) and a 1M sulphuric acid solution (H_2SO_4) were pumped through the system at flow rates of $1.7cm^3$/min. and $6.0cm^3$/min. respectively.
These solutions were mixed thoroughly at the T-piece mixer where the following reaction took place.

$$NaBH_4 + 3H_2O + H_2SO_4 \Leftrightarrow H_3BO_3 + NaHSO_4 + 8H^+$$

The second part of the cycle involved the injection of the arsenic sample via the rotary valve into the acid stream. On mixing, a second reaction takes place generating the volatile arsine species.

$$H^+_{(EXCESS)} + As^{n+} \Leftrightarrow AsH_n + H_2(GAS)$$

Argon gas was continuously pumped through the gas liquid separator at a rate of 600cm^3/min. this provided a means of carrying the arsines into the silica atomisation tube which was heated with an air acetylene flame. Detection of the arsenic species was achieved with an Instrument Laboratory AA/AE spectrophotometer 357 equipped with a hollow cathode lamp emitting monochromatic light at 193.7nm. An absorbance measurement took place over a twenty second integration time with output being continuously measured with a Phillips chart recorder.

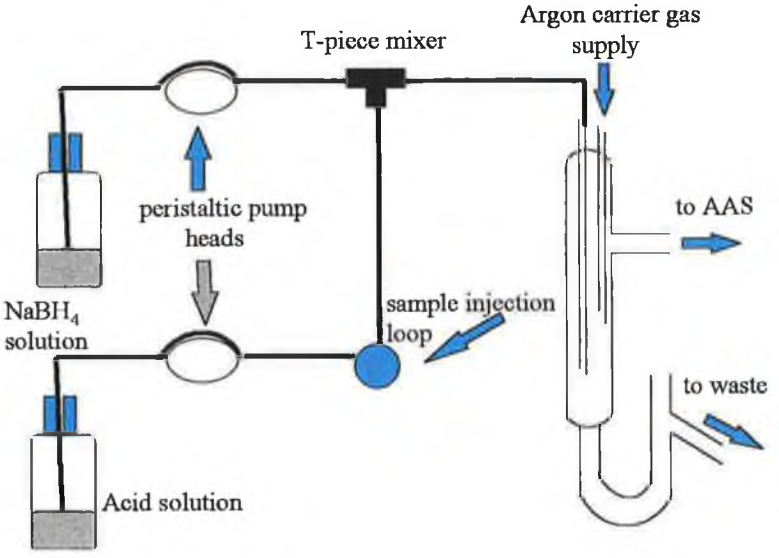


Fig. 2.4; Schematic of Flow injection Hydride Generation Atomic Absorption System

A set of pure standards over an appropriate concentration range were analysed alongside the extracted samples. The percentage recovery was calculated by comparing absorbance peak heights of samples with their corresponding standards. In all cases solutions were injected five times each.

2.2.3. Initial Preparation of packing material.

Prior to carrying out each extraction, the chromatographic packing in question was washed with an appropriate solvent. In the case of the reverse phase type material (C18, CN and C2), packing was first washed with methanol, followed by water, the ion exchange packings required only a water wash. This washing step was carried out by loading approximately 1g of the appropriate packing material into the barrel of a $1cm^3$ plastic syringe plugged with a filter and passing $5cm^3$ of solvent through it. This flow was controlled using a second plastic syringe attached to the tip of the first via a short length of rubber tubing.

2.2.4. Extraction procedure

Each extraction was carried out using 0.04g of tissue sample, this sample was placed in the mortar and spiked directly with an appropriate concentration of specific arsenic species. A preweighed amount of packing material was then added to the fortified tissue which was then blended for 2 minutes using a pestle until the mixture resembled a homogenous powder.

The resultant packing/tissue matrix was then quantitatively transferred using a micro-spatula into the barrel of a $1cm^3$ syringe which was plugged with a filter. After the material was allowed to settle it was washed with $2cm^3$ of a suitable non polar solvent such as hexane which resulted in the removal of cellular debris and other potential organic interferants. In this case flow through the column was gravity controlled. Residual solvent was removed by drying the columns in an oven at 80°C for 1 hour.

Arsenic species were subsequently eluted with a specific volume of sodium dihydrogen phosphate buffer (NaH_2PO_4) adjusted to pH 5.82 with disodium hydrogen phosphate (Na_2HPO_4). Previous work in our laboratories has shown this to be the optimum pH for the separation of arsenicals by ion exchange HPLC[34].

The flow rate of this eluting buffer was controlled as before by means of a $5cm^3$ plastic syringe attached to the end of the column by rubber tubing. Resulting extracts were made up to $10cm^3$ with phosphate buffer and analysed by flow injection hydride generation atomic absorption spectroscopy. Tissue blank controls were treated in the same manner except these were spiked with 10µl of distilled-deionised water. The entire extraction procedure is summarised in fig 2.5.

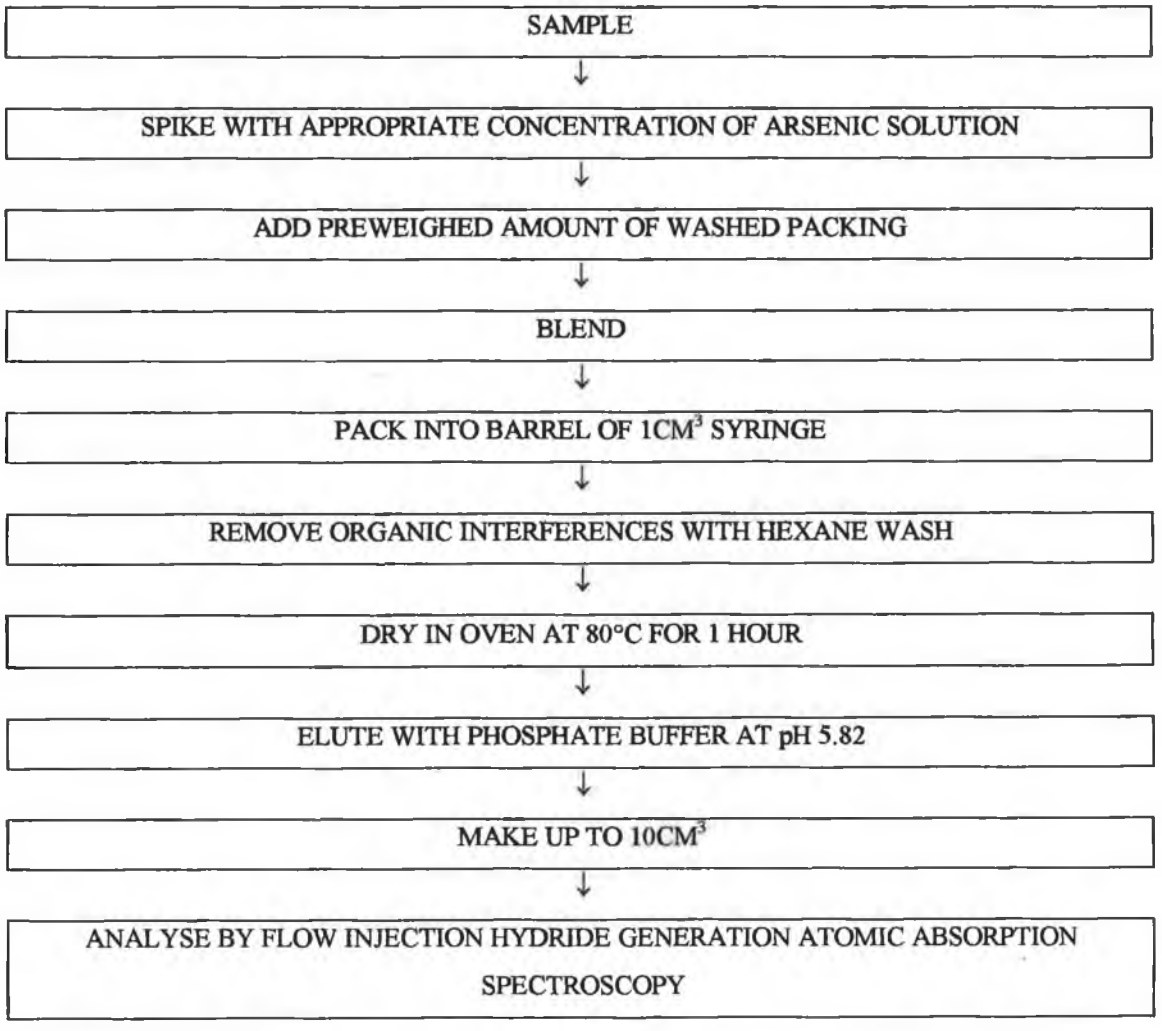


Fig. 2.5 Summary of MSPD extraction of Arsenicals from Fish Tissue.

2.2.5. Stability tests

For certain industrial and environmental applications it may be necessary to carry out a large number of extractions over a period of days and then analyse the extracted solutions together at a later stage.

It was consequently investigated whether solution of arsenic species in phosphate buffer would remain stable over a period of five days. Solutions of arsenite, arsenate, MMA and DMA at a concentration of 300ppb were made up in triplicate in 0.5M phosphate buffer a pH 5.82 every day for five days and stored in glass bottles at a temperature of 4°C. Analysis of all solutions was carried out on day 5.

2.2.6. Preliminary optimisation of extraction conditions

Initial experiments focused on establishing the most suitable parameters for the extraction of arsenic species from fish tissue. This preliminary work was carried out using 0.04g fish tissue and 0.1g of C18 packing. The following arsenic species were investigated: arsenite, arsenate, MMA and DMA. As arsenobetaine requires an additional digestion step to enable its analysis by hydride generation atomic absorption spectroscopy, it was not investigated at this stage.

The necessity for the solvent washing and subsequent drying steps in the extraction process was first investigated using tissue blanks. Once this had been established further experiments were carried out using tissue spiked with 15µl of a 200ppm solution of the appropriate arsenic species this coressponded to a tissue concentration of 75µg arsenic species/g tissue.

The effectiveness of C18 and anion exchange solid-phase extraction packing were primarily examined. The most favourable concentration of phosphate buffer required to elute each arsenic species was established. Following this, the influence of the

amount of packing used on the recovery was studied. Extractions were carried out using 0.1, 0.2, 0.4 and 0.5g of packing.

Chloroform, diethyl ether and hexane were all tested for their suitability as a washing solvent The effect of each solvent on recovery, peak shape and reproducibility was monitored. The length of time required to completely "dry" the MSPD columns without a loss in analyte signal was also determined. Drying times of 30 minutes, 1 hour and 2 hours were examined.

2.2.7. Comparison of solid-phase extraction materials

Initial experiments indicated that C18 was ineffectual in extracting arsenite from fish tissue. Therefore the efficiency of alternative solid-phase extraction materials such as anion (SAX) and cation (SCX) exchange resins and a cyanopropyl stationary phase were studied.

Experiments were carried out using the extraction conditions listed below.

Table 2.1

wt. fish tissue	0.04g
wt. packing	0.40g
level	75µg arsenite/g tissue
washing solvent	Hexane
drying time	60 minutes
conc. phosphate elution buffer	0.5M

The higher buffer concentration was used to ensure complete elution.

Composite solid-phases containing both anion and cation exchange resins and one containing anion exchange mixed with C18 were also tested. In such cases 0.2g of

each packing was used. This ensured that the overall volume of packing was consistent with single mode extractions. These were blended thoroughly before introduction to the spiked fish sample. Although primary investigations were directed at arsenite extractions, recovery of the other arsenic compounds were also studied.

2.2.8. Investigation into the effect of chelating agents on MSPD recoveries

Chelating agents such as ethylene diamine tetraacetic acid and oxalic acid have formerly been used as matrix modifiers in the isolation of various drug substances by the MSPD approach[29]. When mixed with the C18 material, these compounds complex with interfering inorganic ions thus enhancing the recovery of organic residues.

Arising from this it was investigated whether increasing the proportion of complexing agent in the packing would facilitate the improved extraction of arsenite from tissues. An appropriate quantity of chelating agent and packing were thoroughly mixed prior to carrying out each extraction. Tissue samples were spiked with 15µl of a 200ppm arsenite solution, when made up to $10cm^3$ this corresponded to a 300ppb solution assuming 100% recovery. Extraction and analysis were carried out as described previously.

EDTA and oxalic acid were primarily investigated. These were incorporated into the packing in the ratio of w/w 0.05g/0.15g, 0.05g/0.2g, 0.1g/0.2g, 0.2g/0.2g, 0.2g/0.4g. Both C18 and anion exchange packings were investigated. Phosphate buffer at a concentration of 0.2M was used as the eluant. Extractions were also carried out with the omission of complexing agent for comparison purposes and in all instances blanks were included as a means of monitoring interferences.

EDTA and oxalic acid are however very general chelating agents and will readily combine with any other metal ions present in the tissue. A number of dithiocarbamate compounds have been applied to the determination of arsenic in environmental

samples[35]. These provide a greater degree of selectivity, although certain ions such as cobalt, copper and antimony may be extracted concurrently. Diethyl ammonium diethyl dithiocarbamate in particular has been shown to complex with arsenite but not arsenate when present in mineral acid solutions. MSPD extractions of arsenite were consequently carried out in the presence of this organic chelate. In this instance only anion exchange packing was employed. Firstly the dithiocarbamate was combined with the packing as with previous complexing agents. In this case the quantity of packing used was always 0.4g. This was mixed with 0.0g, 0.025g, 0.05g and 0.1g of dithiocarbamate respectively. Extractions were carried out on 0.04g tissue spiked with 15µl of a 200ppm arsenite solution and tissue blanks were also included to monitor interferances

All extractions were carried out in triplicate and repeated over a five day period.

2.2.9. Elution volume tests

For the purposes of optimisation, MSPD was treated as an off-line extraction technique. Arsenic compounds were eluted with 8cm^3 of buffer solution, to ensure maximum possible recovery. However, for routine determinations, it would be preferable if the extraction was carried out on line with the analytical technique, in this case HPLC. This would require the volume of eluting buffer to be kept to a minimum, ideally below 1cm^3. Arsenic compounds could thus be completely eluted from the extraction column and subsequently be introduced to the analytical column in a small plug of solvent.

It was examined whether increasing in the concentration of phosphate buffer would allow for a reduction in the minimum elution volume required to achieve maximum recovery of arsenic species from the solid phase extraction columns. The combined effects of elution volume and phosphate concentration on recovery were initially studied with respect to arsenite. A spiking level of 75µg arsenite/g tissue was

employed. Phosphate buffer at pH 5.82 was used as the eluting buffer and concentration of 0.5M, 1.0M and 0.2M were investigated. In each case, the percentage recovery of arsenite after $1cm^3$, $2cm^3$ and $5cm^3$ elutions was recorded. All eluted solutions were made up to $10cm^3$ in phosphate buffer of the appropriate concentration prior to analysis. Subsequent elution experiments were carried out using 0.2M phosphate buffer and the effects of increasing elution volume on the recoveries of arsenate and MMA were investigated. As in the previous investigation, a spiking level of 75μg arsenic species /g tissue was employed for each arsenic species investigated. All elution volume tests were carried out in triplicate and repeated over a two day period.

2.2.10. Validation of the extraction procedure

The MSPD extraction of arsenic species from fish requires statistical validation. This was accomplished by methods analogous to those used for validating chromatographic techniques, using intra (within day) and inter (between day) variability assays[36]

Extractions were carried out using 0.04g of tissue samples as before and for each arsenic compound, a spiking range of 5 - 25μl of a 200ppm solution was employed. The tissue samples therefore were fortified with arsenic in the concentration range of 25 - 125 μg / g tissue. Comparison of extracted spiked samples for each concentration of arsenic to that of its respective pure standard run under identical conditions allowed the calculation of percentage recoveries.

Intra-assay variabilities were determined as follows; The percentage recovery for three replicate extractions at each concentration all measured within one day were averaged resulting in mean ± standard deviation. Each mean was then divided into its respective standard deviation which gave the relative standard deviation (RSD). When this value is expressed as a percentage it is termed the coefficient of variation (CV). The mean percentage coefficient of variation ± the standard deviation was then defined as the

intra-assay variability. Mean percentage CV relates to the precision to the method and its corresponding standard deviation relates to the error associated with it.

For intra-assay variability, a separate calibration curve was generated and the extraction procedure carried out using a spiking level of 75µg/g tissue for each compound, every day over a three day period. The percentage coefficient of variation for the mean of these replicates was then calculated as the inter-assay variability.

Once the intra and inter-assay variabilities and their standard deviations were below 10%, the isolation technique was deemed valid.

2.2.11. Application of MSPD to the extraction of arsenobetaine from fish tissue.

It is imperative that any technique involving the extraction of arsenic species from fish should be valid for the isolation of arsenobetaine (Asbet) the principal form in which it is found.

The highly stable nature of this organometallic compound however makes the molecule inert to the action of reducing agents such as $NaBH_4$ making analysis by straightforward hydride generation AAS impossible. A number of methods have been employed to convert Asbet into a hydride active form including a hot base digestion procedure outlined by Kaise et al.[14], a thermochemical hydride generation interface[16], and by irradiating the compound in the presence of potassium persulfate ($K_2S_2O_8$) with a UV light source [18]. The latter technique has proved to be highly successful for on-line conversion of organoarsenicals for use in tandem with HPLC - HGAAS flow systems.

For the purposes of this work however a batch digestion technique in which arsenobetaine solutions were heated in the presence of NaOH and $K_2S_2O_8$ was applied. This protocol was adapted from that outlined by Heng-Bin et al.[37]

The ideal conditions for the digestion of arsenobetaine were established using a 5ppm stock solution made up in 0.2M phosphate buffer pH 5.82. Initially approx. 0.1g of NaOH was completely dissolved in $10cm^3$ of this stock solution and 0.1g $K_2S_2O_8$ was then added. This mixture was heated to boiling and maintained at this temperature until it had reached near dryness. The resulting digest was then made up to $5cm^3$ with 0.2M phosphate buffer.

In order to monitor the conversion of arsenobetaine, $10cm^3$ portions of a 5ppm arsenate standard solution in 0.2M phosphate buffer were also digested. Interferences were monitored by digesting solutions of 0.2M phosphate buffer containing 1% NaOH and 1% $K_2S_2O_8$. The conversion efficiency of the digestion was calculated by comparing the peak heights obtained from the digested arsenobetaine solutions with the corresponding arsenate "digests". "Undigested" arsenate standard solutions in 0.2M phosphate were analysed alongside the digested solutions to observe any losses in arsenate that may occur in the course of the reaction.

This procedure was then repeated using 2 and 3% $K_2S_2O_8$ solutions to investigate whether this would improve the efficiency of the conversion.

2.2.12. Validation of the MSPD technique for arsenobetaine

The extraction of arsenobetaine from fish was validated as before except in this case the tissue was spiked with 5 - 25µl of a 1000ppm arsenobetaine solution this gave rise to a tissue concentration of 125, 250, 375, 500, 625µg/g tissue. For the purposes of inter-assay variability a spiking level of 625µg/g tissue was employed over a 5 day period.

This increased spiking levels were required so that an adequate response could be achieved by the atomic absorption instrument.

2.2.13. Comparison with existing extraction techniques

The efficiency of the MSPD extraction was compared with an existing liquid-liquid extraction technique. This protocol was initially described by Edmonds and Francesconi for the isolation of arsenobetaine from the lobster tail muscle tissue[6]. In addition to recovery of arsenicals by each extraction, factors such as the amount of time required and the work involved were also taken into consideration.

Extractions were initially carried out using 1.00g (wet weight) fish tissue, this was spiked with 125µl of a 200ppm arsenic solution, giving an overall tissue concentration of 25µg arsenic / gram tissue. This fortified sample was mixed with $10cm^3$ of a methanol / water 1/1 v/v solution and sonicated for twenty minutes. The solution was then removed and the tissue re-extracted with a fresh $10cm^3$ aliquot of solvent, this was repeated a further three times. The combined extracts were then evaporated to dryness on a hot plate and made up to $10cm^3$ with distilled-deionised water. Cellular components were then removed by extraction with $10cm^3$ of diethyl ether. The aqueous phase was subsequently acidified with $10cm^3$ of 2M HCl and shaken with three $10cm^3$ portions of liquefied phenol (90% in water). The phenolic fractions were then combined, diluted with diethyl ether and shaken with three $5cm^3$ portions of distilled-deionised water. This final aqueous solution was washed with diethyl ether and made up to $25cm^3$ with distilled-deionised water.

Extractions were primarily carried out using arsenite and arsenate, for each compound extractions were carried out five times, blanks were also included.

2.3. Results and Discussion

2.3.1. Stability Tests

The variations in peak height with time for arsenate, arsenite, MMA and DMA are listed in table 2.2. It can be seen that arsenite (AsIII) solutions exhibited an appreciable drop in signal intensity with time. This was probably due to the fact that at pH 5.82 and above, arsenite is susceptible to oxidation to the more stable pentavalent species. Arsenate does not form hydrides as readily and hence a large decrease in signal intensity is observed. Signals due to MMA also dropped steadily as the age of the solution increases with the signal produced by a 5 day old solution being 31.42% lower than that produced by a fresh solution of identical concentration. The signal intensities produced by solutions of arsenate and DMA are slightly more consistent with time however in both cases, the degree of variation in signals produced by increasingly older solutions still exceeds 10%.

Table 2.2; Peak heights obtained for 1-5 day old solutions of each arsenic species

arsenic species			peak	heights (mm)		max. % drop
	1 day	2 day	3 day	4 day	5 day	
As III	69.80	49.80	41.80	39.80	45.00	42.97%
As V	23.25	21.20	20.50	21.50	22.60	11.80%
MMA	14.00	13.10	11.25	11.20	9.60	31.42%
DMA	7.60	6.40	6.80	9.80 *	7.40	15.78%

* not counted as the signal is substantially higher than 1 day old solution, probably due to a pipetting error.

As outlined in section 2.2.10, one of the criteria for method validation is that the between day variation in the signal intensity must be below 10%. The inconsistency in the response yielded by progressively older standard solutions of each arsenic species investigated over a five day period means that the MSPD extraction of arsenic species

from tissues and the analysis of the resulting extracts could not be carried out on separate days if the method is to be deemed valid.

2.3.4. Preliminary optimisation of extraction conditions

The initial conditions chosen for the extraction of arsenite arsenate, MMA and DMA from fish tissues are listed in table 2.3.

Table 2.3; Most suitable conditions for MSPD extraction of arsenic species from fish tissue

Weight of tissue used for extraction	0.04g
Weight of packing	0.40g
Washing solvent	Hexane
Drying time	60 minutes
Concentration of eluting buffer	0.01M with C18 packing 0.5M with anion exchange.

The initial studies with tissue blanks revealed that when the washing step was omitted, organic components of the tissue matrix gave rise to an interfering signal on the AA. When the extraction columns were washed and not dried, a large background signal resulted in the eluant due to the presence of residual organic solvent. This interfering signal diminished slightly when the columns were placed in an oven at 80°C for 30 minutes and disappeared completely when this drying time was increased to one hour. Additional experiments with longer drying times revealed that increasing the amount of time the columns spent in the oven beyond 60 minutes did not result in any further improvements.

When investigating the most suitable solvent to use it was discovered that when the columns were washed with chloroform, there was still a slight interference signal in the blank tissue extracts even when the columns had been dried in the oven for 60 minutes. This effect was also observed when columns were washed with hexane and diethyl ether. These experiments were also carried out using tissue samples spiked with 15µl

of a 200ppm solution of arsenic species. Arsenite, arsenate MMA and DMA were all investigated at this point. Findings indicated that the columns washed with hexane afforded more consistent recoveries of MMA and DMA. This may have been due to the fact that these compounds are very slightly soluble in the diethyl ether and therefore are eluted to a small extent in the washing steps.
In the preliminary stages of the project, a phosphate buffer at a concentration of 0.01M was chosen to elute the arsenic species. This was chosen due to the fact that 0.01M phosphate is commonly used as the mobile phase in anion exchange chromatography of arsenic species. It was subsequently investigated whether increasing this concentration to 0.05M and 0.1M would effect the recovery of arsenic. In the case of C18 packing, maximum recoveries of in the region of 70%,65%,50% and 20% were achieved for arsenate, MMA, DMA and arsenite respectively, upon elution with 0.01M buffer. Increasing this concentration to 0.05M had no effect on the recoveries and when the concentration of elution buffer was increased to 0.1M, recovery of arsenate and DMA decreased by about 30%.

When investigating the recovery of arsenic species from anion exchange packing, it was found that buffer concentrations of 0.01M and 0.05M were not sufficiently strong enough. A 0.1M buffer yielded a 50% recovery of arsenate which improved to 80% when this was increased to 0.5M. In the case of MMA and DMA, due to their more organic nature a slightly lower concentration of buffer was needed to elute them from the anion exchange packing. Both compounds gave a maximum recovery of between 60-70% upon elution with 0.2M buffer. It was found that in the case of both packings, when the quantity of packing used for each extraction was increased from 0.1g to 0.2g an approx. 20% increase in all recoveries resulted. A further 10% was achieved when this quantity was doubled. However when 0.5g of packing was used there was no significant improvement in recovery. In addition to this increasing the amount of material in the solid-phase extraction columns increased the time required for washing and elution as the flow rate decreased.

Due to the fact that 0.01M buffer systems are more compatible with on-line HPLC extractions than 0.5M buffer systems, MSPD extraction using C18 packing was further investigated at this point. Using the conditions outlined in table 2.2, and a spiking concentration 125μg/g tissue, the reproducibility of the extraction was assayed for arsenite, arsenate, MMA and DMA. Extractions were carried out in triplicate and repeated each day over a three day period. Mean percentage recoveries were found to be ; 67.27 ± 10.19%, 69.60 ± 1.00% and 53.20 ± 2.72% for As V, MMA, and DMA respectively. .

The MSPD technique unfortunately proved to be unsuccessful in the extraction of arsenite from the fish tissue at this stage with a maximum recovery of 20% being achieved with the C18 packing. The main objective of all subsequent work was therefore to concentrate on modifying the extraction conditions so as to improve recovery of arsenite while maintaining acceptable recovery levels for the remaining arsenic compounds.

2.3.5. Comparison of solid-phase extraction materials.

The variation in percentage recoveries for arsenite, arsenate, MMA and DMA obtained from anion exchange (SAX) packing, cation exchange (SCX) packing, anion/cation exchange (SAX/SCX) packing mixture, anion exchange/octadecyl silica (SAX/C18) packing mixture and cyanopropyl (CN) packing are illustrated in fig 2.6. In all cases extractions were carried out using 0.04g of tissue spiked with 15μl of a 200ppm standard solution of the appropriate arsenic species and a total packing volume of 0.4g. The resultant columns were eluted with 0.2M phosphate buffer pH 5.82.

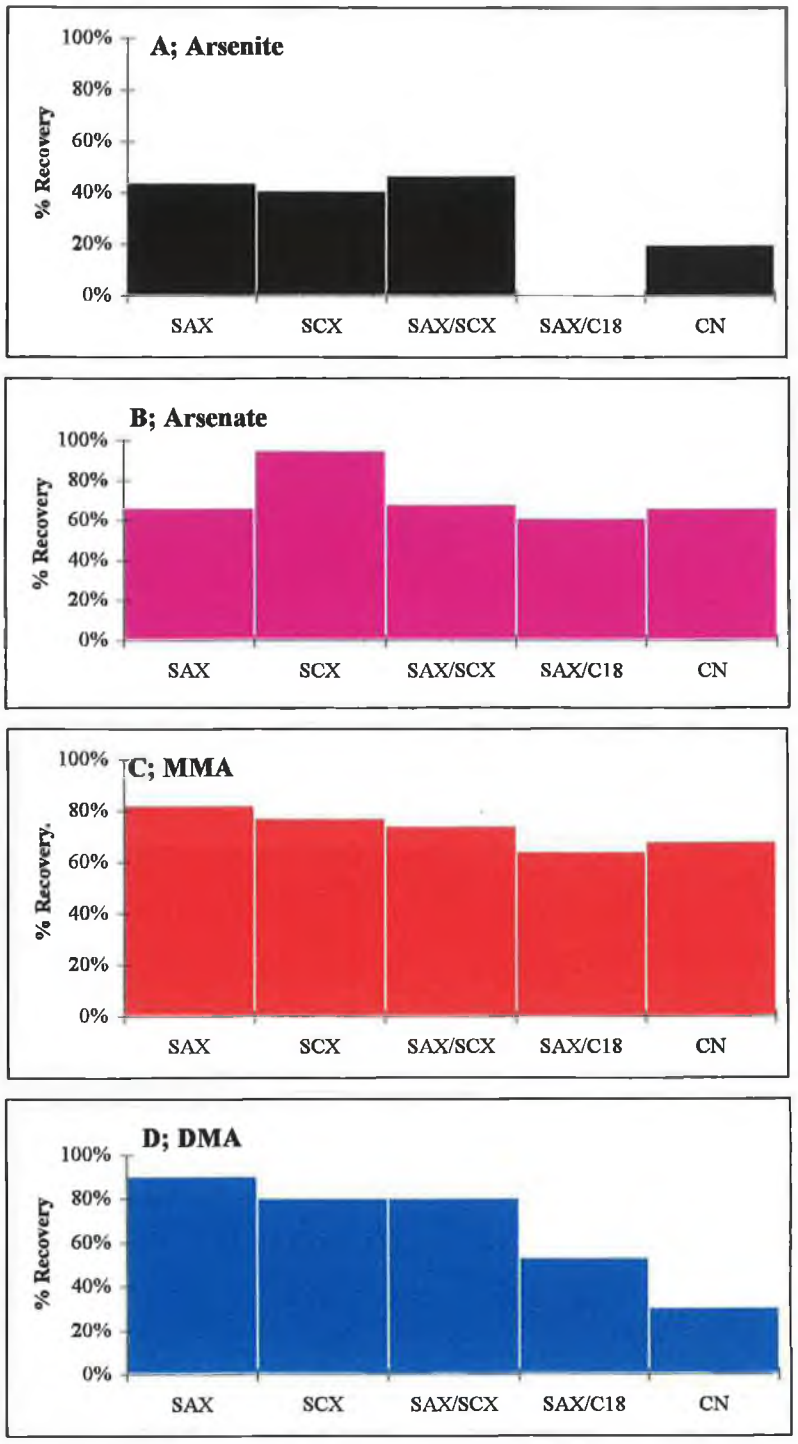


fig 2.6; Variation in percentage recoveries for A;arsenite, B;arsenate, C; MMA and D;DMA obtained from each of the packings investigated. Extractions were carried out using 0.04g of tissue, a spiking level of 75μg arsenic species/g tissue and a total packing weight of 0.4g. Elutions were carried out using 8cm^3 of 0.2M phosphate buffer pH 5.82.

As can be seen, acceptable recovery levels for arsenite were not attained with any of the packings investigated. The non-polar type stationary phases (C18 and CN) gave particularly low recoveries. Even when used in combination with anion exchange materials, recovery never exceeded 30%.

An attempt was made to account for the remaining 70%. One theory suggested that the arsenite ion being a charged moiety was not retained by the C18 but merely became loosely dispersed between the hydrocarbon chains and was subsequently displaced by the flowing liquid in the washing stage. Therefore extractions were carried out as before and the n-hexane washings were retained and subjected to a number of liquid-liquid extractions with 0.5M phosphate pH 5.82, a 30% nitric acid solution, and a methanol/water 60/40 (v/v) solution. These aqueous solutions were then analysed for arsenic.

No traces of the element were found in any of these solutions. As trivalent arsenic is known to react strongly with sulfhydrl groups in cells, it was therefore concluded that added arsenite was probably becoming bound to cellular proteins in the tissue matrix and these complexes were consequently strongly retained by the stationary phase. Use of stronger eluents such as 1M HCl did not give an appreciable recovery, but when tissue coated C18 material was digested in a 9/1 $HNO_3/HClO_4$ v/v solution for 3 hours a signal of about 50% that of the corresponding standard resulted. This explanation was further supported by the fact that when the tissue was excluded and the arsenite solution was simply ground up with the packing, and elution carried out as usual, a 80% recovery could be achieved.

Employment of ion exchange type resins led to slightly improved recoveries of arsenite. Results were roughly similar for anion and cation exchange resins and mixtures of the two with an average recovery of 40% being attained in each case. In addition to this these ion exchange packings proved to be superior to C18 in the extraction of arsenate, MMA and DMA from tissue. The anion exchange (SAX) material gave most favourable results for the extraction of MMA and DMA with

percentage recoveries of 82 % and 90% achieved for MMA and DMA respectively. The cation exchange material yielded almost 100% recovery for arsenate. It would therefore seem that a mixed anion/cation solid-phase would universally be the most suitable for the simultaneous extraction of all arsenic compounds studied. Unfortunately, use of cation exchange packing proved to be problematic. Solutions eluted from cation exchange columns were cloudy in appearance. In addition to this a considerable amount of frothing was observed in the gas liquid separator following injection of these solutions, giving rise to very noisy inconsistent signals. Blanks run alongside these samples also caused an increase in the noise level upon injection. In order to eliminate these interferences, the cation exchange resin was pre-washed with 0.5M buffer. This reduced the amount of interference, however, noise and frothing was still a problem. The resin was also pre-washed with NaOH, but this did not lead to an improvement. A small amount of C18 material was placed as a plug at the bottom of the column, which reduced interferences slightly, but the arsenic signal was also suppressed.

For this reason, the use of cation exchange resins for the MSPD extractions were not pursued further and anion exchange resin was deemed to be the most suitable solid-phase for further study.

2.3.6. Effect of Chelating agents on extraction of Arsenic species by MSPD.

As outlined in section 2.2.8, it was investigated whether the incorporation of chelating agents into the C18 and anion exchange packing materials would improve the extraction efficiency for arsenite. The effects of EDTA, oxalic acid and diethyl ammonium diethyl dithiocarbamate (DTC) were all examined. Initial findings indicated that inclusion of oxalic acid in both the C18 and anion exchange packing made no difference to the end result. The percentage recovery of arsenite from C18/EDTA matrices proved to be highly inconsistent varying from 20% - 60% for extractions carried out within the same day. Experiments using tissue blanks revealed the presence of a high background interference in the solutions eluted from the C18/EDTA

extraction columns. This interference was absent, however, when EDTA was combined with the anion exchange packing. This was probably due to the fact that the EDTA was strongly retained by the ion exchange resin and therefore not eluted by the phosphate. Unfortunately, there was no significant difference in recovery of arsenite from anion exchange in the presence or absence of EDTA.

The inclusion of DTC in the packing matrix also gave rise to high background interference in the resulting eluants with absorbance signals from tissue blank extracts up to 10 times more intense than that of the corresponding 300ppb arsenite standard solution. In the majority of cases there was no observed difference in the signals obtained from blank tissue extracts and those obtained from extracts of tissues spiked with 15µl of a 200ppm arsenite solution. In addition to this, the mechanical grinding involved in combining the tissue with solid-phase extractant resulted in the DTC compound which initially existed as a moist powder being liquefied, this would inevitably lead to inconsistencies in the composition of the solid-phase.

The introduction of chelating agents into the packing material thus proved to be ultimately ineffective in improving the MSPD extraction efficiency of arsenite. All remaining experiments were carried out on 0.04g of tissue spiked with 15µl of a 200ppm solution of appropriate arsenic species using 0.4g of anion exchange solid phase extraction packing.

2.3.7. Effects of elution volume on recoveries of arsenic species from MSPD columns

The effects of increasing the concentration of the eluting buffer on recovery was primarily investigated for the extraction of arsenite from the MSPD column. Elution was carried out using $1cm^3$, $2cm^3$ and $5cm^3$ respectively of 0.5M, 1.0M and 0.2M phosphate buffer at pH 5.82. All solutions were made up to 10cm3 with the appropriate concentration of phosphate buffer prior to analysis. The percentage

recoveries obtained with each elution volume at each phosphate concentration investigated are compared in fig 2.7.

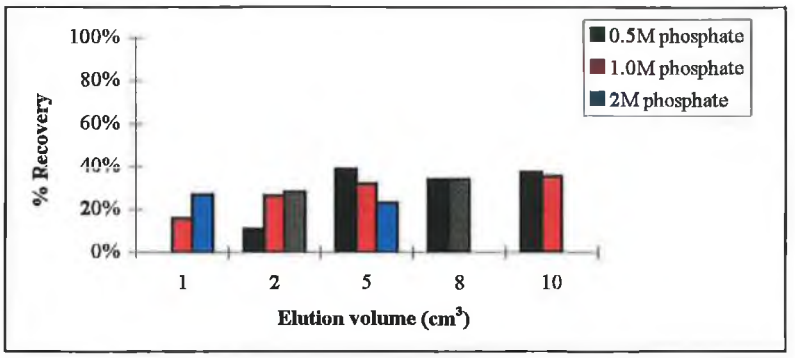


Fig.2.7; Comparison of percentage recoveries of arsenite achieved using $1cm^3$, $2cm^3$ and $5cm^3$ respectively of 0.5M, 1.0M and 0.2M phosphate buffer at pH 5.82. In all cases extractions were carried out using 0.04g of tissue, a spiking level of 75μg arsenite/g tissue and 0.4g of anion exchange packing material.

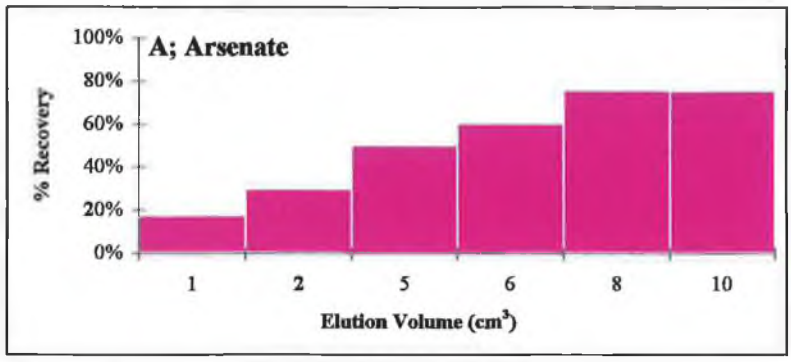
Although, in the case of arsenite, percentage recovery was consistently below 40%, these findings indicate that on increasing the concentration of eluting phosphate buffer, a lower volume of liquid was required to achieve maximal recovery. In the case of a 2M buffer solution, recovery peaks at an elution volume of $1cm^3$. Use of these more concentrated buffers is however hampered by the fact that at these levels phosphate tends to suppress the arsenic absorbance signal quite strongly. It was observed that below concentrations of 300ppb, signals due to arsenite were barely distinguishable from noise when present in phosphate buffer solutions exceeding 0.5M concentration. Furthermore, detection limits of arsenate, MMA and DMA were as high as 1ppm in this media.

For the purposes of these elution experiment volume experiments, this problem was overcome by the introduction of a dilution factor for the phosphate buffer. Eluted arsenic solutions were made up to the $10cm^3$ volume with distilled-deionised water instead of phosphate buffer, thus when elutions were carried out with $1cm^3$ of 2M

buffer, a 1 in 10 dilution of phosphate was effected leading to an overall buffer concentration of 0.2M.

Although maximum recovery of arsenite could be achieved with $1cm^3$ of 2M NaH_2PO_4, it was not feasible to use these conditions for further validation and work on on-line extractions. In addition to the difficulties with signal suppression, it was discovered that phosphate solutions were completely saturated before reaching a concentration of 2M and therefore the salt tended to recrystalise when solutions were left standing at room temperature leading to inconsistencies in the final concentration of the eluting buffer. Moreover, it was decided that for the purposes of on-line extractions / determinations, the use of highly concentrated buffers may prove to be ultimately damaging to the analytical column resulting in a decrease in its lifetime.

Additional experiments on the effects of elution volume on percentage recovery were carried out using a phosphate buffer at a concentration of 0.2M. In this case arsenate and MMA were the species under investigation. With this buffer concentration, employment of a dilution factor was not necessary and resultant solutions could be accurately made up to a final volume of $10cm^3$ with 0.2M phosphate buffer. The percentage recoveries for each of these species achieved following elution with $1cm^3$, $2cm^3$, $5cm^3$, 6 cm^3, 8 cm^3 and $10cm^3$ are illustrated in fig 2.8.



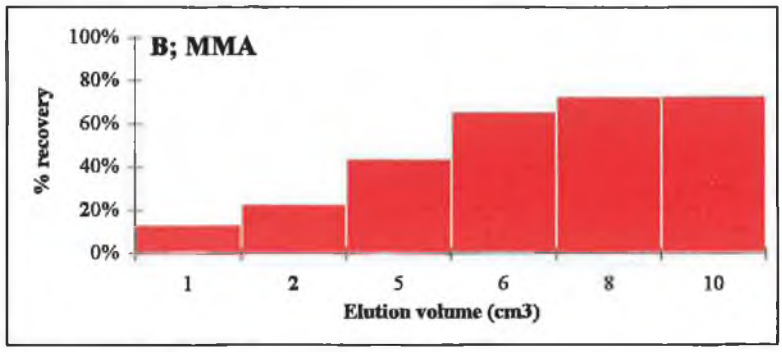


Fig.2.8; Variation in percentage recoveries of A; Arsenate and B; MMA achieved using 1cm^3, 2cm^3, 5cm^3, 6cm3, 8cm^3 and 10 cm^3 respectively of 0.2M phosphate buffer at pH 5.82. In all cases extractions were carried out using 0.04g of tissue, a spiking level of 75μg arsenic species/g tissue for each species investigated and 0.4g of anion exchange packing material.

It can be seen from fig 2.8 that the minimum volume of eluting buffer required for maximum recovery of arsenate and MMA was 8cm^3. This volume was too high to allow elution from the extraction column to be accurately included on-line with the analytical method and therefore extracted arsenic species must be eluted separately prior to analysis.

2.3.8. Optimisation of persulphate conversion of arsenobetaine into a hydride active derivative.

Initial digestions were carried out using 5ppm solutions of arsenate and arsenobetaine in 0.2M phosphate buffer heated to boiling in the presence of 1% NaOH and 1% $K_2S_2O_8$. Findings illustrated that arsenate remained unchanged by the digestion and

100% conversion of arsenobetaine was achieved. Analysis of the blank digests confirmed the absence of background interferences and comparison of the signal intensities achieved by digested and undigested arsenate standard solutions indicated that no appreciable losses occurred in the course of the digestion..

When digestions were carried out over the concentration range of 1 - 10 ppm Asbet the response was only linear in the range 1 - 5ppm, with similar signals arising from solutions of 5ppm, 7.5ppm and 10ppm Asbet indicating that a higher concentration of $K_2S_2O_8$ was probably required to successfully convert higher concentrations of arsenobetaine to arsenate. Increasing the level of persulfate to 3% improved the linearity of the response giving 100% conversion of arsenobetaine over the desired concentration range.

2.3.9. Validation of MSPD extraction.

Validation of the MSPD extraction of arsenite, arsenate, MMA, DMA and arsenobetaine was carried out using the optimised conditions listed in table 2.4.

Table 2.4; Optimum conditions for MSPD extraction of arsenic species from fish tissue

Weight of tissue used for extraction	0.04g
Type of packing	Anion exchange
Weight of packing	0.40g
Washing solvent	Hexane
Drying time	60 minutes
Elution buffer	0.2M phosphate buffer pH 5.82
Volume of elution buffer	8 cm^3

In the case of arsenite, arsenate MMA and DMA the intra assay (within day) variability of the optimised MSPD extraction was carried out using a spiking range of 25-125μg arsenic species/g tissue. In the case of arsenobetaine a higher spiking range of 125-

625μg/g tissue was employed, the reason for this increase in spiking level has been explained in section 2.2.12. The between day variation in recovery (inter-assay variability) which is related to the precision of the method was examined for arsenite, arsenate MMA and DMA using a spiking level of 75μg/g tissue for arsenobetaine this spiking level was again increased to 625μg/g tissue.

The intra and inter-assay variabilities obtained for arsenite, arsenate, MMA, DMA and arsenobetaine are listed in tables 2.5 - 2.14.

2.3.9.1. Intra-assay variability for arsenite

Table 2.5; Recoveries of arsenite from spiked samples of fish tissue

tissue concentration µg/g		% recovery	mean % recovery ± std.dev.	Relative std.dev.(RSD) (std dev./mean)
25	(a)	36.50%		
	(b)	34.30%	36.05 ±1.28%	0.0355
	(c)	37.36%		
50	(a)	35.40%		
	(b)	34.59%	34.70 ± 0.53%	0.0153
	(c)	34.11%		
75	(a)	45.54%		
	(b)	26.90%	35.93 ± 7.62%	0.2121
	(c)	35.36%		
100	(a)	38.29%		
	(b)	43.22%	40.27 ± 2.12%	0.0527
	(c)	39.31%		
125	(a)	25.62%		
	(b)	26.85%	28.01 ± 2.56%	0.0914
	(c)	31.37%		

Mean percentage recovery of arsenite over the concentration range examined :

34.89% ± 5.67%

Mean Coefficient of Variation (Intra-assay variability) for arsenite : 8.14 ± 6.99%

2.3.9.2. Inter-assay variability for arsenite

Table 2.6; Mean percentage recoveries of arsenite over five days

	Mean percentage recovery
Day 1	18.62%
Day 2	22.68%
Day 3	25.83%
Day 4	35.93%
Day 5	36.11%

Mean percentage recovery of arsenite over a five day period : 26.98 ± 6.72%

Relative standard deviation : 0.2490

Coefficient of variation (Inter-assay variability): 24.90%

2.3.9.3. Intra-assay variability for arsenate

Table 2.7; Recoveries of arsenate from spiked samples of fish tissue

tissue concentration µg/g	% recovery	mean % recovery ± std.dev.	Relative std.dev.(RSD) (std.dev./mean)
25 (a)	63.70%		
(b)	72.66%	75.53 ± 11.02%	0.1459
(c)	90.24%		
50 (a)	82.33%		
(b)	91.52%	82.68 ± 7.08%	0.0856
(c)	74.19%		
75 (a)	74.14%		
(b)	66.13%	70.14 ± 4.05%	0.0577
(c)	69.23%		
100 (a)	64.05%		
(b)	62.92%	62.18 ± 1.91%	0.0307
(c)	59.56%		
125 (a)	68.12%		
(b)	65.92%	67.03 ± 0.89%	0.0133
(c)	67.05%		

Mean percentage recovery of arsenate over the concentration range examined :

71.45% ± 9.68%

Mean Coefficient of Variation (Intra-assay variability) for arsenate: 6.66 ± 4.66%

2.3.9.4. Inter-assay variability for arsenate

Table 2.8; Mean percentage recoveries of arsenate over five days

	Mean percentage recovery
Day 1	78.28%
Day 2	72.71%
Day 3	79.55%
Day 4	70.84%
Day 5	70.14%

Mean percentage recovery of arsenate over a five day period : 74.30 ± 3.87%

Relative standard deviation (RSD): 0.0522

Coefficient of variation (Inter-assay variability): 5.22%

2.3.9.5. Intra-assay variability for MMA

Table 2.9; Recoveries of MMA from spiked samples of fish tissue

tissue concentration µg/g	% recovery	mean % recovery ± std. dev.	Relative std. dev. (RSD) (std dev./mean)
25 (a)	72.39%		
(b)	80.43%	80.43 ± 6.58%	0.0818
(c)	88.47%		
50 (a)	75.61%		
(b)	82.53%	81.69 ± 5.72%	0.0700
(c)	86.95%		
75 (a)	84.81%		
(b)	79.63%	84.00 ± 3.29%	0.0392
(c)	87.56%		
100 (a)	89.59%		
(b)	69.66%	81.02 ± 8.37%	0.1033
(c)	83.81%		
125 (a)	77.34%		
(b)	81.36%	79.00 ± 8.07%	0.0221
(c)	80.71%		

Mean percentage recovery of MMA over the concentration range examined :

81.39% ± 5.38%

Mean Coefficient of Variation (Intra-assay variability) for MMA : 6.32 ± 2.9%

2.3.9.6. Inter-assay variability for MMA

Table 2.10; Mean percentage recoveries of MMA over five days

	Mean percentage recovery
Day 1	73.90%
Day 2	78.51%
Day 3	84.45%
Day 4	79.78%
Day 5	84.00%

Mean percentage recovery of MMA over a five day period : 80.12 ± 3.87%

Relative Standard Deviation : 0.0483

Coefficient of Variation (Inter-assay variability): 4.83%

2.3.9.7. Intra-assay variability for DMA

Table 2.11; Recoveries of DMA from spiked samples of fish tissue

tissue concentration µg/g		% recovery	mean % recovery ± std. dev.	Relative std. dev. (RSD) (std dev./mean)
25	(a)	91.50%		
	(b)	105.00%	102.60 ± 9.63%	0.0938
	(c)	101.30%		
50	(a)	82.35%		
	(b)	92.27%	87.90 ± 4.13%	0.0469
	(c)	89.10%		
75	(a)	86.99%		
	(b)	73.56%	80.06 ± 5.49%	0.0685
	(c)	79.62%		
100	(a)	82.15%		
	(b)	83.58%	84.27 ± 2.11%	0.0251
	(c)	87.15%		
125	(a)	77.45%		
	(b)	82.41%	82.52 ± 4.19%	0.0518
	(c)	87.71%		

Mean percentage recovery of DMA over the concentration range examined :

86.81% ± 8.38%

Mean Coefficient of Variation (Intra-assay variability) for DMA : 5.72 ± 2.29%

2.3.9.8. Inter-assay variability for DMA

Table 2.12; Mean percentage recoveries of DMA over five days

	Mean percentage recovery
Day 1	79.59%
Day 2	66.54%
Day 3	86.84%
Day 4	80.06%
Day 5	88.15%

Mean percentage recovery of DMA over a five day period : 80.23 ± 7.67%

Relative Standard Deviation : 0.0956

Coefficient of Variation (Inter-assay variability): 9.56%

2.3.9.9. Intra-assay variability for arsenobetaine

Table 2.13; Recoveries of arsenobetaine from spiked samples of fish tissue

Tissue concentration (μg/g)	% recovery	mean % recovery ± standard dev	Relative std. dev. (RSD) (std dev./mean)
125 (a)	50.40%		
(b)	55.50%	52.95 ± 2.55%	0.4816
(c)	79.06%		
250 (a)	76.83%		
(b)	79.86%	76.86 ± 2.43%	0.0316
(c)	73.90%		
375 (a)	74.66%		
(b)	71.94%	77.47 ± 6.00%	0.0775
(c)	85.82%		
500 (a)	70.42%		
(b)	82.89%	80.34 ± 7.29%	0.0907
(c)	87.73%		
625 (a)	79.49%		
(b)	81.73%	80.94 ± 1.52 %	0.0188
(c)	83.05%		

Mean percentage recovery of arsenobetaine over the concentration range examined : 75.55% ± 10.44%

Mean Coefficient of Variation (Intra-assay variability) for arsenobetaine : 5.33 ± 2.71%

2.3.9.10. Inter-assay variability for arsenobetaine

Table 2.14; Percentage recoveries of Asbet over a 5 day period

	Mean % Recoveries ± std. dev.
Day 1	76.57 ± 1.48%
Day 2	82.14 ± 1.10%
Day 3	71.56 ± 0.98%
Day 4	69.26 ± 3.88%
Day 5	80.94 ± 1.53%

Mean percentage recovery over a five day period: **76.09 ± 5.05 %**

Relative Standard Deviation : **0.0664**

Coefficient of variation (Interassay variability) : 6.64%

It can be seen from table 2.5 that the percentage recoveries of arsenite following extraction by MSPD were low never exceeding 40% over the concentration range examined. This recovery was also very inconsistent with the within day coefficient of variation calculated to be 8.14%. The results in table 2.6 indicate that there was also a substantial between day variation, the percentage recovery on day 1 of these tests was 18.62% compared to a recovery of 36.11% which was achieved on day 5. The between day coefficient of variation for the MSPD extraction of arsenite from fish tissue was calculated to be 24.90%. This far exceeds the criteria for method validation

discussed in section 2.2.10 which states that both the within day and the between day variation should not exceed 10%.

The technique met with a higher degree of success when applied to the extraction of arsenate, MMA DMA and arsenobetaine. For arsenate, recoveries in the range of 62 - 82% were achieved in the concentration range 25 - 125µg/g tissue. These recoveries were still slightly inconsistent; the percentage recovery obtained from tissue spiked with 50µg arsenate/g tissue and 75µg arsenate /g tissue differed by 12% however the overall within day coefficient of variation was calculated to be 6.66 ± 4.66% which is within the limits of the method validation criteria. A greater degree of precision was attained for between day variability with a coefficient of variation of 5.22% being achieved.

The technique was particularly successful in extracting MMA and DMA from the fish tissue. For MMA, percentage recoveries in the range 79-84% were achieved in the concentration range examined with a within day coefficient of variation of 6.32 ± 2.9% being attained. The variation in results for the inter-variability assay were also very favourable giving a final between day coefficient of variation of 4.83%. For DMA, the results varied to a slightly greater extent ranging from 80-102%; however as can be seen the recoveries were consistently over 80%. Both values for within day and between day variabilities were below 10% thus meeting the criteria for the technique to be validated.

It can be seen from the results in tables 2.13 and 2.14, that with the exception of the 125µg/g spike, percentage recoveries of arsenobetaine from tissues were in the region of 76-81%. for the within day variability tests. The within day coefficient of variation was calculated to be 5.33 ± 2.71%. The percentage recoveries for extractions carried out every day over a 5 day period ranged from 69-82% with a between day coefficient of variation of 6.64%. Although further work is required to examine the extraction efficiencies at lower spiking levels the MSPD technique is valid for the extraction of arsenobetaine from fish tissue in the concentration range examined.

2.3.10. Comparison of MSPD approach with existing liquid liquid extraction techniques.

For practical applications this liquid - liquid extraction technique proved to be a highly time consuming and labour intensive process. Some of the principle drawbacks of this technique were associated with the use of phenol. It was found that when shaken with an aqueous solution, an emulsion type mixture was formed with the phenol, which could take up to an hour to separate into its component fractions. It was presumed that this was due to the highly acidic nature of phenol, the proton from the OH group was readily dissociating and partitioning into the aqueous phase, thus stabilising the mixture. This was remedied slightly by making the aqueous solution more acidic prior to extraction with phenol. The increased concentration of H^+ suppressed the ionisation of the phenol to some extent thus making the two solutions more miscible. The highly corrosive nature of phenol would also discourage its use in routine extractions of Arsenic from tissue.

On analysis, the signal due to arsenic was masked by a large background signal arising from residual organic interferants. An attempt was made to eliminate these by passing extracts through packed columns containing approx. 2.0g of C18 or anion exchange materials. "Untreated" solutions gave rise to large absorbance signals which subsequently tailed off slowly giving rise to very broad peaks. In the case of C18 solutions were merely passed through the packing and then analysed. In some cases the intensity of the initial signal was equal to that of corresponding "untreated" solutions but the degree of tailing was reduced indicating that the C18 material had removed some of the organic residue. In other instances the overall signal was lower, in all cases however the interfering compounds were still present in the final solution.

With anion exchange, the extracts were first passed through the column, it was assumed that the arsenicals would be retained by the anion exchange material whereas the organic solvents would simply pass straight through. The columns were then washed with 0.5M phosphate buffer, pH 5.82 to elute the arsenic. Both eluates were analysed. It was found that signals from the washing step were slightly lower than the

original indicating that the organic interferants had been retained by the column to some extent. Analysis of the second buffer elution showed them to be completely blank.

As an alternative to chromatographic clean up, an attempt was made to remove solvent residues by evaporating them under a stream of nitrogen. This proved a very effective and simple way of removing the interferants however analysis showed that there was no arsenic present in these solutions.

It was concluded that all of the arsenic had been lost at some point during the extraction, this was probably a consequence of the large number of steps involved. In addition to this, at the phenolic extraction stage the inorganic arsenic compounds have probably a greater tendency to remain in the aqueous layer. Therefore for the simultaneous extraction of inorganic and organometallic arsenicals the compounds would probably be separated into two final solutions this would further add to the time required for analysis.

The liquid - liquid extraction protocol used in these experiments was initially described by Edmonds and Francesconi for the isolation of arsenobetaine from the lobster mussel tissues[6]. In this instance, the extraction efficiency was low with only 12.1% of the original arsenobetaine concentration being recovered by the extraction.. A similar procedure was employed by Momplaisir et al. for the extraction of arsenobetaine, arsenocholine and tetramethylarsonium ions from seafood[17]. These workers however reported an extraction efficiency of 83% or greater for each of the analytes investigated. These values are comparable with the MSPD extraction efficiencies obtained in the course of this study.

This low recovery efficiency and inconsistency of results would discourage the use of this liquid-liquid extraction technique for routine extractions on a large scale. In addition to this the highly time consuming and labour intensive nature of the procedure

combined with the corrosive nature of phenol and the use of environmentally damaging organic solvents severely limits its application for use in environmental analysis.

2.3.11. Conclusions

The purpose of this study was to explore the feasibility of MSPD methodology for use as an alternative to classical liquid-liquid extraction techniques for the isolation of arsenicals from fish tissue.

In order that it be deemed a valid extraction technique, it is required that values for intra and inter-assay variabilities which are associated with precision and accuracy respectively, be below 10%. With the exception of arsenite, the values obtained met with these criteria. It is the organometallic arsenic species however which are predominant in fish tissues[1]. Trace levels of inorganic arsenic can be monitored by acid digestion procedures used to determine total arsenic concentrations, prior to the speciation study.

MSPD overcomes many of the inherent problems associated with liquid-liquid extractions, thus making it a more effective alternative for isolation purposes. Primarily it is less time consuming and labour intensive. Theoretically there is approx. $100m^2$ surface area per 0.2g packing; therefore the sample is dispersed over a large surface area. As a result as exhaustive extraction whereby a large volume of solvent is passed over a thin layer of sample is achieved using a relatively small volume of buffer. The technique also affords a more economical use of solvents, thus making it more environmentally friendly. Washing the columns with hexane removes any cellular components which are liable to interfere and this combined with the drying step gives rise to a final extraction which is interference free, which gives consistent and high recoveries which are comparable to those published for corresponding liquid-liquid extraction techniques.

For the purposes of this optimisation and validation, spiking levels were rather high with respect to tissue concentration of arsenic, this was necessary due to the detection limitations of the instrument used. In order that MSPD be used as an extraction technique in environmental applications, a pre-concentration step would be required prior to analysis. This would allow for standard additions closer to the actual levels of arsenicals in the tissue.

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Chapter Three.

The Application of Capillary Zone Electrophoresis to Arsenic Speciation.

3.1. Introduction

Capillary electrophoresis (CE) is a rapidly developing technique for the separation and analysis of both neutral and charged substances. This separation is based on the differential migration of analytes in a buffer filled microbore capillary, in the presence of an electric field. One of the principle attractions of CE lies in the fact that it is possible to achieve highly efficient separations analogous to those found in capillary gas chromatography. However, unlike GC methods that are limited to thermally labile compounds, the applicability of CE is extended to substances conventionally analysed by HPLC methods. The low cost of capillary tubing, small reagent consumption and minimal sample preparation requirements makes CE attractive as a complementary technique to HPLC for cost effectiveness and shorter analysis time. However due to the small dimensions of the capillary and low sample injection volumes the technique is hampered by low sensitivity for on line detection. To date, capillary electrophoresis has found successful application in the analysis of various pharmaceutical products and other large organic compounds however there is still a large number of applications for which the potential of this technique has yet to be explored. The majority of current research is aimed at modifying instrument design and developing pre-concentration techniques that will improve the detection limits for CE determinations. This will further broaden the scope of capillary electrophoresis to areas such as metal speciation where detection at trace levels is essential.

3.1.1. The origin of Electrophoretic techniques.

The rapid evolution of capillary electrophoretic techniques over the past ten years has been caused by the development of commercially available instrumentation. However, the foundations of CE were laid down almost a century ago with the formulation of Kohlrauschs' theories on the behaviour of ions in a solution under the influence of an electric field[1]. Kohlrausch initially described how the passage of an electric current through a solution was caused by the independent migration of ions towards the respective electrodes. The conductivity of the solution therefore is a function of the concentration of the ions and the velocity of their migration. This migration velocity

also known as the drift speed of the ion arises due to the combination of two opposing forces acting on each ion. Firstly, the force due to the electric field which is the product of the ionic charge and the magnitude of the field and secondly the frictional force that acts on the ion as it moves through the solution. This latter parameter is the product of the speed of the ion and the friction coefficient which from the Stokes relation [2] is a function of, the magnitude of the solvated ionic radius and the viscosity (η) of the solution. The mobility (μ) of the ion is defined as the ratio of this frictional drag to the electric field and can be calculated from the formula:

$$\mu = \frac{v}{E} = \frac{q}{6\pi\eta r}$$

Where v is the ionic velocity, q is the charge on the ion, r is the hydrated ionic radius and E is the magnitude of the electric field.

An additional factor that can strongly influence the electrophoretic velocity of an ion through a glass tube is electroendosmosis. This arises because the inner surfaces of glass or quartz tubes acquire a negative charge when filled with an aqueous salt solution and subjected to an electric potential. Positively charged ions from the solution are subsequently attracted towards the interface and form a series of electrical double layers as described by the Stern model[2]. The cations that are closest to the interface are very tightly bound to the wall and are unaffected by the electric potential. At a further distance from the wall there exists a layer which though still essentially cationic in character, is less rigid and more diffuse. Upon application of a voltage, the cations in this diffuse layer migrate towards the negative electrode. As these ions are hydrated, water and other buffer molecules are dragged along with them which creates a bulk flow of solution towards the negative electrode. This phenomenon is termed the electroosmotic flow (EOF). The potential across the double layers known as the zeta potential is one of the critical parameters in determining the magnitude of the EOF. The other factors being the dielectric constant of the solution and its' viscosity. The presence of an EOF makes it possible to determine cations, anions and neutral compounds in a single run.

3.1.2. The Development of Capillary Zone Electrophoresis as an analytical tool.

Kohlrausch also proposed theories on the behaviour of a boundary between two salt solutions with a common counter ion in the presence of an electric field[1]. He suggested that under the influence of an electric field, the concentration ratio of two ions A and B at the boundary reached a steady state which was related to the mobilities and charges of the ions by the function:

$$\frac{C_A}{C_B} = \frac{\mu_A}{\mu_B + \mu_x} \text{x} \frac{\mu_B + \mu_x}{\mu_B} \text{x} \frac{Z_A}{Z_B}$$

Where μ_A μ_B and μ_X denote the mobilities of the two ions and the common counter ion X, Z is the charge on each ion and C refers to the concentration of the ions. If the mobility of A exceeds that of B and the initial concentrations are equal, the shape of the zone occupied by B ions will adjust accordingly forming a concentration gradient within the zones until the equilibrium is reached. This is when the concentration of each ion at the boundary obeys the Kohlrausch equation. The same set of rules can be applied when a number of different ions are present in the solution.

This theory prompted a series of investigations into the separation of ionised species by exploiting the differences in their electrophoretic mobilities. In the 1930s Tiselius[3] succeeded in separating proteins into specific boundaries by performing "moving boundary" electrophoresis in a quartz U tube. These protein boundaries were detected by photography using UV light. A particular problem with the method was that a substantial amount of heat was generated in the quartz tube during the separation. Without effective heat dissipation, a radial temperature gradient was established within the tube which caused inhomogenities in the viscosity of the support electrolyte. The resultant parabolic flow caused considerable blurring of the boundaries. In a later publication, Tiselius[4] modified the experiment by introducing a rectangular electrophoresis cell with cooling at 4°C. With this apparatus four serum proteins were successfully separated into definite zones.

The problem of removing the excess heat generated during electrophoretic separations to improve resolution continued to be a dominant theme in the early development of the technique. In 1967, Hjerten[5] succeeded in eliminating the problem of broadening due to thermal convection. A series of aromatic carboxylic acids was electrophoretically separated in tubes with an internal diameter of 3mm. Hjerten recognised that although it was not possible to dissipate the heat generated during electrophoresis, broadening of the sample zones could be minimised the by rotating the tube about its longitudinal axis. This caused radial mixing within the tube thus cancelling the convective gradients. To achieve adequate resolution, it was necessary to coat the inner surface of the quartz with methylcellulose, this eliminated the EOF. Separations were carried out in 0.1M Tris/Acetic acid buffer at pH 6.5. The separated compounds were detected by scanning the rotating tube at 280 and 310 nm before and after electrophoresis and comparing transmissions.

A significant advancement towards the development of instrumental capillary electrophoresis was made by Mikkers et al[6]. These workers demonstrated that dispersion of sample zones could be effectively controlled though the use of microbore tubes made of chemically and electrically inert materials such as teflon. The use of capillary tubing suppressed convection in the separation medium since the micro dimensions caused the viscosity near the capillary wall to increase insofar as flow due to convection did not occur, this was termed the "anticonvective wall effect". It was also noted that sample overloading created disturbances in the local electric field gradient and resulted in distorted peak shapes. Ideally sample concentration should be two orders of magnitude lower than that of the carrier to achieve high efficiency.

Jorgenson and Lukacs[7] expanded further on the use of capillaries for efficient electrophoretic separations and designed a capillary electrophoresis system which would become the prototype for all further research and commercial instruments (fig. 3.1). Experiments were carried out in straight lengths of glass tubing 100cm long and with an internal diameter of 75μm filled with 0.05M Phosphate buffer pH 7. The ends of the capillary were dipped in beakers containing buffer solution and were connected via graphite electrodes to a regulated, high voltage dc power supply. The high

voltage end of the system was housed in a Plexiglass box. An interlock system which cut off the voltage in the event of an accident provided protection for the operator. Sample injection was accomplished by removing the injection end of the capillary from the buffer and placing it in a beaker of sample solution. A voltage was applied for a short time and samples were swept onto the column by electroosmosis. The buffer solution was then replaced at the sample end and the voltage applied once more to carry out the separation. Sample zones were detected on-line using a home made fluorescence detector.

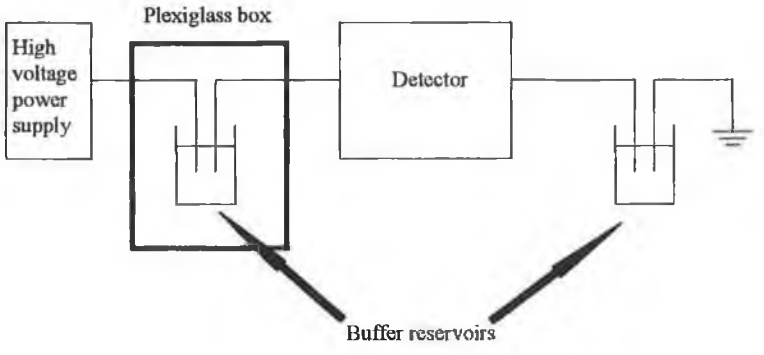


Fig. 3.1; Schematic diagram of the capillary electrophoresis system designed by Jorgenson and Luckas[9].

With this system it was possible to successfully separate a number of amino acids after derivatisation with fluoresamine within a short period of time. The efficiency of the separation was evaluated using a modification of the chromatographic plate theory. The idea that the theoretical plate concept should be extended to capillary electrophoresis was initially proposed by Giddings[8] who adapted the equation to electrophoresis by assuming that molecular diffusion was the sole cause of zone broadening, therefore the number of theoretical plates was a function of the mobility, voltage and diffusion coefficient.

This could be calculated from the formula :

$$N = 5.54 \text{ x} \left(\frac{t}{w_{\frac{1}{2}}} \right)^2$$

Where N is the number of theoretical plates, "t" is the migration time of the analyte of interest and $w\frac{1}{2}$ is the peak width at half height.

Using this formula Jorgenson and Lukacs[7] estimated the efficiency of their electrophoresis system to be in excess of 400,000 theoretical plates. This far exceeded the efficiency of any corresponding liquid chromatography system.

The research of Jorgenson and Lukacs was pioneering in the evolution of capillary electrophoresis as an analytical technique. Following the success of the system, Jorgenson evaluated the performance of capillaries made from Pyrex borosilicate glass, teflon and fused silica for the separation of Dansyl amino acids[9]. Findings indicated that while capillaries made of teflon possessed good transparency to UV light they exhibited poor thermal conductivity which rendered the material unsuitable for general use in capillary electrophoresis. The Pyrex and fused silica capillaries displayed a similar separation efficiency but fused silica was more suited to use in an electrophoresis system due to its superior UV transparency.

Salomen and co. workers[10] described the chemical processes which occur at the surface of fused silica during electrophoresis and discussed how alterations in the electrolyte pH, ionic strength and composition affected the properties of the material. The interaction of cations with the silica wall was assumed to follow the simple adsorption mechanism of;

$$M^+ + SiO^-(H_2O) \rightarrow SiO^- M^+ + H_2O$$

The equilibrium constant for the mechanism was as follows;

$$K_{wall} = \frac{\left[SiO^- M^+\right]}{\left[M^+\right]\left[SiO^-\right]}$$

Using this hypothesis, Salomon et al. derived an expression which related the charge per unit area at the interface (Q) to this equilibrium constant and the cation concentration as follows ;

$$Q = \frac{Q_0}{1 + K_{WALL}\left[M^+\right]}$$

Where Q_0 is the total number of ionised silanol groups at the surface. The magnitude of the EOF could be expressed as a function of this charge per unit area, the viscosity of the medium and the width of the double layer. If the double layer thickness was assumed to be constant it then followed that the electroosmotic mobility was also inversely proportional to the cation concentration. This was confirmed experimentally by demonstrating how the reciprocal of the electroosmotic mobility varied linearly with the cation concentration. The model was only valid however at low cation concentrations, indicting that the double layer thickness also varied with cation concentration. This concentration dependence was an illustration that the electrical double layer did not exist as a single layer of cations but in fact was comprised of two distinct regions, a compact immobile layer of fixed thickness d_0 and a more diffuse outer layer the thickness of which varied with concentration as depicted in Fig.3.2. This was in accordance with the Stern model[2] for the structure of an electrical double layer at an interface.

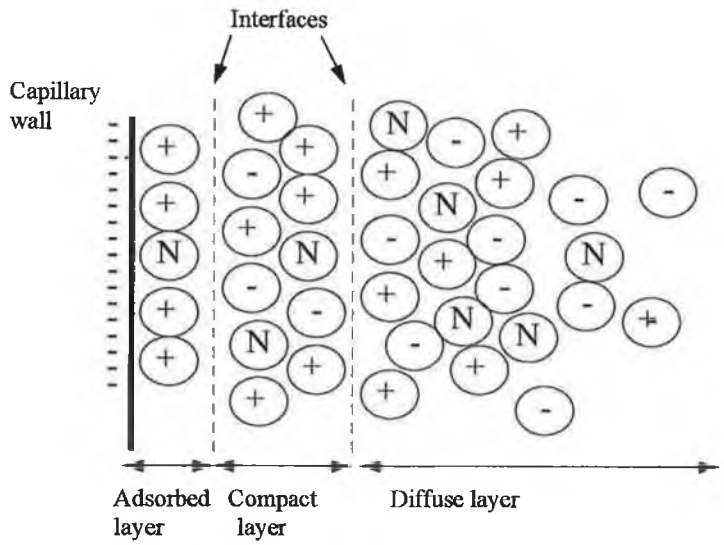


Fig. 3.2; Representation of the double layer at the capillary wall interface according to the model of Salomon et al.[10] The captions +, - and N represent positive, negative and neutral components of the electrolyte respectively.

This body of work was the first attempt to confirm the theory in the context of capillary electrophoresis by fitting experimental data to the model. The interface was more accurately described by assuming that it consisted of a number of layers of ordered hydrated cations interspersed among buffer anions and water molecules. This evaluation of the relationship that existed between the silica interface and the ions of the electrolyte solution led to an understanding of how the electroosmotic flow could be manipulated by the addition of organic solvents and modifiers to the run buffer and alterations in the pH. This would have very important implications in the development of capillary electrophoresis of small ions.

3.1.3. Sample introduction in capillary electrophoresis, the influence of injection volume on separation efficiency.

In the early stages of development, investigators demonstrated how highly efficient electrophoretic separations of analytes could be achieved if carried out in narrow bore capillary tubing. The micro-dimensions of the capillary however placed severe restraints on the maximum quantity of sample which could be analysed by these methods. Mikkers[6] illustrated how differences in the local electric field strengths in the

migrating zone with respect to the background electrolyte resulted in migrational dispersion within the analyte zone. This overloading resulted in asymmetric peak shapes and loss of resolution between closely migrating sample zones. The effect could be reduced by matching the mobilities of the sample constituents to the background electrolyte and eliminated completely by decreasing the concentration of the sample zones with respect to the background electrolyte by a factor of one hundred. In order to achieve this, the length of the initial sample plug had to be kept to a minimum.

It was therefore necessary to derive an expression whereby the optimum plug length which would maximise the quantity of injected sample while maintaining a high degree of efficiency could be calculated. The contribution of the injection plug length to bandbroadening was estimated as follows[11]:

$$\sigma^2_{inj} = \frac{l^2_{inj}}{12}$$

where σ^2_{inj} is the variance due to injection and l^2_{inj} is the injection plug length. An increase in the injection plug length will therefore result in a decrease in the overall separation efficiency. Using the above expression, Grushka and Mc Cormick[12] evaluated the maximum allowable injection plug length as a function of the migration time (t) and the diffusion coefficient (D) of the solute from the equation

$$l_{inj} = \sqrt{(24 D E_h t)}$$

Where E_h is the acceptable increase in the height equivalent of a theoretical plate value (HETP) relative to the minimum, calculated from the expression[13] :

$$H = \frac{2D}{v}$$

Where v is the solute velocity.

A much shorter plug length was thus required for high molecular weight solutes with large diffusion coefficients than for smaller molecules to observe the same decrease in efficiency. This model assumes that longitudinal diffusion within the capillary is the only source of band broadening. When factors such as hydrodynamic pressure, adsorption of molecules to the capillary wall and inefficient heat dissipation contribute significantly to the total peak variance, the model is not strictly valid.

Huang et al.[14] equated the number of theoretical plates with the square of the ratio between the total capillary length to the length of the injection plug:

$$N = 12\left(\frac{L_t}{l_{inj}}\right)^2$$

This is a more generalised approach in estimating the overall contribution of injection plug volume to bandbroadening as effects of diffusion of individual solutes are ignored. However the expression still illustrates the basic principle that as the quantity of sample introduced onto the column is increased, the efficiency of the separation exhibits a significant deterioration.

3.1.3.1. Modes of sample introduction in capillary electrophoresis - Hydrodynamic vs. Electrokinetic injection.

Injection of sample onto the capillary can be achieved in a number of ways but the two most popular options are electrokinetic and hydrodynamic injection schemes[15]. The former of these two schemes relies on the electromigration of the sample ion for introduction onto the capillary. To carry out an electroinjection, the buffer reservoir at the injection end is replaced by sample solution and an electric field is applied for a short period of time. The end of the capillary is then returned to the buffer to allow the separation to proceed. The quantity of material injected is a function of the electroosmotic and electrophoretic mobilities, the field strength, the duration of the applied voltage, the capillary radius and the concentration of the individual ions. To inject a sample hydrodynamically, a pressure differential is introduced across the

capillary. This can be accomplished by simply raising the capillary at the sample end relative to the detection end, sample will consequently enter the capillary by siphoning. In commercial instrumentation this mode of injection is accomplished by applying a pressure on the sample solution or by introducing a vacuum at the far end of the capillary. The volume of material injected depends on the capillary dimensions, the viscosity of the solution and the pressure drop across the column.

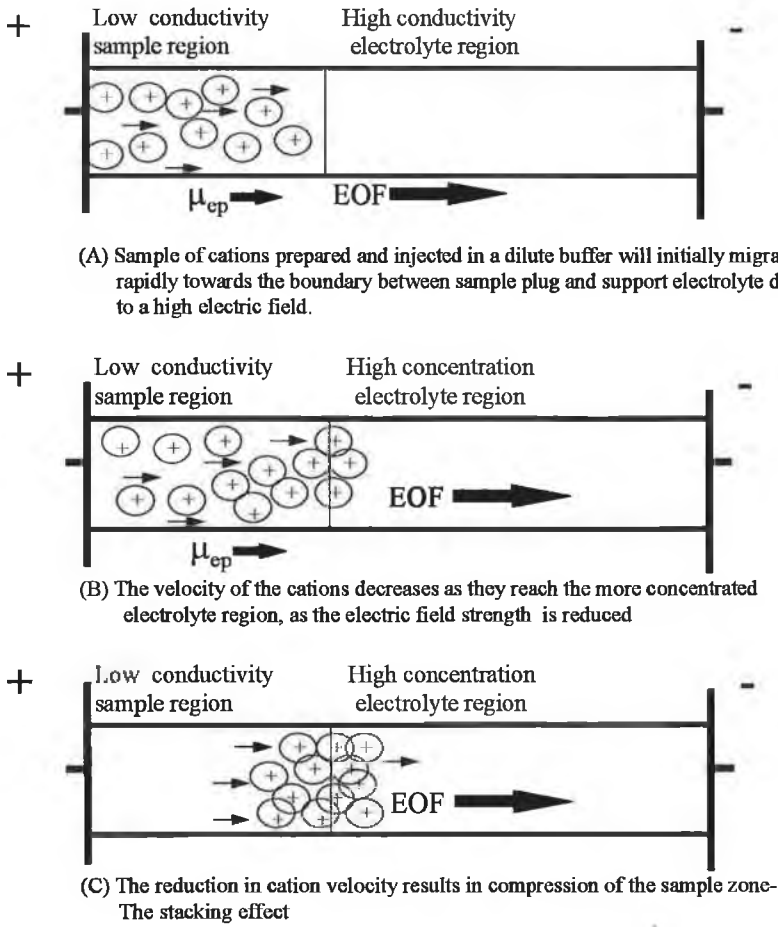
Each technique has its' own merits. With electrokinetic injection, the sample is introduced as a narrow zone which promotes high efficiency. Unfortunately, the dependence of the technique on the individual mobility on the ion, means that ions with high mobilities will be preferentially injected onto the column[16], furthermore, the quantity injected of the same species will vary in different electrolytes due to the dependence on the total ion velocity. Hydrodynamic injection techniques on the other hand, are non discriminatory however the hydrostatic flow generated within the sample plug can contribute to a broadening in the zone, leading to a slight loss in efficiency.

The restrictions due to low injection capacity and small-scale dimensions of the capillary ultimately contributed to lack of detection sensitivity which counter-balanced the advantages offered in terms of superior resolution and shorter analysis time. This rendered capillary electrophoresis unsuitable for many "real life" analytical applications. Methods which would improve the detection limits of capillary electrophoresis therefore needed to be addressed.

3.1.3.2. Improvements in detection limits in capillary electrophoresis; the development of stacking and other injection effects.

In reporting the adverse effects of large injection volumes on peak shape, Mikkers et al.[6] also observed that when the sample was prepared in water, a compression of the sample zone occurred which resulted in sharp symmetrical peaks. When the same injection time was employed for a sample in a buffer matrix, the resultant peaks were broad and exhibited the characteristics of column overloading. The water based sample plug had effectively been compressed into a narrow band the length of which

was conducive to a high separation efficiency. This phenomenon has become known as stacking and arises due to the fact that a sample in a low conductivity buffer will experience a higher electric field strength than in a high conductivity buffer. As a consequence of Ohms law[15], the electrophoretic velocity of an ion is proportional to the local electric field strength, consequently it will migrate more rapidly in the low conductivity region. Upon reaching the boundary between sample buffer and support electrolyte, the ion will slow down abruptly due to the decrease in electric field strength. As a result, a concentration of the sample zone will occur at the sample/buffer interface. This concept is described schematically in fig. 3.3.



(A) Sample of cations prepared and injected in a dilute buffer will initially migrate rapidly towards the boundary between sample plug and support electrolyte due to a high electric field.

(B) The velocity of the cations decreases as they reach the more concentrated electrolyte region, as the electric field strength is reduced

(C) The reduction in cation velocity results in compression of the sample zone- The stacking effect

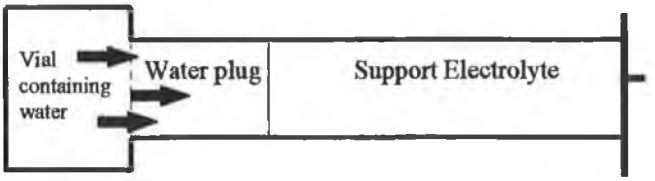
Fig. 3.3; Illustration of cations stacking at a concentration boundary[15].

Research in this specific area has been dominated by Chien and Burgi[17,18,19,20], who described how the Field Enhancement Factor (r.) [17] was equal to the ratio of the resistivities of the injection and buffer regions. When the regions had the same buffer composition this field enhancement factor was also inversely proportional to the ratio between their respective concentrations. If highly dilute buffers or water was used as the sample matrix, the effect of impurities and sample constituents on the total electric field also had to be taken into account. From the theoretical considerations it would seem that the greater the concentration difference between sample and buffer zones, the greater the stacking effect and hence using water as the sample matrix would give the greatest enhancement, however, Chien and Burgi observed that this was not the case[18]. This concentration difference between the zones, gave rise to an electroosmotic pressure at the interface which generated a laminar flow resulting in a loss in separation efficiency. For optimum stacking therefore, the difference in the conductivities of the sample and buffer regions were kept at moderate levels thus reducing the enhancement factor to some extent.

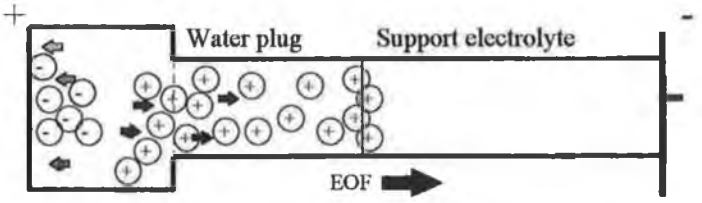
It was subsequently discovered that the stacking efficiency increased substantially if a small plug of water was introduced hydrodynamically onto the column prior to the electrokinectic injection of the sample[18]. This form of stacking was termed "Field Amplified Injection" In the absence of the water plug, physical disturbance of the sample/buffer interface will occur as a consequence of switching directly between high and low concentration buffers. This will cause the field enhancement effect to be diminished. By injecting a water plug prior to the sample, a high electric field strength will be experienced from the beginning of the injection.

With this technique a hundredfold enhancement of signal was observed without loss of resolution. In addition to this, the bias normally observed with electroinjection techniques[16] was not as pronounced when field amplified injection was employed. An explanation for this was that the water zone provided a "void" region where ions of lower mobility could be injected without the diminishing influence from those of higher mobility. Sharper peaks were also observed with field amplified injection.

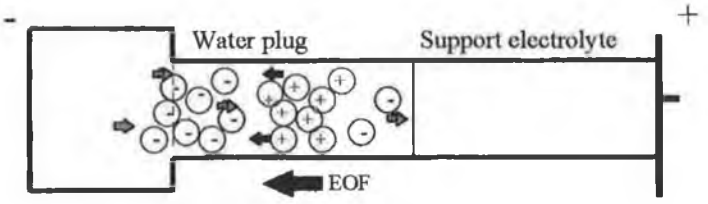
One drawback of this mode of electroinjection was that positive and negative ions had to be determined separately, to overcome this problem Chien and Burgi introduced a field amplified polarity switching protocol[19] (fig. 3.4). This involved injecting the water plug hydrodynamically as before and then applying a positive voltage for a period of time for the introduction of the positively charged species. The polarity of the electrodes was then reversed and a voltage was applied. This caused the EOF to travel in the opposite direction. However, due to the amplified electric field the mobilities of the negatively charged ions were strong enough to overcome this effect and travel onto the column, stacking up at the front of the water plug, while the positively charged ions concentrated at the rear. The reversed EOF resulted in the loss of some of the positively charge ions and therefore the negative voltage had to be applied for a shorter period of time. When both sets of ions had been successfully injected, the electrodes were switched back to their normal configuration and the separation continued. By carefully optimising the duration of each injection equal quantities of positive and negative ions could be introduced onto the column and stacked into narrow zones.



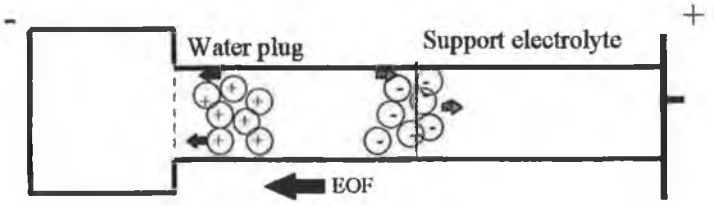
(A) Water plug is injected hydrodynamically onto the column



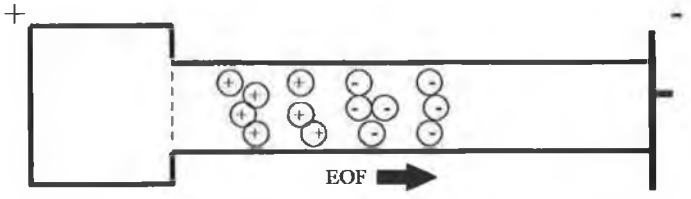
(B) Sample injection phase 1; A positive voltage is applied causing positive ions to migrate onto the column and stack at the concentration boundary while negative ions migrate in the opposite direction



(C) Sample injection phase 2; A positive voltage is applied causing negative ions to migrate onto the column while the positive ions migrate back out towards the injection end



(D) Sample injection phase 3 ; Positive and negative ions stack at opposite ends of the water plug



(E) Polarity is switched back to the original configuration and separation proceeds

Fig.3.4; A schematic diagram of the polarity switching field amplified injection procedure[19].

The advent of stacking allowed larger quantities of sample to be introduced onto the column than conventional injection techniques and thus detection limits could be improved substantially. However the technique still exhibited certain limitations with regard to the maximum volume of sample which could be injected. As mentioned previously, the mismatch in local electroosmotic velocities in the regions due to their conductivity differences caused a pressure difference which led to a laminar flow profile being generated in the capillary. This had a detrimental effect on separation efficiency which increased as the sample load increased. Another restriction to the amount of sample which could be injected was that in a discontinuous buffer system as is employed with stacking, the total electric field strength is distributed unevenly across the capillary, the majority of it being dropped across the region of low conductivity. As the size of this region is increased, the electric field in the region of higher conductivity will be diminished to a level insufficient to drive an electrophoretic separation. Chien and Burgi recognised that in order to inject very large volumes of sample, it was necessary to completely remove the injection matrix before separation[20]. Following on from previous work with polarity switching sample injections[19] a system was devised whereby large volumes of negatively charged ions could be stacked into narrow zones and the sample buffer removed by applying a negative voltage across the capillary. This causes reversal of the EOF which causes the large water plug to be pumped back out the injection end of the capillary. The mobility of the negative ions in the enhanced field are strong enough to overcome the frictional drag of the EOF and they will migrate in the opposite direction, stacking at the sample/buffer boundary. Sample plug removal was monitored by observing the increase in current in the capillary. When this reached a level within 1% of the value obtained when the entire column was filled with support buffer, the polarity of the electrodes was switched back to the normal configuration and the separation was allowed to continue.

This procedure meant that almost the entire capillary could be hydrodynamically filled with sample which was then focused into a narrow band. A maximum fill length existed which was equal to the ratio of the electrophoretic and electroosmotic mobilities, above this value no further improvement in peak shape was observed as when the column was completely filled with water, the field enhancement effect was cancelled. However, from a practical viewpoint, it was more straightforward to

employ whole column injections and stack as much of the sample plug as possible. This technique was only applicable to ions with mobilities opposite in direction to the EOF. For cationic analytes, it was necessary to reverse the charge on the capillary surface through the use of buffer additives, thus switching the direction of the EOF

3.1.4. Capillary ion electrophoresis

The improvements in detection limits brought about by these stacking procedures established capillary electrophoresis as a viable analytical technique particularly in the area of biochemical analysis. Investigators subsequently began to focus on the development of CE methodology for all analytical applications. An area of particular interest was the determination of low molecular mass inorganic and organic ions conventionally analysed by ion exchange chromatography. Ion chromatography is a long established reliable technique which still dominates this area of analysis. However, the high separation efficiency, short analysis times, simplicity of operation and diminished matrix dependence offered by capillary electrophoresis allowed the technique to evolve as an attractive alternative. A further advantage of CE was that ion exchange stationary phases are often expensive and are only applicable to a specific group of ions. Separations by capillary electrophoresis on the other hand do not rely on the interaction of the analyte with a particular stationary phase and therefore a much broader range of substances can be determined simultaneously.

The interest in capillary electrophoresis for the analysis of small ions, led to the emergence of a new electrophoretic technique known as capillary ion electrophoresis. The fundamental drawback of capillary ion electrophoresis however was that the detection problems associated with low loading capacity and narrow optical path lengths were further compounded by the fact that these small ions lacked a suitable chromophore and therefore did not absorb appreciably in the UV region. Even with high volume stacking, detection limits still far exceeded a useful analytical range. The development of a universal, highly sensitive detection scheme was therefore the dominant feature of research into this technique.

A number of workers, introduced a bulk property conductivity detector to the end to the capillary[6,21,22]. This form of detection however was hampered by the high noise levels generated as a consequence of electrochemical reactions at the detector cell electrodes and the fact that the high electric field strength which drove the separation, interfered with the detection process.

The requirement for a universal on-line detector was fulfilled with the introduction of indirect detection methods. Adapted from ion chromatography[23], indirect detection involved the inclusion of a high UV absorbing substance (probe) in the support electrolyte. The migrating ion will cause a displacement of this probe and this will appear as a decrease in background absorbance at the detector as illustrated in figure 3.5. The displaced probe ions appear as a system peak at a migration time equal to that obtained if the probe had simply been injected into a transparent buffer under similar conditions. For optimum results the detection wavelength should be in a region of high absorption for the probe but where the extinction coefficient of the sample is zero

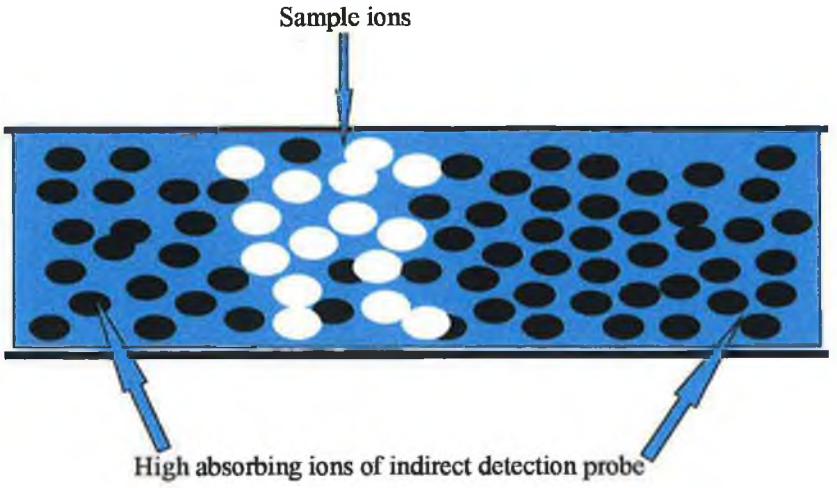


Fig.3.5; Scheme for indirect detection, the mobile phase additive provides a large background signal. In the analyte zone, displacement occurs which is seen as a decrease in absorbance intensity[26].

The concept of indirect UV detection for capillary electrophoresis was introduced by Hjerten et al.[24] for the analysis of inorganic anions. Foret and co. workers[25] however provided the earliest detailed study of the technique examining the effects of the relative mobilities of sample and probe on the displacement process and sensitivity of

the method. As with conventional capillary zone electrophoresis, dispersion of the sample zone due to electromigration, was minimised when the mobilities of the sample and co.-ion were closely matched. In addition to this, the sensitivity of the scheme was determined by the linear dynamic range and the noise levels of the detector. Optimum sensitivity was achieved when a probe of high absorptivity was used at a low concentration. In this particular case a 0.7mM sorbic acid -histidine electrolyte was used to successfully separate a number of carboxylic acids with a detection limit of 0.5pmol.

The underlying principles governing sensitivity of indirect detection of capillary electrophoresis have been outlined by Yeung and Kuhr[26]. The concentration limit of detection for indirect detection was governed by three considerations; firstly the background signal to noise ratio which was a measure of the ability of the detector to measure a small change in signal over a high background, this was known as the dynamic reserve (DR); second was the concentration of the probe, defined as C_m and the third factor was the efficiency of the displacement process which was a measure of the number of probe molecules displaced by a single analyte ion. This was termed the transfer ratio (TR.). An expression for the concentration limit of detection was drawn up as follows:

$$C_{lod} = \frac{C_m}{TR \text{x} DR}$$

These parameters were all inter-connected. With higher concentrations of electrolyte, the increase in background absorbance had an adverse effect on the dynamic reserve of the system. However, more dilute background electrolytes caused a decrease in the transfer ratio. and also had implications for the efficiency of the separation. Therefore for the purposes of method development, a compromise between optimum efficiency and optimum peak area often had to be reached.

Ackermans and co-workers[27] modified the Kohlrausch regulating function[1] to derive a mathematical expression for the transfer ratio in terms of the mobilities of the probe ion (A), its' non UV absorbing counterion (x) and the sample ion (A).

From the Kohlraush theory it can be assumed that when the sample electrolyte boundary reaches a specific zone in the capillary occupied originally by pure electrolyte the respective concentration of the ions A and B adjust so that:

$$C^{C}{}_{A} = C^{S}{}_{A} + C^{S}{}_{B}k_{B}$$

$$\text{where} \quad k_B = \frac{z_B\left[\mu_A\left(\mu_B + \mu_x\right)\right]}{z_A\left[\mu_B\left(\mu_A + \mu_x\right)\right]}$$

Where the superscripts C and S denote the pure carrier and sample zones respectively and k_B is a constant known as the transfer ratio. In the expression for k_B which gives a value for this transfer ratio, μ represents the mobilities of the ions and z refers to their charges. This equation relating transfer ratio to mobility has been verified by Nielen et al.[28] for various alkyl sulphates.

A comprehensive review on the theory of indirect detection in capillary electrophoresis was provided by Buchberger et al[29], who also demonstrated how the change in absorbance which occurred as the sample zone passed the detector could be equated with the product of the sample ion concentration in the detector zone, the molar extinction coefficient of the probe and the transfer ratio, assuming that the analyte absorbance at the detection wavelength is negligible. In addition to this, these workers developed a protocol to determine the transfer ratios experimentally. This procedure involved injecting a series of solutions containing different concentrations of the probe ion into a UV transparent electrolyte and obtaining a calibration plot of peak area versus molar concentration. Secondly, a series of anions were analysed over a concentration range using the probe as the carrier electrolytes and standard curves were obtained as before. The experimental transfer ratio for each ion was then defined as the quotient of the slopes of the sample and probe calibration plots. The suitability of a specific probe was evaluated in terms of the product of its transfer ratio and molar extinction coefficient at the analytical wavelength. With the appropriate selection of probe ion, sensitive determinations of a broad range of cations or anions could

therefore be achieved using capillary electrophoresis with relatively short analysis times.

3.1.4.1. Determination of anions by capillary electrophoresis

The capillary ion electrophoresis technique has been most extensively researched for anionic determinations. There is an additional problem other than detection sensitivity associated with this task. Unlike larger relatively immobile biomolecules which are pulled towards the detector by the EOF even though their inherent migration is in the opposite direction, the mobilities of these low molecular mass anions were strong enough to exceed the EOF, allowing them to migrate back out the injection end of the capillary.

A simple way to avoid loss of anions is to employ support electrolytes at very low pH[30] thus reducing the ionisation of the surface silanol groups affecting the charge density. Alternatively, a very high pH with a low ionic strength buffer will generate an EOF sufficiently high to pull the anion in the opposite direction[31]. These methods however involve very long analysis times and the peaks due to the anions tend to be broad and asymmetrical.

The most effective way to achieve rapid separation of anions, is to include an electroosmotic flow modifier in the support electrolyte. These flow modifiers are long chain alkyl ammonium salts which interact with the capillary surface, cancelling the EOF or in many cases reversing its direction. When the electrode polarity is switched from normal configuration, the altered electroosmotic flow will allow anions to migrate toward the detection end of the capillary. This phenomenon of EOF reversal was first demonstrated by Reijenga et al.[32] using the cationic surfactant, cetyltrimethylammonium bromide (CTAB). Tsuda[33] subsequently found that EOF reversal was complete and stable at a CTAB concentration of 0.5mM and exploited this EOF reversal for the rapid separation of anions. A number of experiments were subsequently carried out to determine the factors which governed this flow reversal. Altria and Simpson[34] investigated the effects of a number of alkyltrimethylammonium

salts on the EOF. Their findings indicated that the alkylchain longer than six carbons was required to completely reverse the flow and the longer the chain length the lower the concentration at which this flow inversion occurred.

The precise mechanism by which this reversal occurs was discussed by Lucy and Underhill[35]. The cationic surfactant molecules are attracted electrostatically to the capillary wall and form a bilayer type structure known as a hemimicelle. There is therefore an excess of cations in the stern layer at the silica interface which causes the resultant zeta potential to be positive. The surplus counter ions in the bulk solution are subsequently attracted towards this pseudo surface causing the EOF to travel in the opposite direction. Two independent pathways for surfactant adsorption onto the capillary wall were proposed. The first model described how individual molecules adsorbed onto the silica surface due to electrostatic attraction. As this adsorbed layer increased in concentration the spacing between the molecules was diminished to the extent that the hydrophobic aliphatic chains of adjacent molecules began to interact with each other. This prompted an increase in adsorption until all adsorption sites were associated with cationic molecule. As the concentration of surfactant in solution increased the Van der Waals attractive forces between the alkyl chains will overcome the electrostatic repulsive forces between the charged head groups and secondary adsorption will occur with surfactant molecules oriented in the opposite direction. The local surfactant concentration at the wall will eventually reach the critical micellar concentration which will complete the hemimicelle concentration and no further surfactant adsorption will occur. As the ionic strength of the electrolyte increases, the electrostatic repulsion between the cationic head groups is lessened and the transition between monolayer coverage and final hemimicelle formation coalesces into a single step. This is the second proposed pathway for surfactant adsorption and describes how the bilayer results from the adsorption of surfactant pairs know as admicelles. This hypothesis is illustrated diagramatically in fig. 3.6.

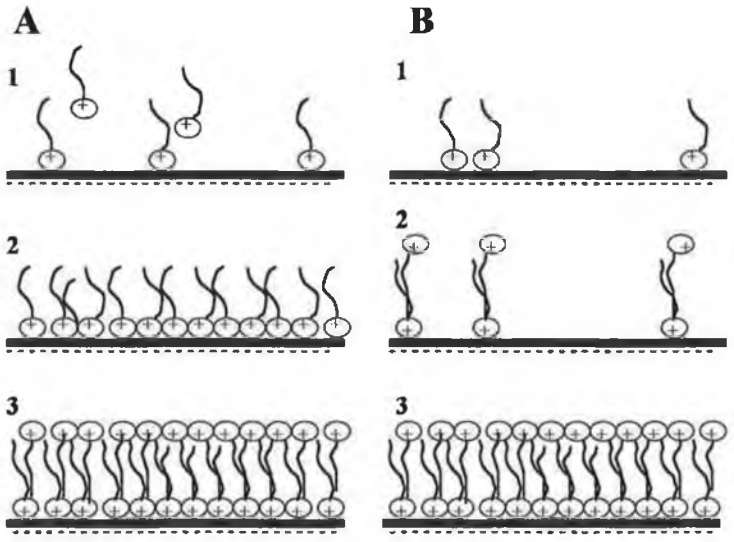


Fig. 3.6; Schematic representation of the adsorption of cationic surfactant showing the orientation of the surfactant molecules on the charged silica surface via monomeric surfactant adsorption (A) and admicelle adsorption (B)[35].

The experiments of Lucy and Underhill[35] supported these adsorption models. At a pH of 3.5, zero EOF was observed over the CTAB concentration range of 0.05mM to 0.1mM. CTAB concentrations above 0.1mM caused the EOF to reverse and the magnitude of this EOF became constant at concentrations above 0.2mM CTAB. The zero flow initially observed is consistent with a monolayer formation and the plateau after 0.2mM suggests that the bilayer formation is complete. These observations indicate that the bilayer formation followed the first adsorption pathway. At high pH where the surface charge density is high, the admicelle pathway was preferred and the EOF switches from normal to reversed in a single transition. Excluding the adsorption mechanism the electrolyte pH exhibited no other effect on the magnitude of the EOF. Adsorption of anionic counterions at the double layer did influence the EOF however. It was noted that as the adsorption affinity of various anions increased, the strength of the EOF decreased. It was also discovered that by increasing the ionic strength of the run buffer, surfactant adsorption was enhanced and this caused an increase in the magnitude of the EOF, However at a certain concentration the double layer began to shrink and this caused a decrease in EOF.

The use of cationic surfactants is sometimes marred by their limited solubility and tendency to form insoluble ion pairs with electrolyte components[36]. Waters have patented an alternative flow modifying compound sold under the trade name of NICE-Pak OFM Anion-BT. This compound has been successfully employed by a number of researchers for anionic determinations[37,38,39]. Harrold et al.[40] proposed that the alkyl diquaternary ammonium salt, 1,6-bis-(trimethylammonium)hexane, as an alternative to CTAB for EOF reversal. This compound was highly soluble in water and showed little interaction with other electrolyte components. For applications which do not require reversal of electroosmotic flow, alkyl amines such as diaminopropane[41] and diethlyenetriamine (DETA)[42,43] have been employed. These buffer additives interact with the silanol groups on the capillary surface but simply mask the negative charge and thus suppress the electroosmotic flow rather than reversing its direction..

The use of electrolytes composed of a flow modifier and an indirect detection probe has proved to be the most successful means of analysing low molecular mass anions. There are a wide variety of probes covering a broad range of mobilities available and their suitability for use with specific groups of anions has been extensively studied. Buchberger et al.[29] evaluated chromate and a number of aromatic carboxylic acids ; benzoate, PA, trimellitate and pyromellitiate, for use as carrier electrolytes and indirect detection agents in the separation of a series of inorganic anions. Chromate exhibited the best suitability for the separation of high mobility ions such as chloride and bromide, with pyromellitate and trimellitate more suited to ions of slightly lower mobilities such as sulphate and phosphate, finally low mobility chromophores such as p-Hydroxybenzoate accommodated the larger sulfonic acid type ions. Dabek-Zlotorzynska and Dlouhy[44] recommended that when a large array of anions with diverse mobilities needed to be analysed it was better to employ two separate buffer systems and separate runs thereby avoiding significant peak tailing for anions of lower mobility and inadequate resolution and fronting for analytes which migrated faster than the probe .

Romano and Krol[38] compared a capillary ion electrophoresis technique with ion chromatography for the determination of chloride, bromide, fluoride, nitrate,

phosphate and sulphate in drinking water, groundwater and wastewater samples. The capillary electrophoretic separations were carried out using a chromate based run buffer containing 0.3mM OFM anion BT flow modifier. There was an excellent correlation between the data obtained from this method and the more established ion chromatography method. However determination of high mobility ions such as fluoride is not possible by anion exchange chromatography as it is normally eluted in the void volume. Furthermore the presence of high levels of carbonate in the groundwater sample has an adverse effect on the resolution of compounds eluting early from the chromatography column. These problems do not arise with capillary electrophoresis where resolution between anions is a function of their electrophoretic mobility and is not as susceptible to adsorption effects. Oehrle[45] applied a similar chromate/OFM electrolyte system to monitor the anion content of sulfonated dyes. Once more, the results obtained from the CE and IC methods were in excellent agreement, however with the capillary electrophoresis method the analysis could be carried out in one third of the time. An additional advantage was that capillary electrophoresis did not exhibit any loss in efficiency with time whereas adsorption of the dye onto the ion exchange column had a deleterious effect on its performance. Making the ion chromatography methods a highly expensive process.

In order to apply this indirect detection/flow modified technique to the analysis of anions at ultra trace levels, a stacking procedure needs to be incorporated into the method. Jackson and Haddad[46] achieved a ten-fold improvement in limit of detection by replacing a standard hydrodynamic injection with field amplified injection. It was emphasised that the differences in the ionic strength of the samples and standards could give rise to inaccurate quantitation due to stacking. The inclusion of an internal standard proved to be the most versatile method of compensating for these ionic strength effects. With this approach a wide variety of environmental samples including sewage effluents, river waters and waste waters could be analysed for various common anions.

Burgi utilised the capacity of diethylenetriamine (DETA) to suppress electroosmotic flow to develop a large volume stacking method for anions which did not require a polarity switching step (fig. 3.7)[47]. With this new alternative procedure, electrodes

remained at reverse polarity and DETA was included in the support electrolyte to eliminate the electroosmotic flow. When a large aqueous sample plug in introduced into the capillary, the water soluble DETA dissolves into the matrix causing an increase in the local zeta potential at the wall. This in turn results in the generation of an electroosmotic flow upon application of a voltage. Due to the configuration of the electrolytes, this flow will be towards the injection end of the capillary, thus the water plug is pumped out while at the same time fresh DETA is pulled into the column. Once the water has been completely removed, the original condition of zero electroosmotic flow will be re-established and the separation can proceed.

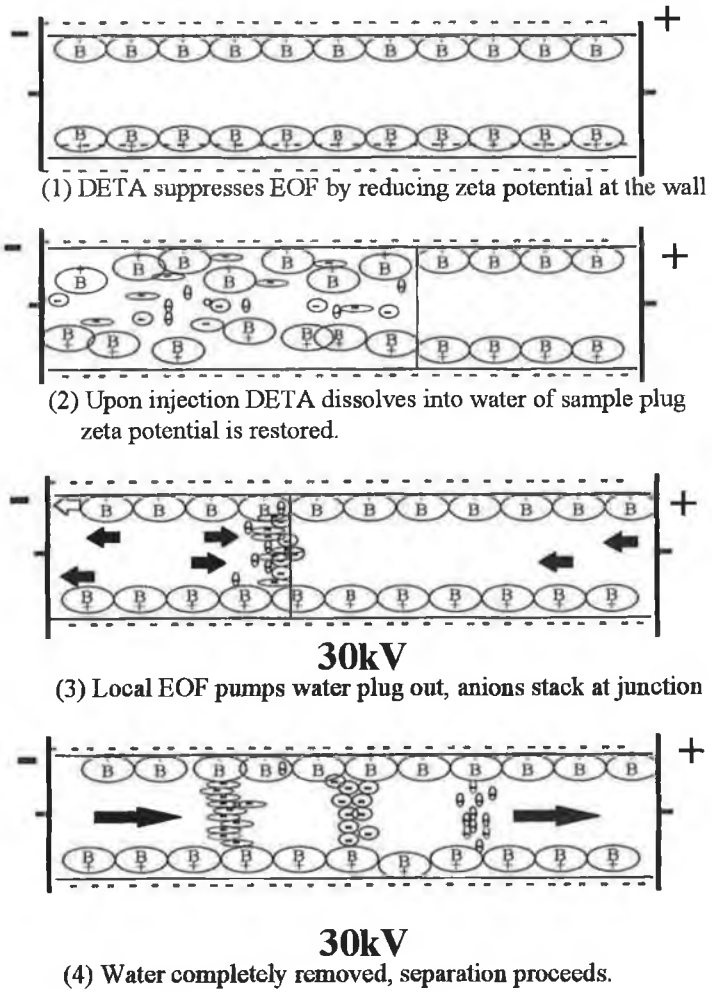


Fig. 3.7; Flow modified large volume stacking of anions[47].

This stacking experiment was carried out for the separation of chloride, nitrate and oxalate anions employing a run buffer of 3mM Pyromellitic acid, 3mM Sodium

Hydroxide and 1mM DETA. The removal of water from the capillary was monitored from the current trace, which rose gradually reaching a steady level when all the water had been pumped out of the column. A hundred-fold enhancement of the signal was achieved when whole capillary injections were employed allowing concentrations as low as 10ppb to be determined. These detection limits were comparable with anything obtained by chromatographic pre-concentration techniques, however a further advantage of this stacking technique was that it was completed in less than sixty seconds while the column concentration technique for ion chromatography was a much lengthier process.

The high efficiency afforded by capillary electrophoresis combined with the sensitivity provided by indirect detection with whole column stacking and the rapid analysis times due to flow modification had firmly established the technique as a complementary if not superior method to ion chromatography for the determination of anions in environmental matrices. The simplicity of the instrumentation and low operating costs may lead to capillary electrophoresis replacing chromatography as the dominant analytical technique in this specific area.

3.1.4.2. Determination of metals and other low molecular mass cations by capillary electrophoresis.

Indirect detection has also been adapted to the capillary electrophoretic separation of metal ions and other low molecular weight cations though not to the same extent as their anionic counterparts. Heterocyclic or aromatic amine molecules are generally employed as indirect detection agents and the same rules regarding transfer ratios and dynamic reserve apply. As the mobility of these cations is in the same direction as the electroosmotic flow under normal conditions, the requirement for flow modifying additives is eliminated. In most cases however metal ions of the same charge possess equivalent ionic conductivities and consequently the difference in their mobilities is too small to allow successful separation. Foret et al.[48] remedied this problem by introducing a weak complexing agent 2-hydroxybutyric acid (HIBA) into the support electrolyte for the separation of a series of lanthanide cations. The mobilities of the

resultant complexes were sufficiently different to allow complete resolution. In this instance creatine was selected as the chromophoric electrolyte. The pH of this electrolyte and the concentration of the HIBA complexing agent were the fundamental parameters governing complex formation and efficiency of the separations. A creatine concentration of 3mM containing 4mM HIBA adjusted to pH 4.0 with acetic acid was chosen as optimum buffer composition for this separation.

Weston and co-workers described how the observed mobility of these metal:ligand complexes was a composite of the individual mobilities of the various metal ligands intermediate complexes, the mobility of the free metal and the electroosmotic flow[49]. These investigators succeeded in separating a series of alkali, alkaline earth and transition metal complexes with a run buffer consisting of 6.5mM .HIBA and a Waters patented indirect detection agent for cations; Waters UVCat-1 at a concentration of 5mM with an overall pH of 4.4. Vogt and Conradi[50] studied in detail how the pH and ligand concentration effected the degree of complexation for α-HIBA and lactate complexes of lanthanide metals. It was found that increasing the pH of the electrolyte or ligand concentration increased the number of ligands complexed to the metal which led to an overall decrease in the electroosmotic mobility of the chelate. Optimum separations were achieved at a pH and ligand concentration where only partial complexation of the lanthanide metals occurred. The higher stability of α-HIBA complexes over lactate meant that a lower concentration of the former was needed for complete separation. Resolution could be further improved if a combination of ligands such as α-HIBA and acetate were used.

The suitability of a number of aromatic amines for use as indirect detection probes was investigated by Chen and Cassidy[51]. A wide array of metal ions ranging from the alkalis to the lanthanide series were separated as their HIBA complexes. Benzylamine and N-methlybenzlyamine based electrolytes were only successful in separating the lanthanide complexes. The incorporation of N-dimethylbenzlyamine into the run buffer with the HIBA proved to be the most successful buffer composition in terms of high efficiency and acceptable signal to noise ratio, for the separation of the metal ions. With a final buffer composition of 6mM DBA and 4.3mM HIBA adjusted to pH 5.0 with acetic acid, the separation of twenty six metal ions was possible within eleven

minutes. Detection limits ranged from 100ppb for the group I and II metal ions to 1ppm for the lanthanide series. Benzylamine and its' derivatives often suffer from poor solubility at lower pH, the heterocyclic imidazole compounds have been shown to be effective alternative chromophores for the separation of alkali and alkali earth metals[52,53,54]. These compounds have the advantage of being highly water soluble and possess mobilities which closely match those of those of the HIBA complexes of transition metals and alkaline earth metals.

One of the drawbacks of this approach is that the capacity of metals to complex with ligands and the rate at which this reaction occurs can vary considerably. Recent investigations have revealed that with the appropriate selection of UV active electrolyte, it is possible to separate certain metal ions without complexation[55]. A buffer consisting of 15mM 2-aminopyridine adjusted to pH 5.0 with acetic acid was capable of separating potassium, barium, calcium, sodium, magnesium, zinc, cadmium, lithium, and chromium in under ten minutes. Ligands such as HIBA or lactic acid were not required.

The need of an indirect detection agent for the determination of metal cations can also be dispensed with if they are complexed with a ligand of high molar absorptivity. This approach has been adapted from an equivalent reverse phase HPLC method. Swaile and Sepaniak were one of the first exponents of this technique for electrophoresis[56] and separated a number of group II metal ions complexed with 8-hydroxyquinoline-5-sulfonic acid. This ligand is capable of forming fluorescent complexes with a variety of metals which enabled the use of highly sensitive laser based fluorimetric detection. Detection limits for calcium, magnesium and zinc were in the low ppb region and technique proved to be a successful method for the determination of these metals in blood serum with few interferences.

4-(2-Pyridylazo)resorcinol (PAR) is a commonly used chelating agent for this purpose. Iki et al.[57] reported the separation of cobalt, vanadium, copper, iron and nickel as PAR chelates in a phosphate/borate buffer with UV detection at 550nm. In addition to this, when the PAR reagent was also included in the run buffer, less stable complexes of cadmium, manganese and zinc could also be analysed. The use of PAR reagents was

further explored by Regan et al.[58] who developed an on-line complexation procedure where a highly concentrated plug of PAR reagent was introduced into the capillary by pressure injection followed by a fifteen second electroinjection of metal ion solutions. Upon application of the voltage, the contramigration of the negatively charged free PAR and metal cations allowed the zones to mix and complexation to occur. This reaction was further enhanced if the metal ions were prepared in water in addition to the stacking effect, a more efficient mixing between the rapidly migrating metal ions and slow PAR reagent was achieved. Separations of cobalt(II) , copper(II), iron(II) and zinc (II) were carried out in 10mM N-(tri(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) buffer at pH 8.4. with detection limits in the 10^{-8} M range.

EDTA will form complexes with the majority of metal ions which makes it a popular choice for metal determinations by capillary zone electrophoresis[59,60] though the molar adsorptivity of these chelates is usually insufficient for low level environmental determinations. Alternatively 2-(5-bromo-2-pyridylazo)-5-(N-propyl-N-suifopropylamino)phenol (5-Br-PAPS)[61] and 1,10, phenanthroline[62] have also proved to be highly effective chromophoric chelating agents for metal ions.

Complexation with high absorbing ligands has proved to be a highly selective and sensitive method for the determination of metal ions by capillary electrophoresis. The technique serves a dual purpose by minimising interactions between the metal ions and the negatively charged capillary wall while at the same time converting the metal ions into complexes which absorb strongly in the UV -Vis region usually at a wavelength where interferences are at a minimum. The ability of metal ions to interact with certain ligands and the stability of the resultant complexes vary considerably. The indirect detection approach is more universally applicable though consequently more prone to interferences .

3.1.4.3. Application of capillary zone electrophoresis to arsenic speciation.

The evolution of capillary ion electrophoresis for simple inorganic cations and anions led to the adaptation of the technique to other areas of inorganic and organometallic analysis. Its' simplicity and high efficiency being particularly appealing for the purposes of trace element speciation. Morin et al.[63] were the first to implement capillary electrophoretic techniques for the separation of arsenic species. Working with a phosphate based buffer system, these investigators studied the effects of pH, temperature and voltage on the separation of four commonly occurring arsenicals; Arsenate (AsV), Arsenite (AsIII), Monomethylarsonic acid (MMA), and Dimethylarsinic acid (DMA). The migrating arsenic species were detected using direct absorbance readings at 190nm. Optimum resolution of the four compounds was achieved with 25mM phosphate at a pH of 5.6. The efficiency of this separation was calculated to be 132000 theoretical plates which was at least an order of magnitude higher than most conventional HPLC methods. Increasing the temperature had little effect on the actual separation selectivity but the peaks were sharper and the analysis time showed a significant decrease.

Lopez-Sanchez et al. studied the quantitative aspects of arsenic speciation by capillary electrophoresis[64]. Arsenate, Arsenite, MMA and DMA were separated using a 25mM phosphate buffer support electrolyte pH 6.8. at 40°C at 25kV. Hysteresis effects were overcome by flushing the capillary with sodium hydroxide and buffer between injections, this led to a high degree of reproducibility with the relative standard deviation over 25 injections below 2%. The sensitivity of the detection was poor however and concentrations below 2ppm were undetected.

Amran and co. workers[65] took advantage of the anionic nature of these arsenic species and demonstrated how the limit of detection could be reduced somewhat by reversing the electroosmotic flow and applying a negative potential. Dodecyltriethylammonium dihydrogenphosphate (Q_{12}) was employed as the flow modifier, a 10mM solution adjusted to pH 8.0 with sodium hydroxide allowed the four arsenic species to be separated within 6 minutes when a voltage of 25kV was applied across the capillary. All standard solutions were made up in water this gave rise to signal enhancement due to stacking. The limit of detection for the four arsenic species under these conditions

was found to be 250ppb with the method showing excellent linearity in the range 0 - 3ppm. Although these levels still far exceed those required for the majority of analytical applications they are a substantial improvement on those obtained when a conventional phosphate buffer system is employed.

Lin et al. applied the popular combination of flow modifier and indirect detection agent to the speciation of these arsenic anions[66]. An electrolyte consisting of 6mM chromate adjusted to pH 8.0 with sodium hydroxide and containing the Waters NICE-Pak OFM Anion-BT flow modifier at a concentration of 0.15mM was found to give the maximum separation efficiency for the four arsenic compounds with an applied potential of -28kV.

This system had a linear dynamic range in excess of two orders of magnitude however detection limits were reported to be 650ppb, 440ppb, 1.95ppm and 1.95ppm for Arsenate, Arsenite, MMA and DMA respectively which is still too high for the majority of applications.

The polarity switching field amplified injection protocol developed by Chien and Burgi[20] was modified by Li and Li to stack large injection volumes of arsenic and selenium compounds before separation[67]. Separations were carried out in a phosphate buffer at pH 7.8 at 20kV, the voltage was reduced to 10kV for the purposes of water matrix removal and stacking as higher voltages led to a slight decrease in the peak areas of arsenate and DMA. With the removal of the water plug, the deleterious effects of peak broadening due to the laminar flow were eliminated and subsequently injection volumes equal to the capacity of the entire capillary could be employed. The electroneutrality of arsenite and selenate made it impossible to determine the compounds when the polarity switching field amplified injection was employed as they were removed from the capillary with the water plug, however, the detection limits for the remaining arsenic and selenium compounds were as low as 13ppb. This method was applied to the analysis of a mineral spring water sample where the arsenic and selenium species were successfully resolved from other interfering anionic constituents. These investigations have demonstrated that techniques of indirect detection and flow modification developed for the determination of simple inorganic ions by capillary

electrophoresis are also applicable to more complex anionic constituents of environmental importance. Although the use of capillary electrophoresis for arsenic speciation is still in its' preliminary stages, the combination of indirect detection and stacking has already provided detection limits approaching those obtained by HPLC-HGAAS techniques. The simplicity of the CE instrument design compared to the cumbersome chromatography - atomic absorption spectrometer interface makes the technique a less labour intensive lower cost alternative for this purpose. However, the potential of capillary electrophoresis for arsenic speciation needs to be further explored in terms of improving limits of detection in order for the technique to be useful for environmental arsenic determinations.

3.1.5 Conclusions

In just over a decade, capillary electrophoresis has evolved from being a research curiosity to a highly efficient automated instrumental technique applicable to all areas of analysis. The development of on-column concentration and indirect detection procedures have surmounted the sensitivity problems arising from loading constraints and minute capillary dimensions to a certain extent and has established the technique as viable alternative to HPLC methods. The low cost of capillaries and ease of automation are additional advantages over HPLC methods where expensive columns and associated problems of blockages and leaks are a continuing drawback. The sensitivity of detection in capillary electrophoresis requires further improvement however before the technique can by successfully employed in the area of trace metal speciation in environmental analysis.

3.2. Experimental

3.2.1. Introduction.

Preliminary research has demonstrated the feasibility of capillary zone electrophoresis for the separation of four of the main arsenic compounds in the environment[63-64]. Furthermore, the techniques of flow modification and indirect detection initially developed to allow capillary electrophoretic determinations of small inorganic anions have been successfully adapted for this purpose[65]. While the use of indirect detection and whole column stacking[67] have led to improvements in concentration sensitivity of these arsenic speciation methods, the benefits of using a combination of these enhancement techniques have yet to be realised. To date, arsenobetaine, the most prevalent arsenic compound found in fish tissues, has not been included in these capillary electrophoresis separation studies. The majority of arsenic speciation studies have been concerned with the marine environment, therefore in order to evolve as a practicable alternative to HPLC-AAS for arsenic speciation studies, capillary electrophoretic separations must be modified to incorporate this important arsenic compound. As outlined in chapter 2, the determination of arsenobetaine by existing HPLC-HGAAS methods necessitates an additional derivatisation step which requires further modification of the apparatus to include a specially designed reaction coil or interface. This increases the analysis time and complicates the procedure thus discouraging the use of arsenic speciation as a standard environmental analysis procedure. The development of a simple, highly sensitive and efficient capillary electrophoresis method where all five environmentally important arsenic species can be separated within a reasonable time frame would consequently be particularly advantageous for the advancement of routine arsenic speciation techniques.

In the course of this work, the flow modified stacking procedure with indirect detection developed by Burgi[47] for the separation of chloride, nitrate and oxalate was adapted to the separation of the arsenate, DMA, MMA and arsenobetaine. In order to evaluate this method in terms of reproducibility and sensitivity, a straightforward separation without flow modification and using direct UV detection was also developed for these arsenic species.

3.2.2. Apparatus

The analysis was performed on a Beckman P/ACE capillary electrophoresis system equipped with a fixed wavelength detector this was fitted with a 190nm optical filter obtained from the Acton research corporation. The CE base unit and the detector were both controlled by an IBM workstation computer using System Gold ™ software. The separations were carried out in untreated fused silica capillaries 50μm i.d. X 57cm (total length), with a detection window burned 50cm from the injection end. Between each set of runs, this capillary was conditioned by rinsing for three minutes with a 0.1M sodium hydroxide solution, followed by a three minute rinse with distilled deionised water. The column was then flushed for ten minutes with run buffer. Unless otherwise stated sample introduction was carried out by hydrodynamic (pressure) injection.

3.2.3. Reagents

The following arsenic compounds were investigated ; Sodium arsenite (NaAsO2); Dimethlyarsinic acid (DMA) ($(CH_3)_2AsOOH$), both from Aldrich; Disodium hydrogen arsenate (Na_2HAsO_4) - BDH; Monomethylarsonic acid (MMA) $CH_3AsO(OH)_2$ and Arsenobetaine (Asbet) $(CH_3)_3AsCH_2COOH$, both obtained from the European BCR program as 1000ppm stock solutions in distilled deionised water. The run buffers employed were Disodium tetraborate (BDH), Boric acid (Merck), Diethylenetriamine (DETA) and Pyromellatic acid (PMA) both from Aldrich. All solutions were prepared in distilled deionised water and filtered and degassed before use.

Freeze dried samples of mussel and oyster tissues were donated by the Fisheries Research Centre, Abbottstown, Dublin 15.

3.2.4. Optimisation of the separation of arsenicals in untreated capillaries with direct UV detection.

Initially, the separation of the five arsenic compounds in the absence of flow modification and indirect detection was optimised with regard to pH and concentration of run buffer, voltage and injection time using a 50ppm standard mixture. This approach was similar to that employed by Morin et al.[63]. and Lopez-Sanchez et al.[64]. These workers used phosphate as their run buffer with a pH in the range of 5-6. Over this pH range, however, arsenite remains uncharged and subsequently migrates with the electroosmotic flow. This becomes a problem when the standard matrix is more dilute than the electrolyte or when a sample solution with additional neutral constituents is injected. In this case, arsenite will co.-migrate with the water plug and other uncharged moieties making accurate quantitation impossible. It was therefore decided to carry out the separations at a pH above 7, Amran et al.[65] reported than when a phosphate buffer above pH 7 was employed the peak due to arsenate disappeared from the electropherogram. For the purposes of this study a sodium tetraborate/boric acid buffer which operates in the pH region of 8.0-10.0 was used as the carrier electrolyte. With the buffer concentration fixed at 25mM, the effect of pH on the apparent and actual mobility of each ion was investigated over the range 8-9.5. The variation of mobility with run buffer concentration was then examined at a constant pH. In both cases a 5 second injection time was employed and separation was carried out at 20kV. The optimum separation voltage was selected by performing an Ohms law plot of voltage versus current[15]in the voltage range 0 - 30kV, an upward deviation from linearity was an indication that the rate of heat production exceeded that of removal and hence any operating voltage beyond this inflection was unsuitable. The most suitable injection time was selected as that which maximised peak area while maintaining resolution and high efficiency.

3.2.5. Optimisation of the DETA/PMA based separation with flow modified stacking.

In the case of the DETA/PMA based flow modified stacking and separation procedure the effects of, pH, concentration of the individual buffer constituents, voltage, injection time and temperature were examined

Prior to optimising the separation, the effect of the DETA/PMA run buffer on the electroosmotic flow was studied. For this initial experiment, the instrument was operated in forward polarity mode (i.e. with the negative terminal at the detector end.) and arsenite was used as a neutral marker. In the presence of an EOF, a peak due to this uncharged compound would appear on the electropherogram. The pH of the buffer was varied from 7-10 and DETA concentrations of 1, 5, 10, 15, 25, and 30mM were studied. It was subsequently investigated whether a reversal of the EOF occurred at higher concentrations of DETA, by repeating this set of experiments with the polarity of the electrodes reversed.

For the purposes of pH and ionic strength optimisation, the run buffer composition was kept as simple as possible by carrying out pH adjustments with the addition of PMA to the DETA solution. In this way, interferences due to the presence of additional anions such as hydroxides and chlorides could be avoided. The concentration of PMA added to reach the required pH was calculated by titration, hence the exact composition of the electrolyte for each pH and DETA concentration was known. Optimisation was carried out using a 5ppm standard mixture of arsenate, MMA, DMA and arsenobetaine. Separations were carried out at 20kV and a thirty second injection time was employed as recommended by Burgi[47].

Once the most suitable pH and ionic strength of the buffer had been established, the individual contributions of the DETA and PMA were evaluated by varying the concentration of one while maintaining the concentration of the other at a constant value. In this case additional pH adjustments were carried out using sodium hydroxide or hydrochloric acid.

Voltage optimisation was carried out using the Ohms law plot in the range 0 - 30kV as before and injection times from 5-90 seconds were investigated.

In these primary experiments, the run buffer combination of DETA and PMA as utilised by Burgi for the capillary electrophoretic separation of chloride nitrate and oxalate[47] was adhered to, even though the concentrations of each differed dramatically from those recommended in the original work. However the structural differences between the arsenic species and these anions, may give rise to substantial differences in their abilities to displace the PMA indirect detection probe for detection. Hence, chromate (Cr) and phthalate (PA) additives were also explored as alternative chromophoric agents. The suitability of each indirect detection probe was evaluated on the basis of peak size and symmetry and the degree of background noise.

3.2.5. Validation of the separation methods.

The reproducibility and linearity of each method was determined using the standard procedures of intra (within day) and inter (between day) variability tests[68].

For the intra variability assay, standard mixtures over the appropriate concentration range were separated by each optimised method. For the borate based separation a calibration curve was prepared over the concentration range of 10-100ppm, a much lower concentration range was required for the DETA method and standards from 1-10ppm were injected onto the column. Each standard mixture was injected six times. The linearity of both methods was assessed on the basis of the R^2 regression value calculated from the calibration graph. The within day reproducibilities of the methods were calculated by dividing the mean migration for each arsenic species into its' respective standard deviation, this gave the relative standard deviation. This value was known as the coefficient of variation when expressed as a percentage and was a measure of the precision of the method within a single day.

The between day variation was assayed by injecting a single standard mixture six times a day over a period of five days. The relative standard deviation and coefficient of

variation were then calculated as before. This value gave an indication of the long term reproducibility of the method. A 50ppm standard mixture was used to evaluate the between day variability of the borate based separation. For the indirect detection/stacking method a 5ppm standard mixture was sufficient for this purpose.

3.3.Results and Discussion.

3.3.1 The effect of borate pH on the mobilities of arsenic species.

The pH of the carrier electrolyte is probably the most important parameter governing separation efficiency in capillary zone electrophoresis. The electrophoretic mobility of an ion is dependant on its' charge which in turn is determined by the dissociation constant and the pH of the solution. The optimum pH should be one which maximises the selectivity and efficiency of the separation while allowing separation of the analytes within a reasonable time frame.

For the purposes of this experiment the effect of pH in the range 8-9.25 on the migration of the five arsenic species was examined. The apparent mobility of each ion was calculated from the migration time using the formula :

$$\mu_{app} = \frac{L_d L_t}{V t_m}$$

Where L_d and L_t are the total capillary length and length to the detection window respectively, V is the applied voltage and t_m is the migration time. The electroosmotic mobility was calculated in the same manner using the migration time for the water peak which acted as the neutral marker. The actual mobility of each ion could then be calculated by subtraction.

The influence of pH on the migration times of the arsenic species is illustrated in figure 3.8. There is an almost linear increase in the migration time of each arsenic compound over the pH range 8.00 to 8.75, after which the mobility of arsenate exhibits a pronounced deviation from those of the other four species. It was originally expected that the increase in electroosmotic flow brought about by the increase in pH would overcome the tendency of these arsenic species towards the positive injection end, resulting in an overall decrease in analysis time. The results illustrated in fig 3.8 show that this however was not the case.

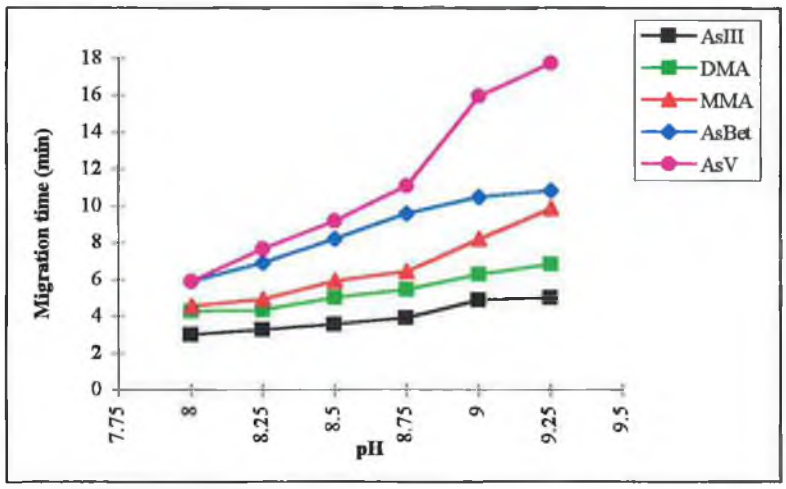


Fig 3.8; Effect of Borate buffer pH on the migration times of the arsenite (AsIII). DMA, MMA, arsenobetaine and arsenate (AsV).

In theory, the electrophoretic mobilities of DMA, MMA and arsenobetaine should be invariant at pH levels greater than 8.20 because their associated pK_a values (listed in table 3.1)[69] are all below this value. The observed increase in mobility for these species is possibly due to the fact that at these high pH levels the previously neutral arsenite species will begin to acquire a partial negative charge which will allow it to migrate independently of the EOF in the opposite direction. The magnitude of the arsenite mobility will gradually increase as the pH approaches its' pK_a value of 9.2. As a consequence of the Kohlrausch regulating function[1], the migration velocities of the other analyte zones will adjust accordingly to maintain the concentration ratio at each boundary at its equilibrium value. This will give rise to an increase in migration times for DMA, MMA and arsenobetaine even though they are ionised to their full potential over the entire pH range.

Arsenate, unlike the other four arsenic compounds investigated, is not fully ionised over this pH range. The increase in electrophoretic mobility above pH 8.75 can be explained in terms of a rapid increase in charge at pH levels close to its' pK_{a3} value.

This mobility increase also allows for an improvement in resolution between arsenobetaine and arsenate. At pH 8.0 the compounds co.-migrate, however separation is achieved above pH 8.25 as the arsenate species becomes progressively more negative.

Table 3.1; pKa values for each arsenic species

Arsenic species	pKa
Arsenite (AsIII)	9.3
Dimethylarsinic acid (DMA)	6.2
Monomethylarsinic acid (MMA)	3.6 8.2
Arsenobetaine (Asbet)	4.7
Arsenate (AsV)	2.3 6.9 11.4

These theories can be more effectively explained by plotting the electrophoretic mobility against pH. It can be seen from fig. 3.9, that the electrophoretic mobilities for DMA, MMA and arsenobetaine increase only very slightly over the pH range examined. The mobility of arsenite exhibits a marked increase at pH 9.0 as it becomes increasingly more ionised, the steady increase in the mobility of arsenate can also be explained in terms of an increase in charge as the ion approaches its' pK_{a3} value.

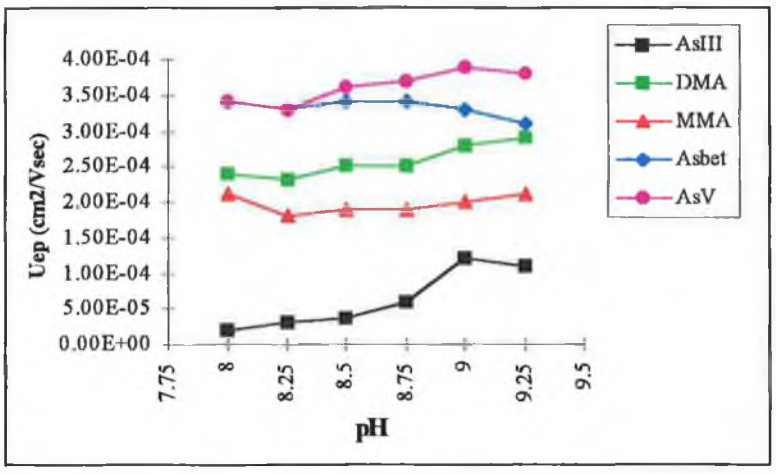


Fig. 3.9; Effect of borate buffer pH on the electrophoretic mobilities of the arsenite (AsIII). DMA, MMA, arsenobetaine and arsenate (AsV).

Electropherograms recorded with 25mM borate buffer at pH 8.25, 8.50 and 9.00 are illustrated in fig.3.10. It can be seen how the increase in pH influences the migration times of all the arsenic species. Increasing the pH has a particularly adverse effect on arsenate, the migration time of this compounds increases considerably and the peak shape becomes so distorted by pH 9.0 that it is useless for quantitative purposes.

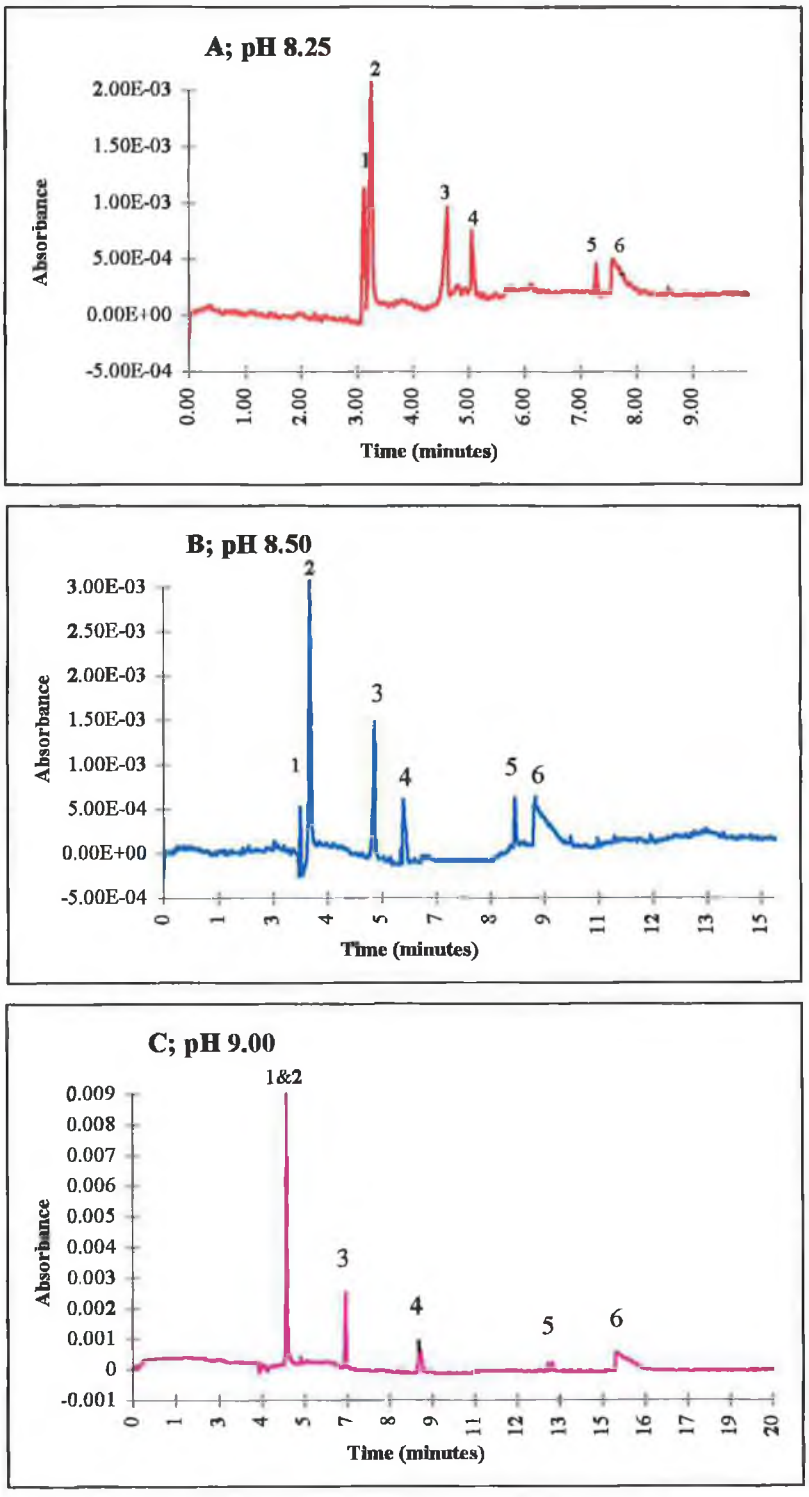


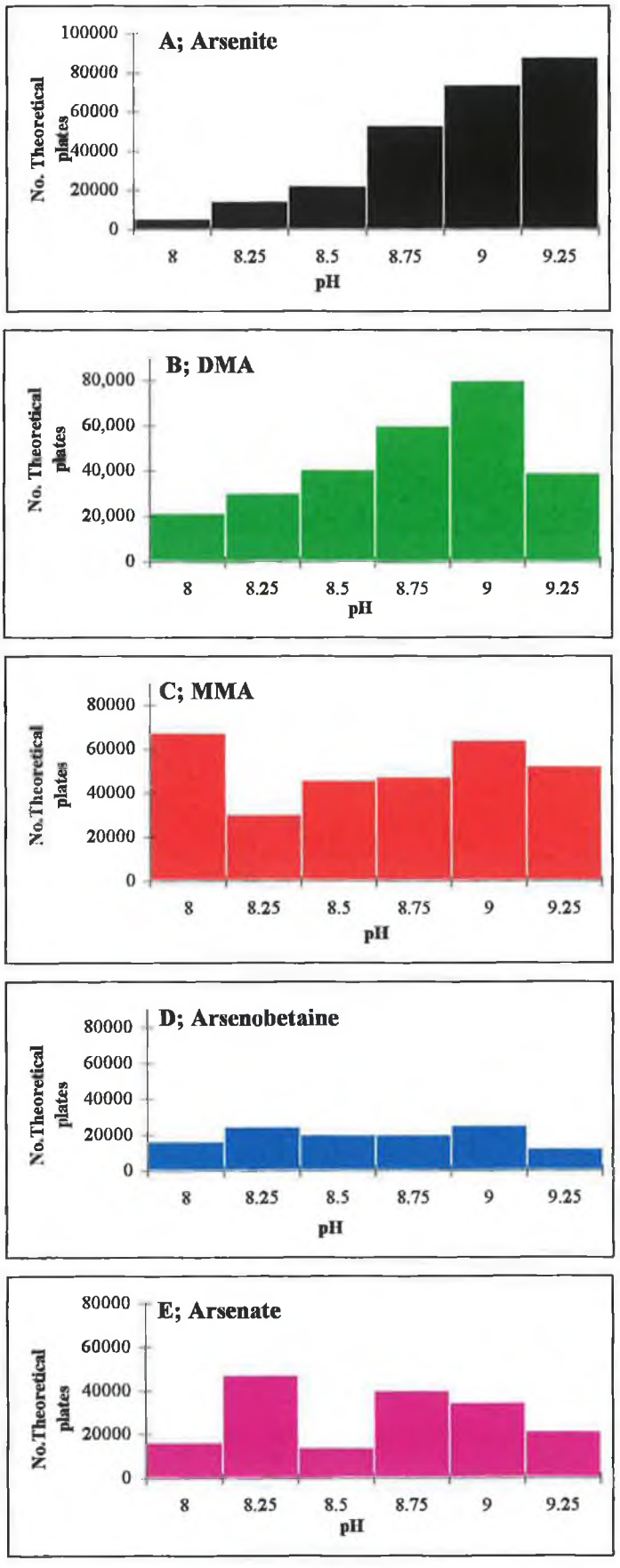
Fig 3.10; Electropherograms of a 50ppm standard mixture of arsenic species separated using 25mM borate buffer at A; pH 8.25, B; pH 8.50 and C; pH 9.0. Separations carried out at 20kV using a 5 second injection time. Migration order; 1. water matrix peak, 2. Arsenite , 3. DMA, 4. MMA, 5. Arsenobetaine, 6. Arsenate.

The separation efficiencies for each arsenic compound were calculated using the formula :

$$N = 5.54\left(\frac{t_m}{w_{\frac{1}{2}}}\right)^2$$

Where $w_{1/2}$ is the peak width at half height. The variation in efficiency with pH for each arsenic species is illustrated in fig 3.11. For arsenite and DMA the efficiency increases steadily with increasing pH, this is due to the fact that the peak widths remain relatively constant as the migration times increase. The decrease in efficiency for DMA at pH 9.25 is caused by a marked increase in peak width. The drop in efficiency of MMA above pH 8.0 is a consequence of the increase in mobility of this ion towards the injection end as the pH approaches its' pK_{a2} value. The resultant increase in migration time leads to bandbroadening. The calculated efficiencies for MMA above this pH follow a similar trend to DMA, with a second decline in efficiency at pH9.25 due to increased peak width. The efficiencies of arsenate and arsenobetaine do not seem to follow a particular pattern, the slowest migrating ion arsenate exhibiting lower efficiency due to the fact that considerable band broadening occurs in the course of the separation.

In spite of the high separation efficiencies which were achievable in the pH range 8.50 -9.00, the resolution between the water matrix and arsenite peaks was the deciding factor in selecting the most suitable buffer pH. The separation of these two peaks was incomplete at pH 8.75 and above and at pH 8.25 and below, therefore a pH of 8.50 was ultimately selected as the optimum for the separation of the five arsenic species. With this pH, it was possible to completely resolve the five arsenic species within 10 minutes. This pH was employed for all further optimisation experiments.



Fig;3.11; Variation in calculated efficiency values for each arsenic species in the pH range 8.00 to 9.25

3.3.2. Optimisation of borate buffer concentration.

The magnitude of both the electroosmotic flow and the electrophoretic mobility of an ion is inversely proportional to the viscosity of the buffer medium[15]. Van Orman et al.[70] confirmed experimentally that the electroosmotic flow decreases with the square root of the buffer concentration. This trend can be observed in fig 3.12 where the migration times of the arsenic species increase steadily with increasing ionic strength. As the mobilities of these ions are opposite in direction to the electroosmotic flow, the increase in migration time is not as pronounced as the corresponding decrease in the electroosmotic flow, the diminished electrophoretic mobilities exhibiting a reduced resistance to the flow towards the negative terminal.

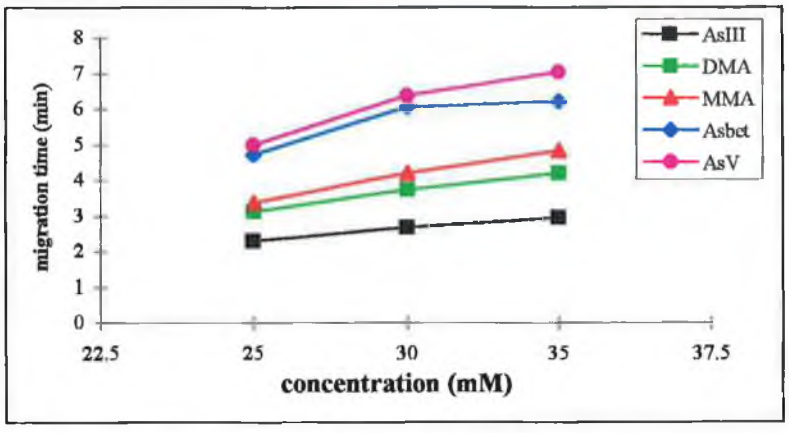


Fig 3.12; Variation in migration time with borate buffer concentration

Electropherograms obtained using 25mM and 30mM borate buffers both at pH 8.5 are compared in fig. 3.13. It can be seen that the increase in migration time is not uniform for each arsenic compound. The ionic strength alters the electrophoretic mobility of each ion to a different extent thus the increase in migration time for arsenate is far greater than the corresponding increase in the migration of arsenite.

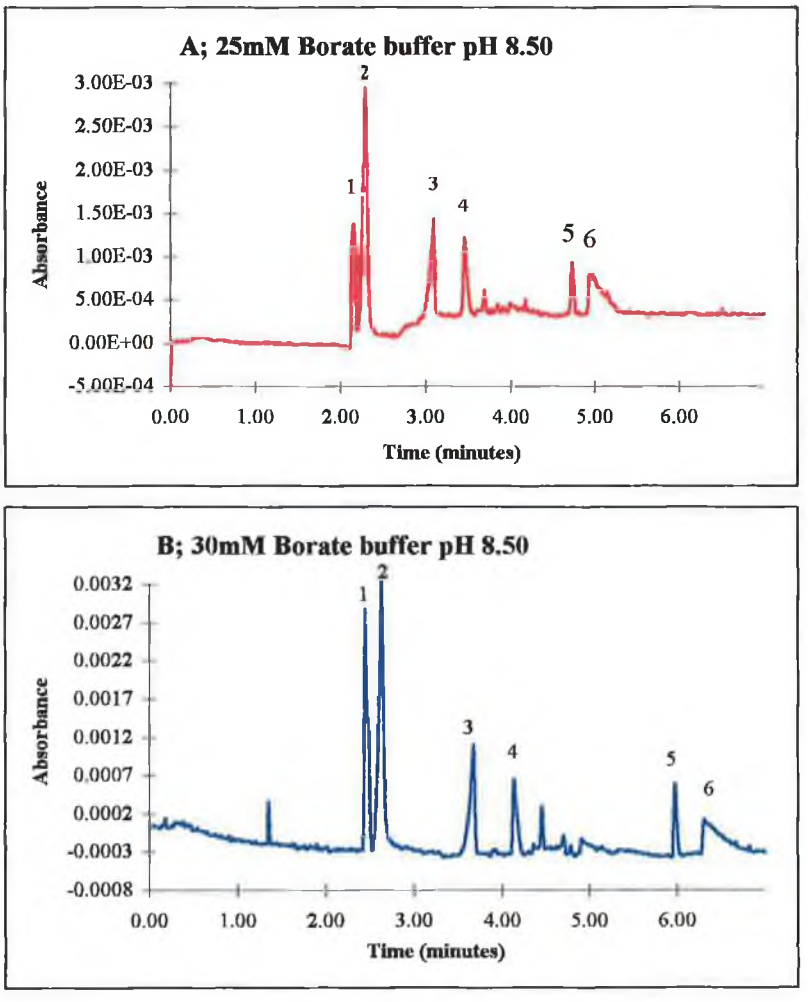
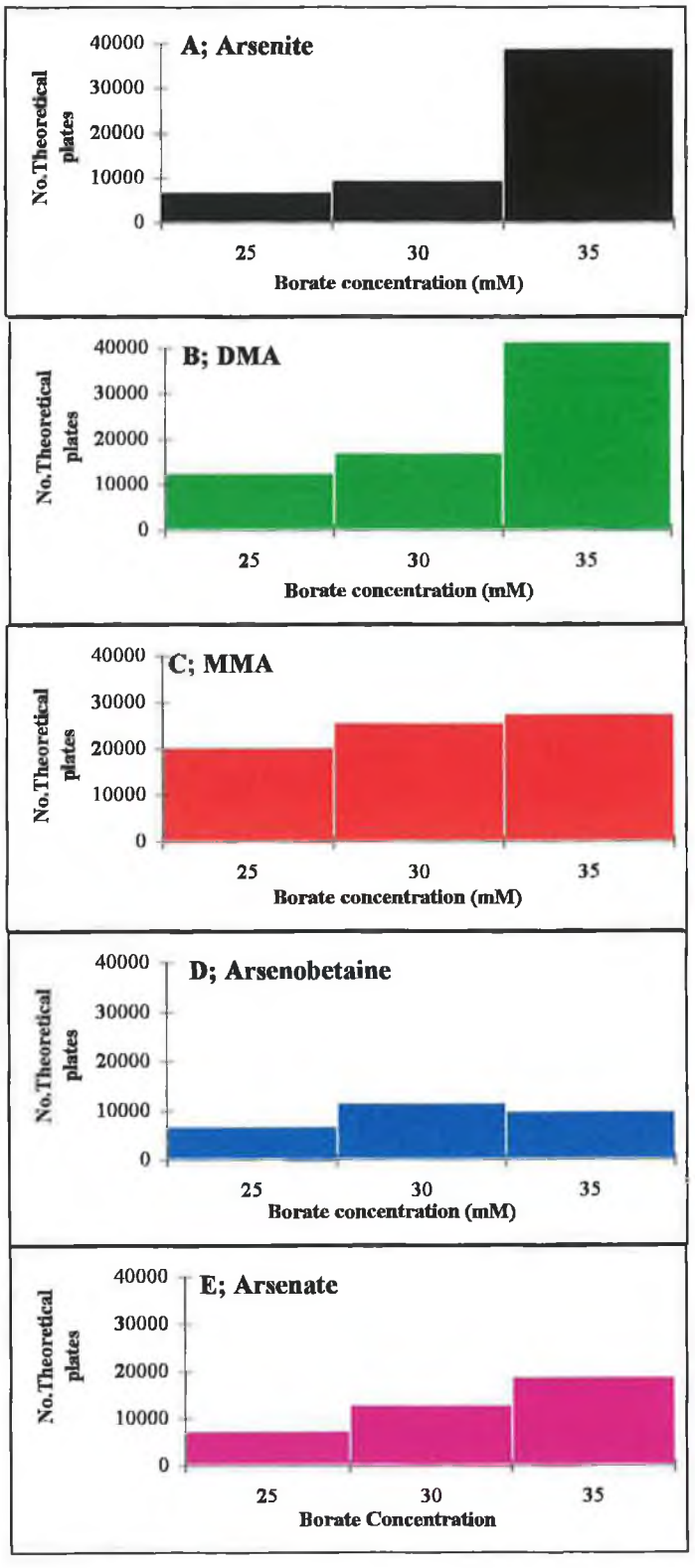


Fig 3.13; Electropherograms of a 50ppm standard mixture of arsenic species using A; 25mM and B; 30mM borate buffer both at pH 8.50, with a 5 second injection time and a 20kV operating voltage. Migration order; 1.water matrix peak, 2. Arsenite , 3. DMA, 4. MMA, 5. Arsenobetaine, 6. Arsenate.

The effect of borate concentration on the calculated separation efficiencies for each arsenic species is illustrated in fig.3.14. It can be seen that in all cases, the number of theoretical plates increased steadily with increasing ionic strength, this is due to the fact that the sample solution matrix is slightly more dilute than the run buffer and hence a narrowing of the analyte zones will occur due to a stacking effect.

Increasing the buffer concentration however led to increased analysis times and higher separation currents which further limited the maximum operating voltage. Therefore a compromise between superior efficiency and analysis time had to be made. All

subsequent separations were carried out using a 25mM run buffer pH 8.50. This permitted separation within 7 minutes while still maintaining a high level of efficiency.



Fig;3.14; Variation in calculated efficiency values for each arsenic species in the concentration range 25-35mM

3.3.3. Optimisation of separation voltage

As the migration velocity of an ion is linearly dependant on voltage, the higher the applied electric field the more rapid the separation will occur. The maximum operating voltage is limited however by the ability of the system to dissipate the joule heat which arises as a consequence of resistance to electrical conduction in the capillary. The maximum operating voltage can be ascertained from an Ohms law plot (fig. 3.15) of voltage vs. current. A positive deviation from linearity would be an indication that the rate of heat production exceeded that of heat removal, rendering any voltage above this value unsuitable. As can be seen from fig. 3.15. the current increases linearly with voltage for the entire instrument range of 0-30kV. Therefore it was possible to carry out the separation at the maximum operating voltage. This allowed separation within 7 minutes.

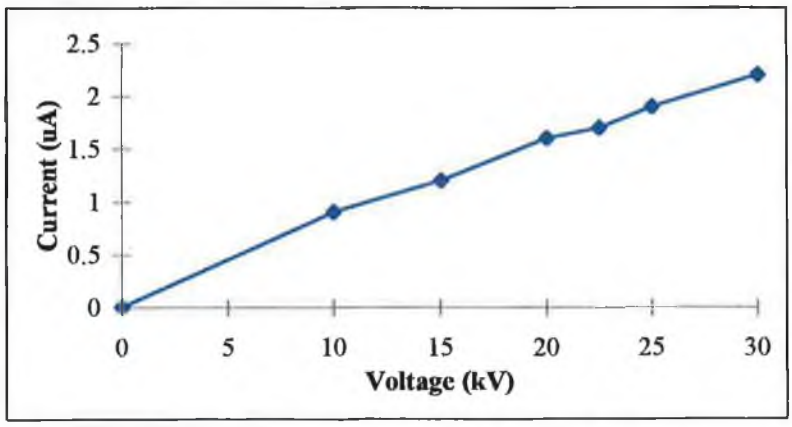


Fig. 3.15; Ohms law plot for a 25mM Borate buffer pH 8.50.

3.3.4. Optimisation of injection time for borate based separation.

The efficiency constraints on sample load have been outlined in section 3.1.3. In selecting the most suitable injection volume, high efficiency must be compromised to a certain extent in favour of the increase in peak area offered by larger plug lengths. It should be stressed however that the quantity of sample introduced onto the column must be such that a reasonable level of efficiency is maintained for the separation.

This increase in peak area with injection time can be seen in fig.3.16. With the exception of arsenate which shows a deviation at 7 seconds, a linear relationship appears to exist between the two parameters for the other arsenic species studied. For each arsenic compound the peak area increases to a different extent. As a hydrodynamic pressure injection is employed, this is not due to a sampling bias, but rather to differences in the molar extinction coefficients of the individual species.

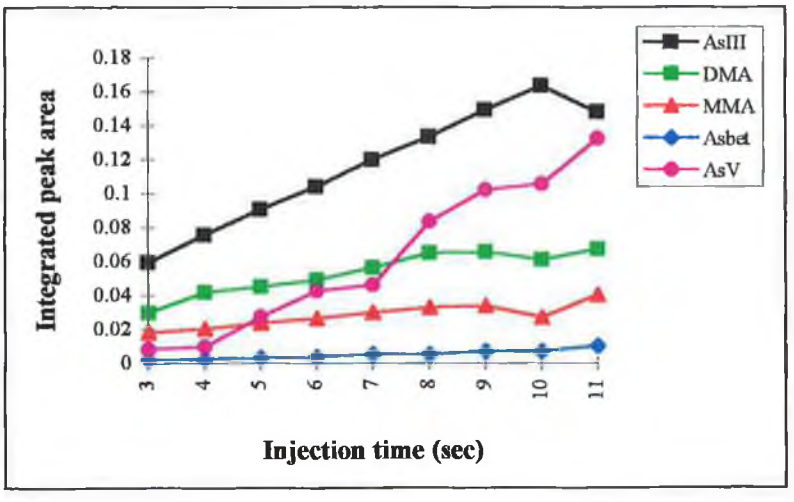


Fig. 3.16; Variation of integrated peak area with injection time.

Electropherograms obtained using a 3 second and a 9 second injection time are compared in fig. 3.17. The increase in peak area for the longer injection is apparent however the increased broadening of the peaks should also be noted especially for the DMA and arsenate peaks which have also experienced a loss of symmetry.

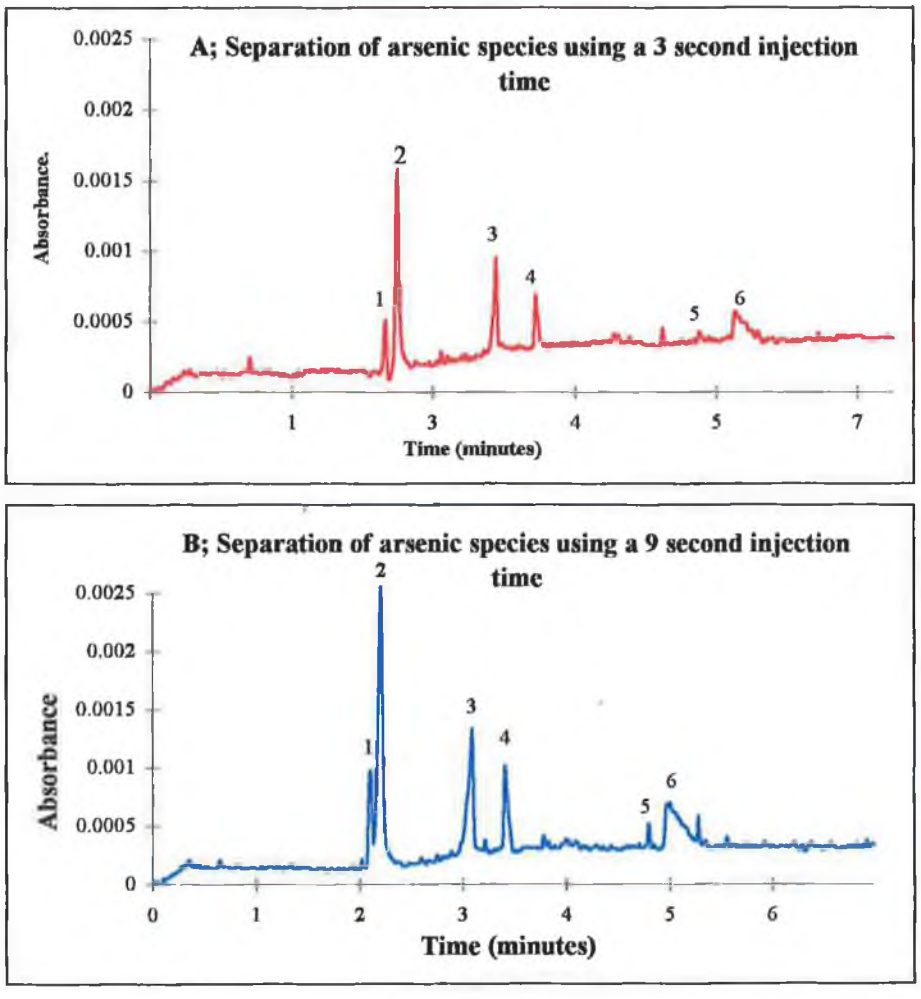


Fig 3.17; Comparison of electropherograms with a 3 second and 9 second injection time. Separations achieved using 25mM borate at pH 8.5, V = 30kV. Peak order; water, arsenite, DMA, MMA, Asbet & arsenate.

The increase in bandbroadening inevitably results in a decline in separation efficiency as reported in fig. 3.18. With the exception of DMA this decrease in efficiency appears to follow a pattern. The calculated efficiency shows a very gradual decrease from the three to five second injection, it then drops significantly when a six second injection is employed. This indicates the maximum allowable plug length has probably been exceeded with this six second injection time giving rise to more erratic decreases in efficiency. The efficiency for DMA on the other hand drops by 16% between a three and four second injections with a further 10% decline when a 5 second injection is employed. The efficiency value then remains relatively constant until another sharp decrease between a nine and ten second injection. The DMA peak also exhibits a

greater loss in symmetry with increasing injection volumes. This denotes a much lower limit for injection plug length then the other arsenic compounds.

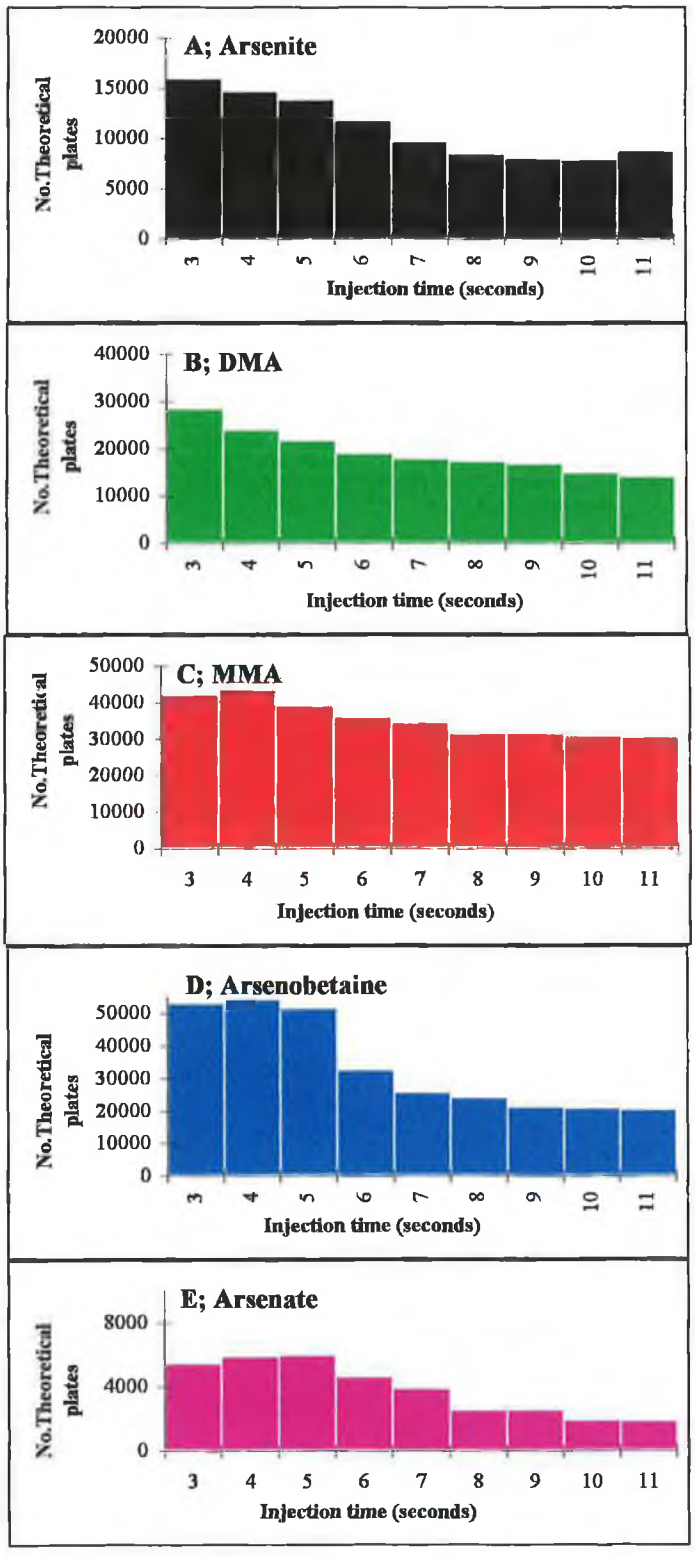


Fig. 3.18; Variation in calculated separation efficiencies with increasing injection time.

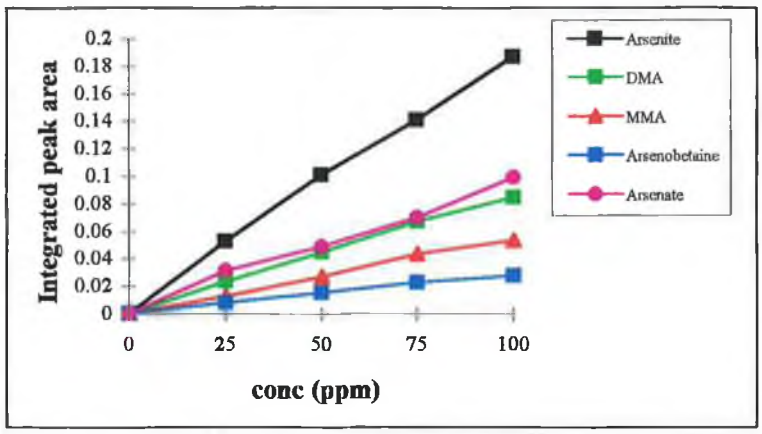


Fig. 3.19; Calibration curves for arsenite, DMA, MMA, arsenobetaine and arsenate.

Table 3.3; Limits of Detection and standard curve regression values for each arsenic compound separated in borate buffer under optimum conditions.

	R^2	Limit of detection (ppm)
Arsenite	0.9975	1
DMA	0.9981	17
MMA	0.9962	14
Arsenobetaine	0.9949	11
Arsenate	0.9912	6

Table 3.4 summarises the statistical validation figures for within day and between day variations.

Table 3.4; Statistical validation data for borate based separation.

	Within day variability (n = 6)	Between day variability (n = 6)	Mean number of Theoretical Plates.
Arsenite	0.52 %	4.67%	13,106
DMA	0.82%	4.25%	20,019
MMA	1.50%	5.40%	42,872
Arsenobetaine	0.99%	7.00%	11,394
Arsenate	1.17%	8.10%	4,418

The benefits offered by longer injection times in terms of detection ultimately outweighed the disadvantages due to lower separation efficiencies and therefore a nine second injection time was finally chosen for all further quantitation experiments.

3.3.5. Validation of the borate based separation of arsenic species.

The parameters used to carry out all further validation and quantitation work are listed below ;

Table 3.2; Optimum conditions for borate based separation of arsenicals

Column dimensions	**50μm (i.d.) X 57cm**
pH	**8.50**
Concentration	**25mM**
Voltage	**30kV**
Injection time	**9 seconds**

As can be seen from fig. 3.19, all five arsenic compounds responded in a linear fashion in the pH range 25-100ppm with regression (R^2) values greater than 0.99 (see table 3.6). Below 25ppm, detection sensitivity was too low to discriminate between solutions of similar concentrations. The limits of detection, defined as the concentration which gave rise to a signal equal to three times the noise level are also listed in table 3.3. though in reality it was not possible to distinguish peaks from noise at these levels.

The degree of variation within a single run is extremely low, with all percentage relative standard deviation values under 2.0%. The between day variation is slightly greater but nevertheless is below 10 % thus meeting the criteria for a valid precise separation method.

The separation efficiency of the method varies by almost a factor of ten, from 4,418 theoretical plates for arsenate to 42,872 for MMA. This substantial variation probably arises due to a disparity in the individual diffusion coefficients of the arsenic species, the arsenate zone undergoes considerable broadening in the course of the separation, while MMA migrates as a sharp narrow band. These calculated efficiency values in terms of number of theoretical plates compare favourably with corresponding values reported by Morin et al following the separation of arsenic species by ion pair- reverse phase liquid chromatography[71]. With this HPLC method, the number of theoretical plates calculated for arsenite, arsenate, MMA and DMA were 2,250, 6,500, 1.300 and 2,800 respectively. With the exception of arsenate which experiences a drop in efficiency due to zone broadening, the efficiency of the capillary electrophoresis separation is thus far superior with a five fold improvement in the theoretical plate number for arsenite and a fifteen fold improvement in the calculated plate number for MMA and DMA.

The high efficiency, satisfactory linearity and acceptable reproducibility levels recommends this method as a viable means of separating arsenic species. Unfortunately, the calibration range and high limits of detection render it unsuitable for the majority of environmental applications.

3.3.6. Optimisation of DETA/PMA based separation; Initial experiments.

In order to compensate for the detection shortcomings of this straightforward CE separation with direct UV detection, Burgis' protocol of flow modified stacking[47] with indirect detection was adopted for the purposes of arsenic speciation.

Prior to optimising this separation, the exact effect of DETA on the electroosmotic flow was investigated with the electrodes set at forward bias. The DETA was initially

investigated as a buffer additive with a phosphate buffer at pH 8.0. In this instance, a 100ppm solution of arsenite was employed as a neutral marker with detection at 190nm. In each case, a 25mM solution of phosphate buffer was employed at pH 8.0 and the DETA concentration was varied from $1x10^{-5}$M to $1x10^{-3}$ M. It was found that when incorporated into these systems the DETA merely brought about a reduction in electroosmotic flow rather than eliminating it completely. The extent of this flow retardation increased with increasing DETA concentration, however as the quantity of DETA in the phosphate buffer system increased the buffering capacity of the system was diminished making accurate pH determination impossible. As a result the use of these DETA/phosphate buffer systems was not investigated further.

The run buffer composition of 3mM PMA/3mM NaOH/1mM DETA originally used by Burgi for the flow modified stacking and separation of chloride, nitrate and oxalate[47] was subsequently examined. Both forward and reverse polarity modes were investigated. The absence of a peak due to arsenite after sixty minutes in both directions was an indication that the flow had been completely stopped. This phenomenon was also observed when DETA was used at concentrations of 10mM, 20mM and 50mM. Consequently, a DETA/PMA electrolyte in various combinations was used for all further investigations.

The separation of DMA, MMA, arsenobetaine and arsenate was originally attempted using this 3mM PMA/3mM NaOH/1mM DETA buffer composition. This gave a final pH of 3.5. As recommended in the original work[47], the electrodes were set at reverse polarity, detection was set at 254nm and a 30 second injection scheme was employed. Arsenite was not included at this point due to the fact that it will remain neutral and thus not migrate independently at pH levels below 9.2. This buffer composition proved unsuitable for the separation of the four arsenic species by virtue of the fact that only one large broad peak between two and six minutes, was obtained in the electropherogram. When the compounds were analysed individually, the all appeared at the same time in the electropherogram with the exception of DMA which was uncharged at this pH (see table 3.1).

To overcome these ionisation problems, the pH of the solution was increased by reducing the level of PMA in the buffer. This was accomplished by making up a solution of 1mM DETA and adding PMA drop wise to it until a pH of 8.50 was reached. This pH was chosen as it was found to be optimum for the previous separation. In this case, separation of the four compounds was observed but the peaks were extremely broad and a run time of 30 minutes was required before the appearance of the peak due to DMA. Improvements in peak shape and analysis times were noted when the DETA concentration was increased to 10mM. Separation of arsenobetaine, arsenate, MMA and DMA was finally achieved within 12 minutes when a 25mM solution of DETA adjusted to pH of 8.50 with a 25mM solution of PMA, was employed. At this initial stage, the peak areas obtained with this separation were up to one hundred times larger than those obtained with the borate based separation.

3.3.7 Optimisation of DETA/PMA buffer composition in terms of pH.

The separation of arsenobetaine, arsenate, MMA and DMA was optimised over the pH range 7.50 to 9.25. The concentration of DETA was held at 25mM throughout these experiments, pH adjustments were carried out, by titrating with a 25mM solution of PMA. The exact composition of the run buffers for each pH are listed in table 3.5.

Table 3.5; Composition of run buffer for each pH; PMA concentration calculated by titration with 25mM DETA.

pH	DETA concentration (mM)	PMA concentration (mM)
7.50	25	10.40
8.00	25	10.15
8.25	25	10
8.50	25	9.70
8.75	25	9.40
9.0	25	8.90
9.25	25	8.25
9.5	25	7.28

The effect of pH on the migration times of the four arsenicals investigated are illustrated in fig 3.20. In the absence of an electroosmotic flow, migration of the arsenic species is purely driven by their individual electrophoretic mobilities, therefore, the influence of pH on migration time can be more accurately predicted. As expected from the pK_a data listed in table 3.1. there was only a minor deviation in migration times of arsenobetaine and arsenate over the pH range studied. This was also observed for the borate based separation, however it was necessary to calculate the actual electrophoretic mobility and correct this value to allow for EOF effects before it could be illustrated. The migration time for MMA decreases steadily as the pH approaches its' pK_{a2} value after which the mobility did not vary greatly. The migration time for DMA decreases and subsequently levels off almost in tandem with that of MMA. As DMA is fully ionised after pH 6.2 this observed trend is due to the fact that the migrating zone obeys Kohlraushs' regulating function altering its' mobility in accordance with that of the preceding zone. The increase in DMA migration time above pH 8.75, was possibly due to an interaction between excess DETA in the run buffer and the slow migrating DMA molecules resulting in intermediate neutral complexes. This theory of transient complex formation between the arsenic anions and DETA may also be used to explain the reversal in migration order between arsenobetaine and arsenate. The arsenate molecule is much smaller than the bulky arsenobetaine molecule and more negatively charged therefore it is more likely to interact with DETA either in solution or on the wall, migrating at a slower rate than expected. This hypothesis, however, was not proven in the course of this project.

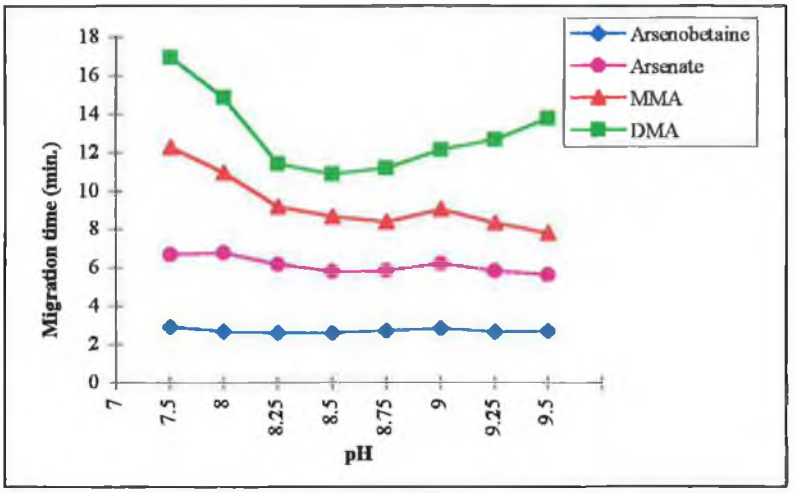
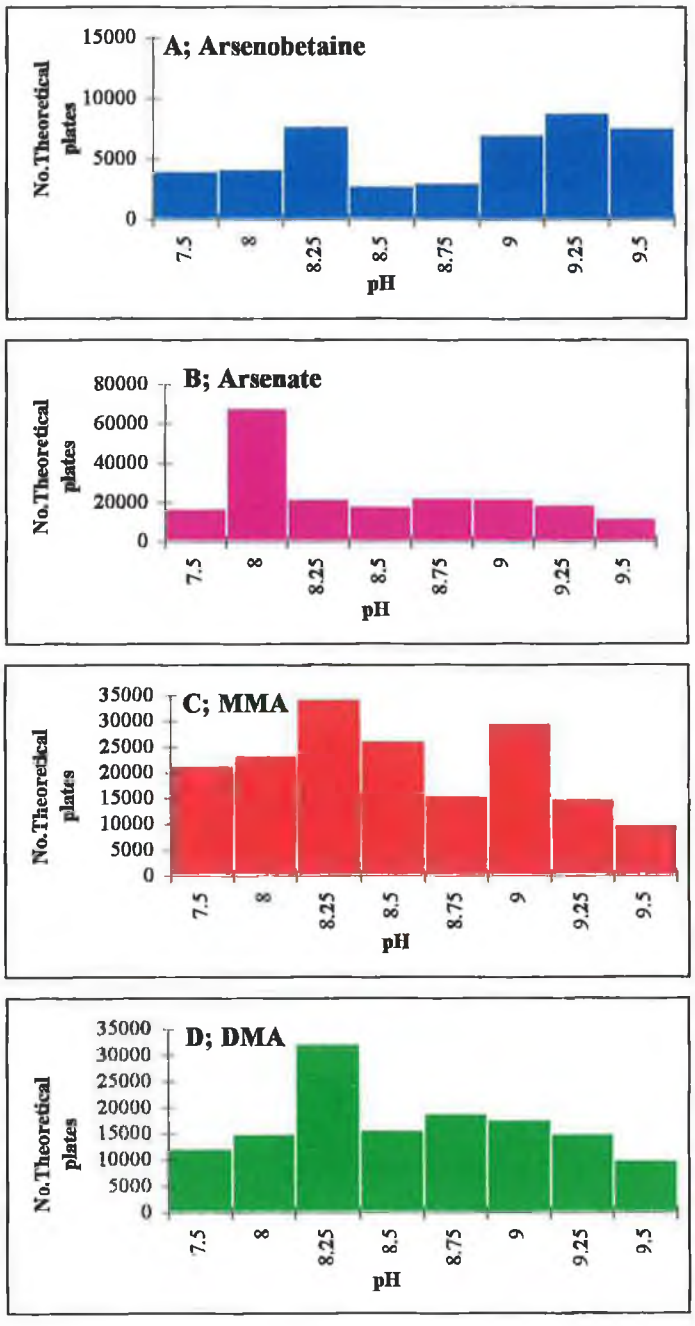


Fig 3.20; Variation in migration times with pH of DETA/PMA run buffer.

Due to the high dissociation constant for arsenite, it was not possible to determine this compound with a DETA based run buffer at a pH less than 9.50. The effects of using a very high pH run buffer were investigated, however even at high pH values the mobility of arsenic was too low to allow it to migrate through the capillary in under 40 minutes, furthermore the use of a higher pH necessitated the use of lower concentrations of PMA, which had adverse implications for detection. The variation in calculated separation efficiencies for each arsenic compound over the examined pH range is illustrated overleaf in fig. 3.21



Fig; 3.21; Variation in calculated efficiencies over the pH range 7.50-9.50 for each arsenic species for the DETA/PMA separation.

This efficiency data does not appear to follow a particular trend with pH. With the exception of certain outlying values, the peak efficiency for each compound is relatively constant throughout the pH range. It would therefore seem that the selection of the optimum pH required for the separation is very much an arbitrary matter, with any value in the range 8.25 to 8.75 being suitable. This would be the case if it were not for the presence of an additional system peak which migrates just ahead of

arsenate. As the pH increases the separation of this peak from that of arsenate deteriorates. The resolution between these two peaks ultimately became the factor which dictated the optimum pH for the separation.

The effects of increasing pH on the separation of the four arsenic species is more apparent in fig. 3.22, which compares electropherograms obtained at pH 8.0, 8.5, and 9.0. The initial decrease in migration time of MMA and DMA between pH 8.0 and 8.5 can be seen, the loss in resolution between the system peak and arsenate is also evident. At pH 9.0 (fig .3.22 C), these peaks are no longer baseline resolved. The peak due to DMA has also become broader leading to a decline in efficiency. Finally the adverse effect on peak size due to insufficient PMA levels at this higher pH can also be observed.

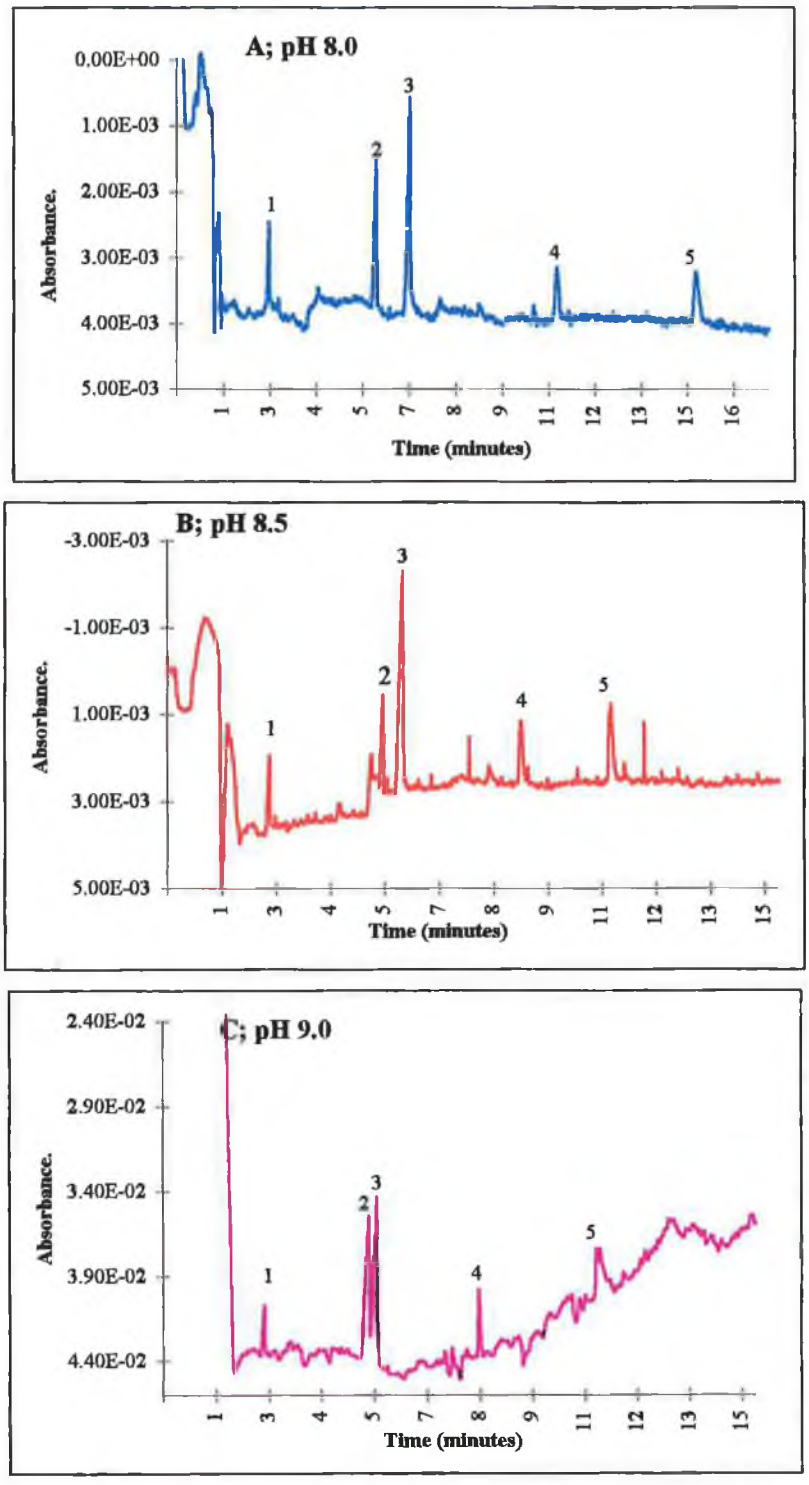


Fig 3.22; Comparison of electropherograms of a 5ppm standard mixture of arsenic species obtained in the presence of 25mM DETA adjusted to A; pH 8.0, B; pH 8.50 & C; pH 9.0 with PMA. In all cases separations were carried out at 20kV and a 30 second injection time was employed. Migration order; 1. arsenobetaine, 2. "system peak", 3. Arsenate, 4. MMA & 5. DMA.

In light of these resolution problems a pH of 8.50 was finally chosen as the most appropriate for the separation of the four arsenicals with DETA/PMA run buffer.

3.3.8. Optimisation of ionic strength of run buffer.

When considering the most suitable run buffer concentration, it must be taken into account that both constituents contribute to different aspects of the overall separation. The concentration of DETA primarily influences the separation of the arsenic anions, it has already been demonstrated that DETA concentrations as low as 1mM are capable of stopping the electroosmotic flow[47], further increases in concentration merely serve to provide a more viscous matrix to carry out the separation and also to enhance the overall stacking effect. The PMA concentration plays a more influential role in the overall detection of the migrating arsenic species, its' function as an indirect detection agent means that the shape and size of the individual peaks are determined by the PMA concentration in the run buffer.

For the early ionic strength experiments, the DETA/ PMA buffer was treated as a single entity. The exact composition of each run buffer was determined by titrating a specific concentration of DETA to pH 8.5 with a PMA standard solution. The results of this are listed in table 3.6. The combined effect on efficiency and peak size was then assessed. Once the optimum buffer composition had been established, the individual contributions of PMA and DETA could be evaluated.

Table 3.6; Run buffer composition for ionic strength optimisation experiments

[DETA] mM	[PMA] mM	separation current μA
5	1.69	4.6
10	3.64	8.6
15	5.50	12.0
20	6.80	15.9
25	10.00	18.60
30	10.54	20.1

As can be seen from fig. 3.23, in the absence of an electroosmotic flow, the ionic strength of the run buffer has a minimal effect on the migration of the analytes. Increasing the ionic strength will however cause the separation current to increase which may have a negative influence on the maximum separation voltage due to joule heating effects.

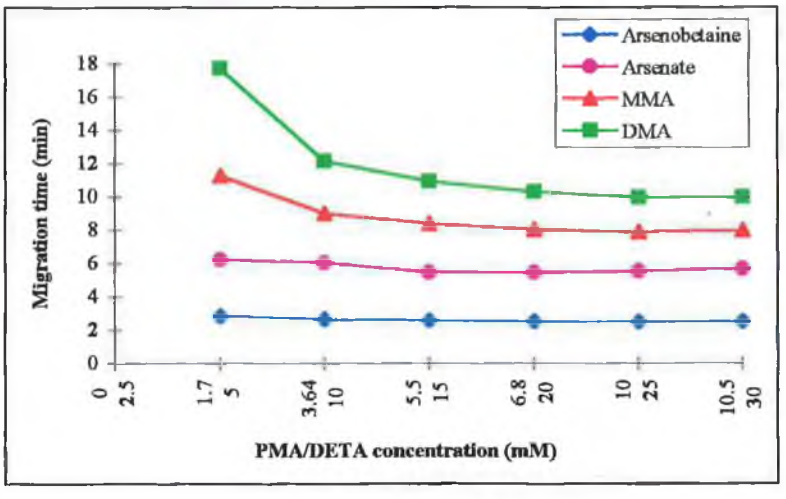


Fig 3.23; Effect of increasing DETA/PMA concentration on migration times of arsenic compounds.

The effect of increasing the ionic strength of the run buffer on the separation efficiency can be seen from fig. 3.24. In all cases the number of theoretical plates increases steadily as the concentration of the run buffer is increased. The increase in run buffer concentration further enhances the compression of the dilute sample zone resulting in narrower migration bands and consequently greater efficiency.

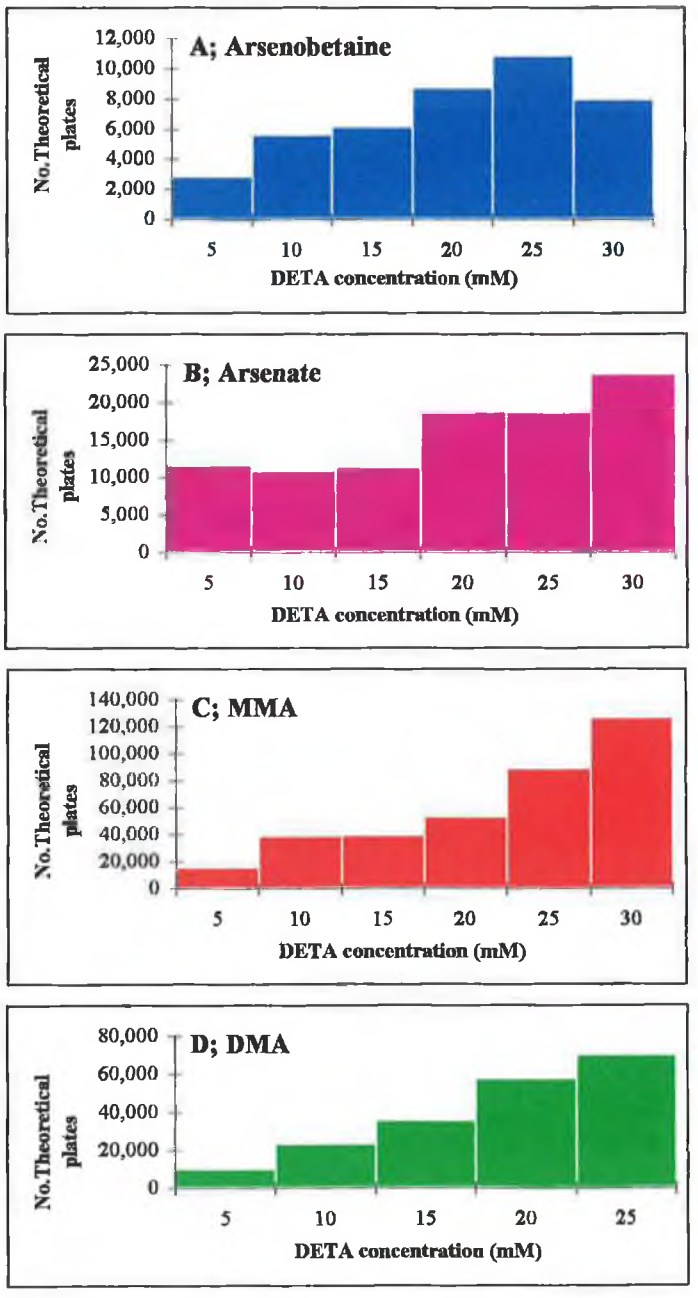


Fig 3.24; Variation in calculated efficiencies for each arsenic species with increasing DETA concentration.

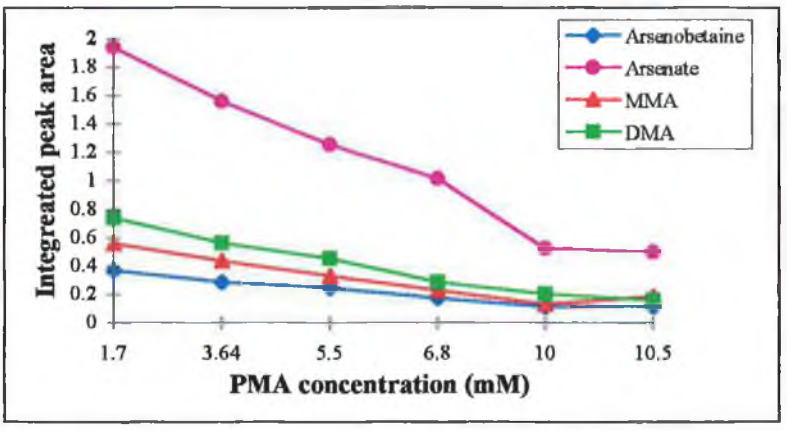


Fig 3.25; Influence of PMA concentration on peak area

The effect of increasing PMA concentration on peak size is much more pronounced. It can be seen from fig 3.25 that there is an approximately linear decline in peak area with increasing PMA concentration. These observations are in agreement with the expression derived by Yeung and Kuhr[26] directly relating limit of detection with the concentration of the background electrolyte. The decrease in peak size is also due to the fact that higher concentrations of probe reduce the dynamic reserve as described in section 3.1.4. This further diminishes the limit of detection.

The combined effect of peak area reduction with decreased migration time brought about by increasing the ionic strength of the run buffer can be clearly seen from the comparison of the electropherograms obtained with the 5mM/1.69mM and the 30mM/10.5mM DETA/PMA run buffers in fig. 3.26. When the higher ionic strength run buffer is employed, the total analysis time is reduced by half. Coinciding with this however is a substantial decrease in peak size, it can be seen that the peak due to arsenate is at least five times larger with the lower ionic strength run buffer.

While the influence of PMA on peak size is self evident, the exact contribution of each of the buffer constituents to the separation efficiency was not so obvious. These individual inputs were investigated by holding one of the buffer components at a constant concentration while varying the other, additional pH adjustments being carried out by the addition of either NaOH or HCl. Findings indicated that although the concentration of PMA was the predominant factor in the separation, migration

times and peak widths also decreased with higher DETA concentrations. This was particularly evident for the MMA and DMA peaks. This implied that the function of the DETA was not solely to stop the electroosmotic flow, but it also played a minor role in the actual separation of the species.

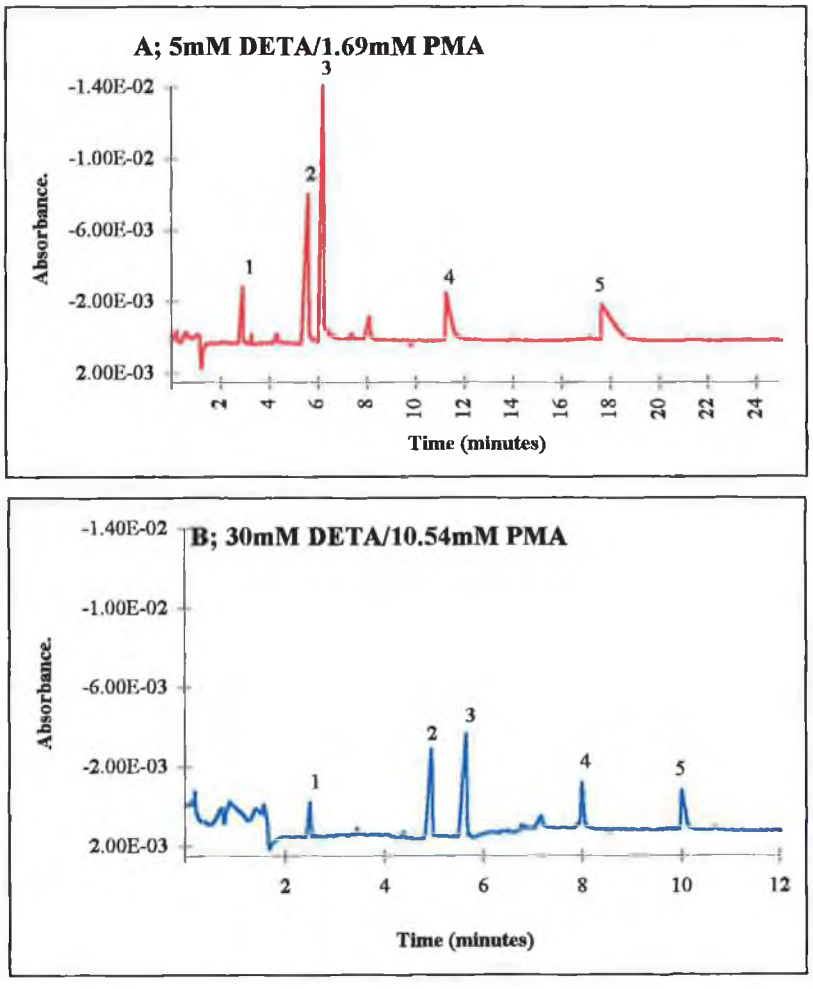


Fig 3.26; Comparison of electropherograms generated using 5ppm standard mixture of arsenic species in the presence of A; a 5mM DETA/1.69mM PMA run buffer and B; a 30mM DETA/10.54mM PMA run buffer both these buffers were at a pH of 8.50 In all cases separations were carried out at 20kV and a 30 second injection time was employed. Migration order; 1. arsenobetaine, 2. "system peak", 3. arsenate, 4. MMA & 5. DMA.

The many aspects to be considered in optimising the run buffer concentration for an indirect detection protocol such as this, means that the final choice is often a compromise between high efficiency and maximum peak size. In this case, a lower concentration of PMA gave rise to very large peaks at the expense of increased bandbroadening and migration times. Ultimately a run buffer composition of 15mM DETA and 5mM PMA was deemed to fulfil the requirements of high detector sensitivity while allowing the separation of the four arsenicals within 16 minutes.

3.3.9. Evaluation of phthalate (PA) and chromate as alternative indirect detection probes.

The initial optimisation steps were carried out using the buffer composition of DETA flow modifier and PMA indirect detection agent. Chromate and phthalate (PA) are also commonly used as indirect detection probes for anion determinations, therefore, once a suitable pH and ionic strength had been established, these chromophores were evaluated as possible alternatives to PMA, for the purposes of arsenic speciation.

The suitability of each probe was judged on the basis of its' effect of migration times, separation efficiency and size and shape of the individual peaks.

From the outset, the use of the chromate electrolyte with DETA was problematic, the separation currents were double those obtained when PMA or PA were used and the baseline was extremely noisy, making it difficult in certain cases to distinguish the peaks from the background noise. For this reason, the data generated from these chromate runs was not processed further.

The migration times, peak areas and separation efficiencies for each arsenic compound using PA and PMA buffers in the concentration range 2.5-12.5mM are compared in figures 3.27 - 3.29.

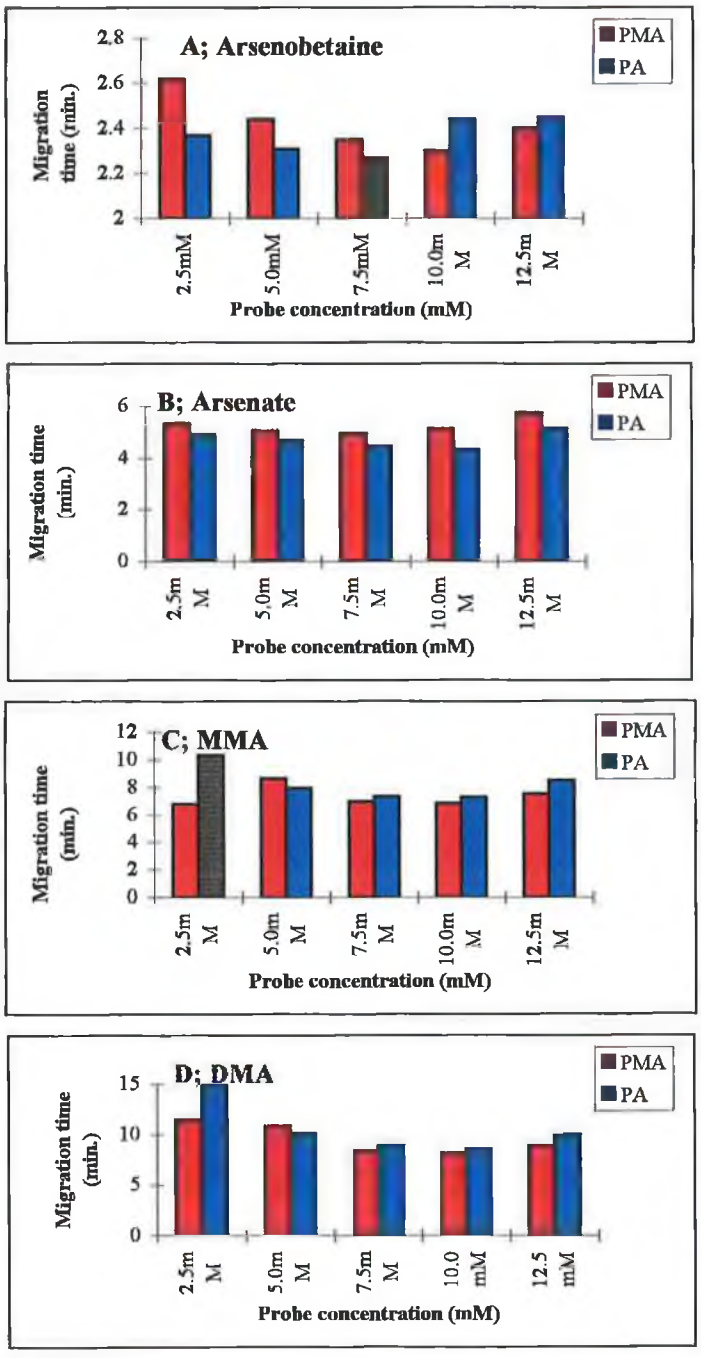


Fig 3.27 Comparison of migration times for each arsenic species in the presence of PMA and PA over the concentration range 2.5 - 12.5mM.

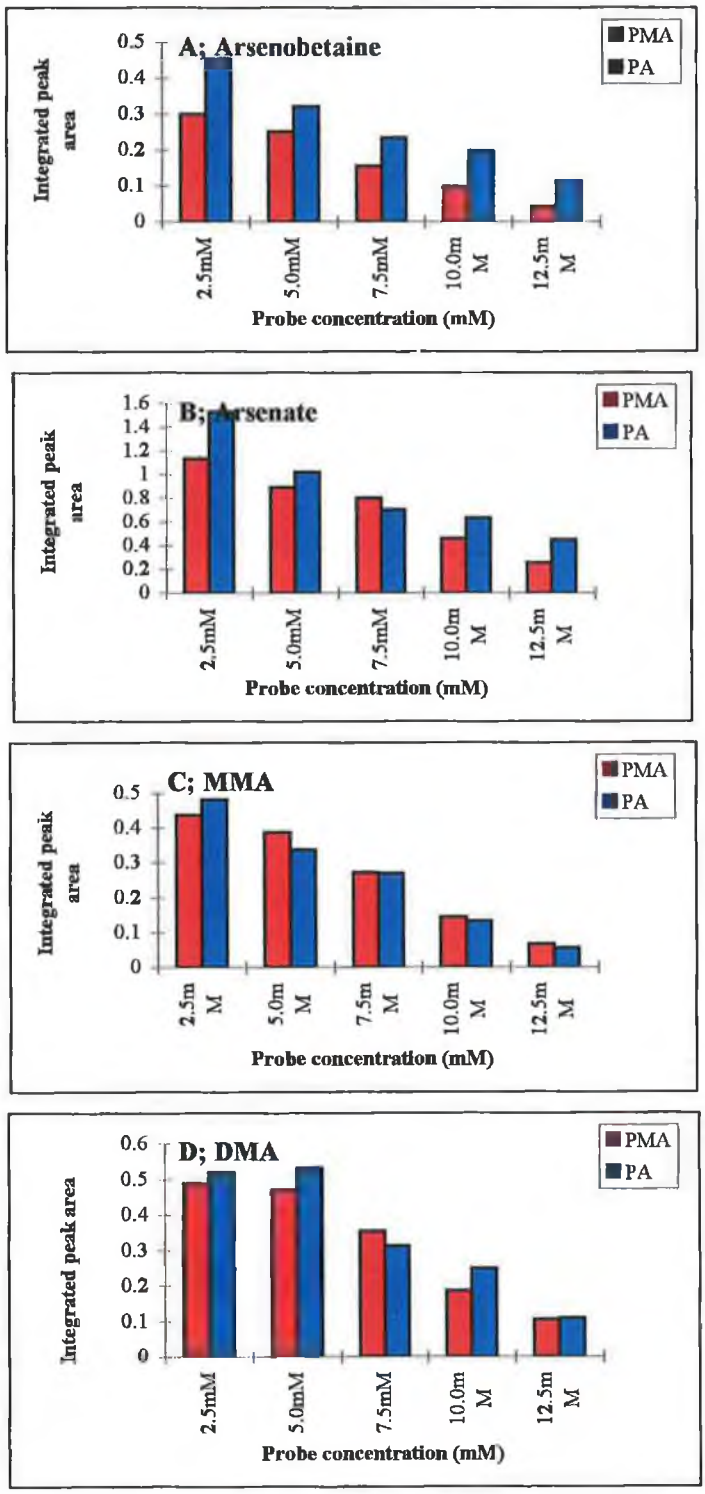


Fig 3.28; Comparison of integrated peak area values for each arsenic species in the presence of PMA and PA over the concentration range 2.5 - 12.5mM.

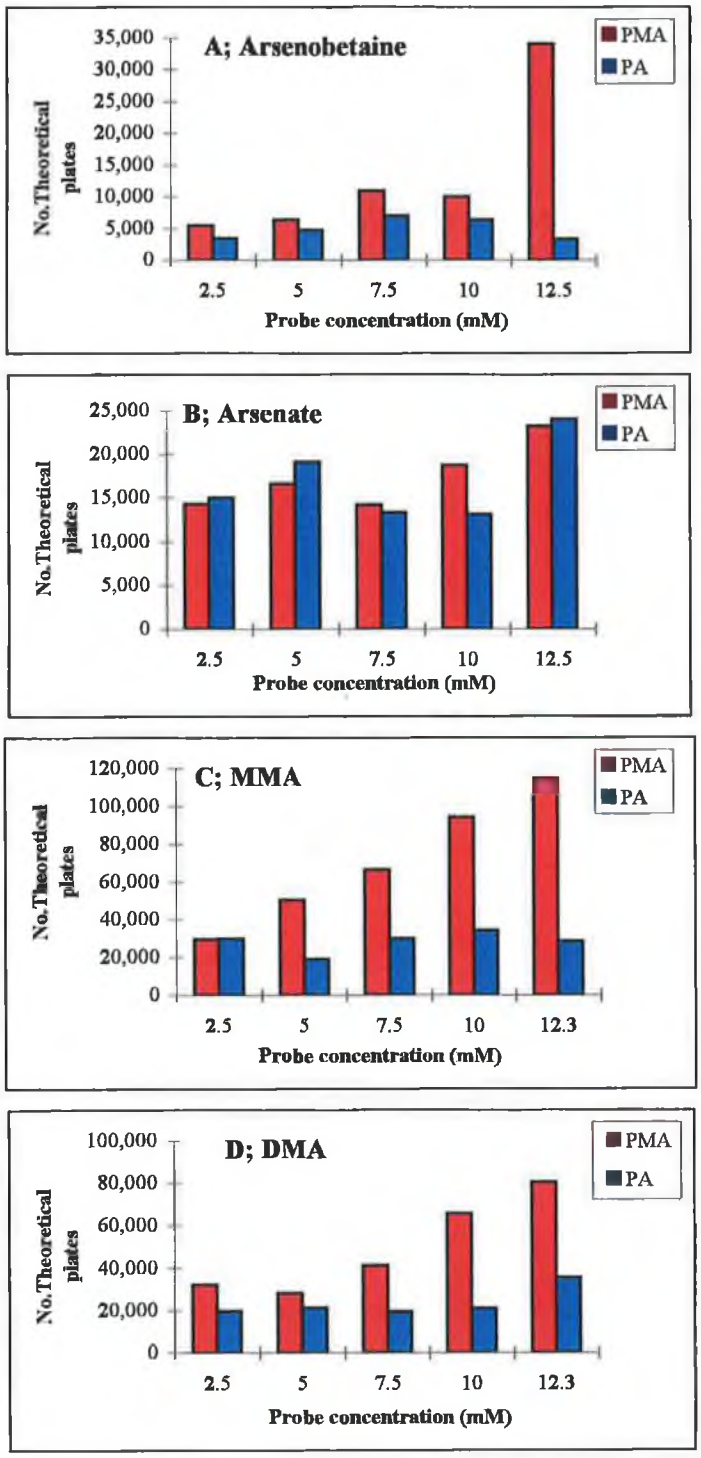


Fig 3.29 Comparison of calculated separation efficiencies for each arsenic species in the presence of PMA and PA over the concentration range 2.5 - 12.5mM.

The observed similarities in migration times and peak areas for each arsenic compound using PA and PMA electrolytes arise from the similarities in the actual mobilities of the probes under these conditions[29]. Slightly higher peak areas are obtained however when PA is used as the carrier electrolyte, this is an indication that the a more efficient displacement process exists between the arsenic anions and this probe. The increase in separation efficiency and lower overall analysis time obtained with PMA electrolytes however compensates for the marginal reduction in peak area. Therefore the original buffer composition of PMA and DETA was retained for all further modifications of the technique for arsenic speciation.

3.3.10. Optimisation of separation voltage.

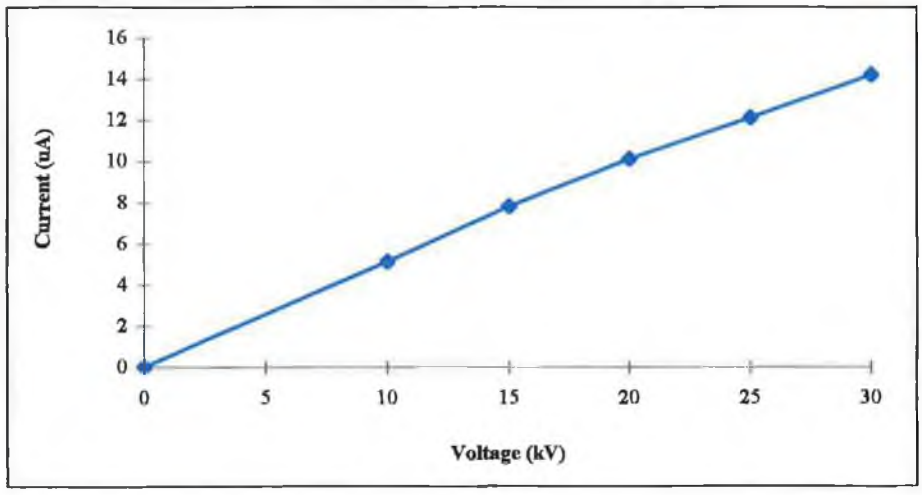


Fig 3.30; Ohms law plot for 10mM DETA/5.5mM PMA run buffer system, pH 8.5

The use of Ohms' law plots to establish the maximum separation voltage, have been discussed in section 3.3.3. As with the borate based separation, in this instance the operating current also increases linearly with voltage over the entire instrument range of 0 - 30kV making it possible to carry out separations at the maximum available voltage. With a 30kV operating voltage, separation of the four arsenicals is completed in 10 minutes.

3.3.11 Optimisation of Injection time.

In the original work on the DETA/PMA based separation of chloride, nitrate and oxalate, Burgi employed a 30 second injection time[47], it was reported that this corresponded to half the column volume for a 50μm x 57cm capillary. In order to exploit the detection benefits of flow modified stacking to their full capacity, the feasibility of whole column injections were investigated. The increase in peak area as the injection time was increased from 5 to 90 seconds over 5 second intervals was monitored. The outcome of these experiments is displayed in fig 3.31. As would be expected the peak area increases as the quantity of sample introduced onto the column is increased. This increase is steady up to an injection time of 45 seconds after which the peak areas become very erratic. In the case of arsenate, the peak area increases more rapidly when injections longer than 50 seconds are employed. The increase is due to a greater degree of bandbroadening rather than an actual increase in peak size. The peak areas for the other arsenicals show only a minor variation with injection times above 50 seconds. This would indicate that a 50 second injection time is sufficient to completely fill the capillary, after this no further sample loading will occur. This effect is more clearly demonstrated in fig. 3.32 where electropherograms obtained using 30, 60 and 90 second injections are compared. It can be seen that there is a marked difference in size between the peaks obtained with a 30 second injection and those obtained with a 60 second injection. The electropherograms obtained with a 60 and a 90 second injection however are very similar.

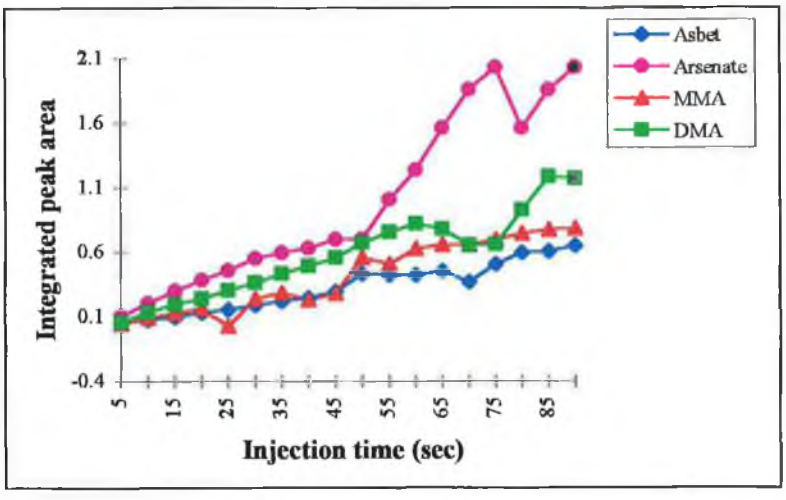


Fig. 3.31; Effect of increasing injection time on integrated peak area.

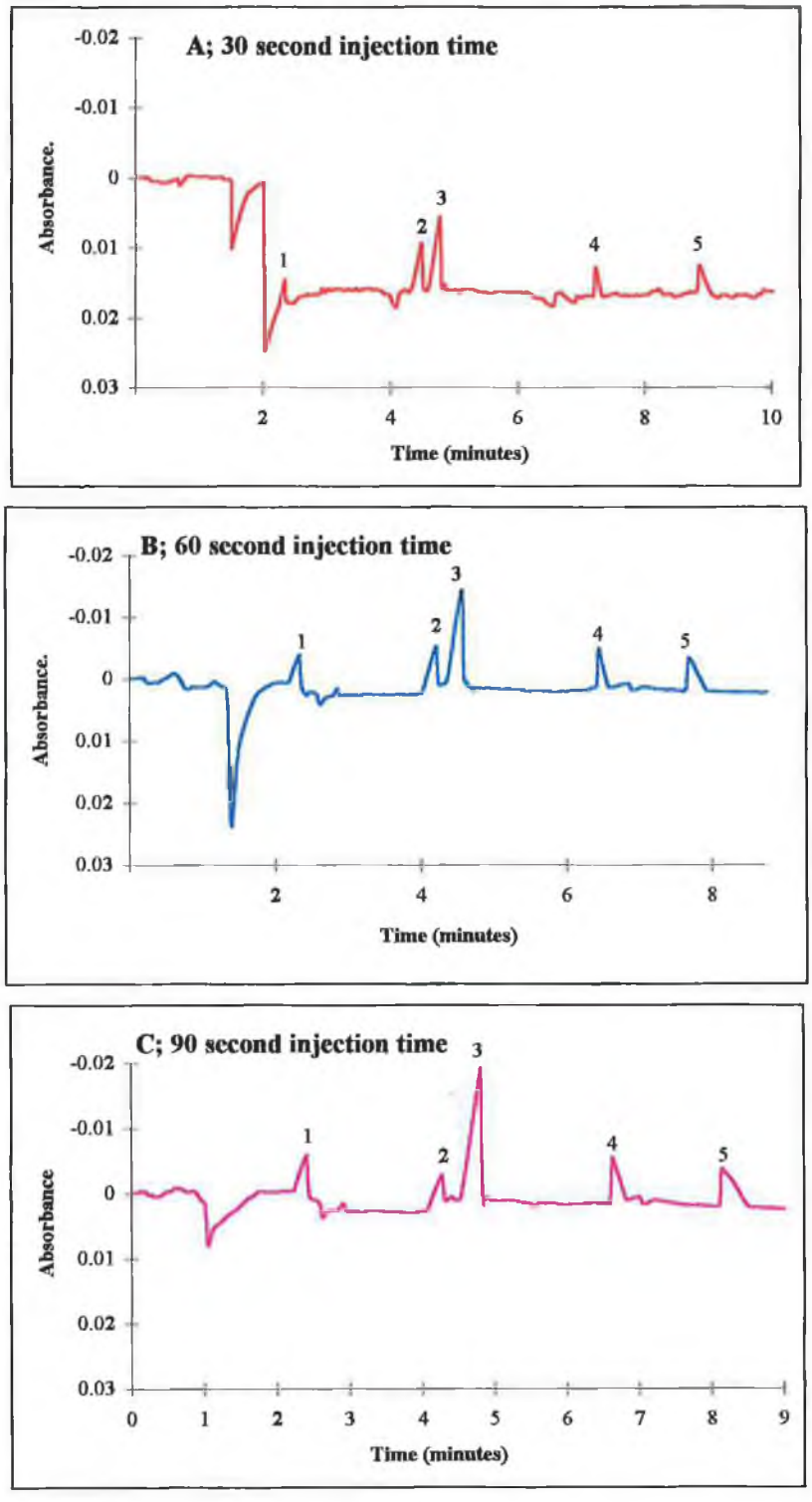


Fig 3.27; Comparison of electropherograms generated using a 5ppm standard mixture of arsenic species employing A; 30,B; 60 and C; 90 second injection time. In all cases separations were carried out using a 15mM DETA/5.5mM PMA run buffer pH 8.50, with a 30kV separation voltage at 40°C. Migration order; 1. arsenobetaine, 2. "system peak", 3. arsenate, 4. MMA & 5. DMA.

The theory that the column is completely filled with a 50 second injection is further supported by the fact that the calculated efficiency values drop steadily until this value is reached after which they remain practically unaltered this trend is illustrated in fig. 3.33.

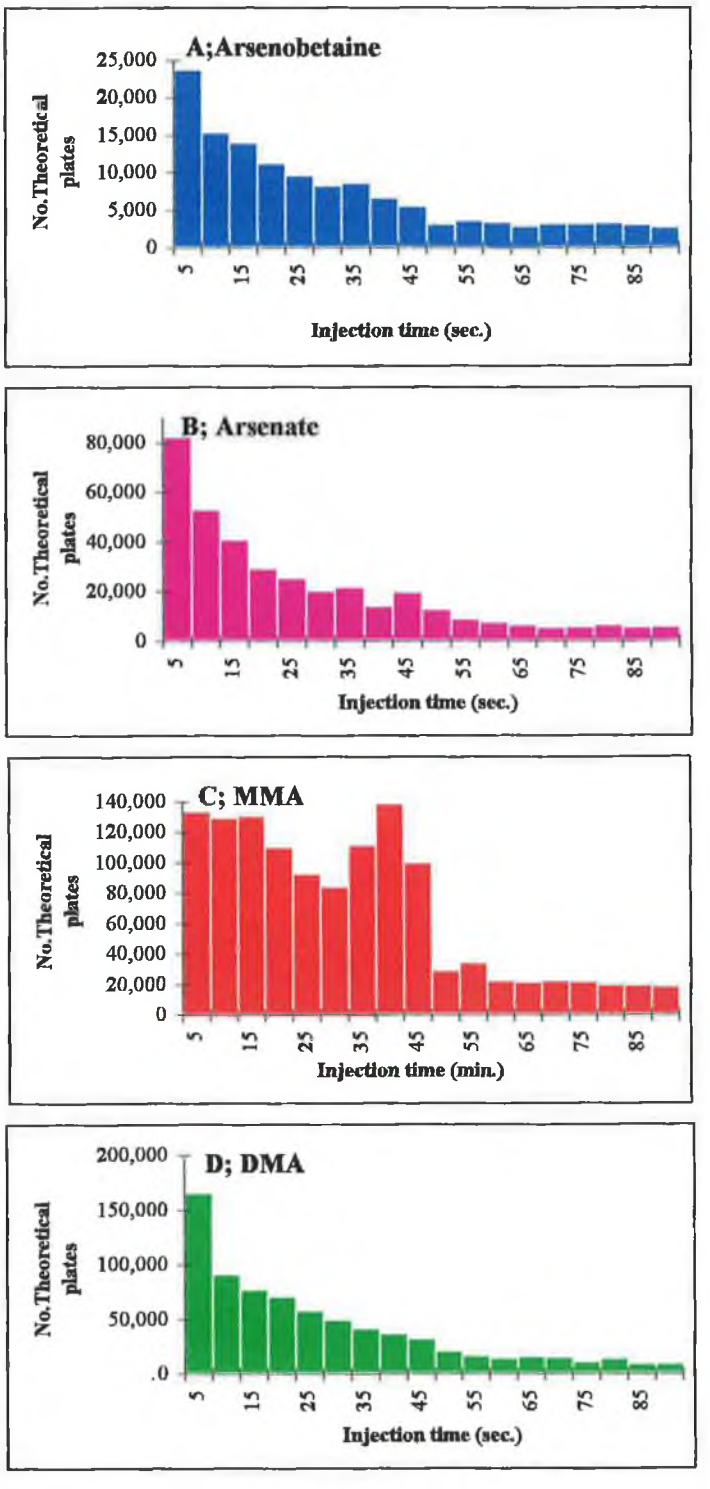


Fig 3.33; Variation in calculated efficiencies for each arsenic species with increasing injection time.

As was the case with the borate based separation, efficiency was sacrificed in favour of increased peak area. However, the erratic response obtained for peak areas with longer injection times combined with the reduced separation efficiency was discouraging for the use of whole column injections. Therefore a slightly shorter injection time of 45 seconds was adopted which combined the advantages of more reproducible larger peaks with an acceptable separation efficiency.

3.3.12. Investigation of temperature effects on migration times.

The relationship between ion mobility and buffer viscosity are outlined in section 3.3.2. as a direct consequence of this, migration times of the analytes should increase if the temperature of the buffer in the capillary is increased, thereby decreasing the viscosity. This concept was explored in the temperature range 25-40° C. The instrument is capable of operating up to 50° C but the low boiling point of DETA prevented the use of such high temperatures.

The effect of temperature on the individual migration times of the arsenic species is illustrated in fig. 3.34. With the exception of arsenobetaine, the migration times of the other arsenicals experience the largest decrease as the temperature is raised from ambient to 30°C and then continue to decrease steadily with increasing temperature.

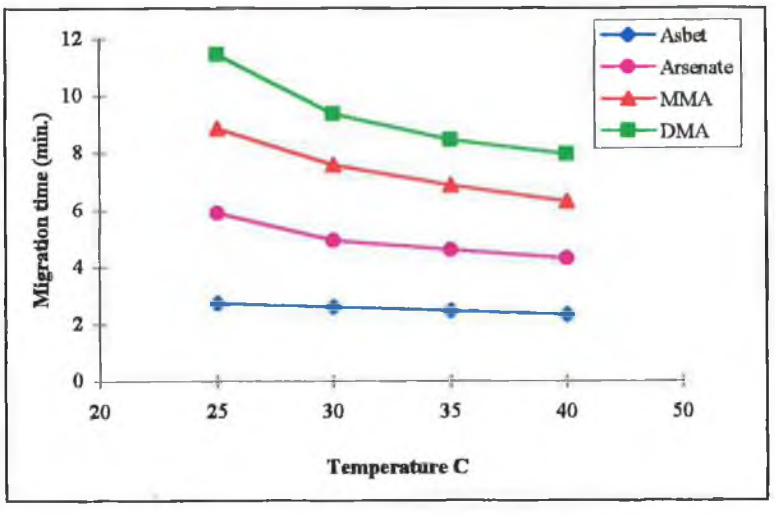


Fig 3.34; Effect of temperature on migration times of arsenic species.

Increasing the run temperature did not generate any adverse joule heating effects, therefore all validation work was carried out at 40°C which allowed for separation in 8 minutes.

3.3.13 Validation of DETA/PMA based separation.

Validation of this flow modified separation method was carried out using the following optimum conditions:

Table 3.7; Optimum conditions for DETA/PMA separation of arsenic species

Column dimensions	**50μm x 57cm**
pH	**8.50**
Run buffer composition	**15mM DETA/5.5mM PMA**
Voltage	**30kV**
Injection time	**45 seconds**
Temperature	**40°C**

Table 3.8 summarises the statistical data obtained from this validation work. While the regression values for each of the calibration curves (displayed in fig. 3.35.) are slightly lower than the corresponding values obtained from the borate based separation the degree of linearity is still at an acceptable level. This slight decline in R^2 values is probably due to the fact that the replacement efficiency of the arsenic ions may not vary with concentration in a linear fashion. As the borate buffer system utilised a direct detection scheme the linearity of the calibration curve was solely effected by differences in the concentration of the standards .

Table 3.8; Summary of validation data with respect to linearity, reproducibility and efficiency

	Linearity (R2)	Mean Migration time (min)	Within day variation (%RSD) (n = 6)	Between day variation (%RSD) (n = 6)	Theoretical plates
Asbet	0.9832	2.33	2.35	2.40	4,613
Arsenate	0.9903	4.64	0.56	0.95	18,466
MMA	0.9904	6.96	2.84	4.93	57,766
DMA	0.9900	8.91	1.49	2.92	24,387

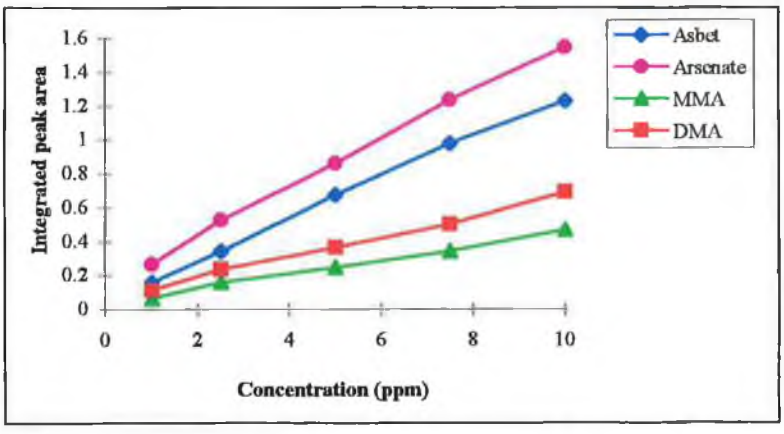


Fig. 3.35; Calibration curves for arsenobetaine, arsenate, MMA and DMA obtained following separation of standard mixtures under optimum conditions.

With this DETA/PMA based separation the migration times of the arsenic species are very reproducible with the majority of within and between day variation values below 3%. The migration times for MMA show a greater fluctuation for between day analyses but the %RSD nevertheless remains below 5%.

The range of calculated theoretical plates is almost identical to that observed with the borate based separation, varying from 4.613 to 57,766 theoretical plates. Again, this broad variation in separation efficiency can be attributed to the fact that the individual analyte zones will all diffuse to a different extent in the course of the separation.

Thus the high efficiency reported for the borate based separation has been translated to this flow modified stacking / indirect detection procedure. The substantial increase in peak size however recommends this method more favourable for use in environmental analyses.

3.3.14 Determination of transfer ratio and concentration limit of detection.

The expression derived by Yeung and Kuhr[26] for determining the concentration limit of detection (C_{lod}) for an indirect detection method relates the limit of detection with the concentration of indirect detection probe (C_m), the transfer ratio (TR) and the dynamic reserve (DR), as follows;

$$C_{lod} = \frac{C_m}{TR \text{x} DR}$$

The protocol for determining transfer ratio and dynamic reserve developed by Buchberger et al.[29] and outlined in section 3.1.4, was adopted in this instance for determining the limit of detection of the DETA/PMA separation method.

A calibration curve for the pyromellitate ion was obtained by running standard solutions in a phosphate buffer pH 8.5 at 30kV with detection at 254nm. As recommended by Buchberger[29] the peak areas obtained were multiplied by the apparent velocity of the ions prior to plotting against concentration. This functioned as a correction factor for the discrepancies in response due to differences in the rate at which the analyte zone migrates past the detector. A similar calibration plot was obtained for each arsenic anion, in the PMA/DETA run buffer. The transfer ratio was then defined as the quotient of the slope of the sample calibration plot and the probe calibration plot. The dynamic reserve was defined as the signal to noise ratio of the background electrolyte. Table 3.9 summarises the experimentally determined transfer ratios and resulting concentration limits of detection for each of the arsenic species.

Table 3.9; Experimentally determined transfer ratios and limits of detection for the arsenic species.

	Arsenobetaine	Arsenate	MMA	DMA
Transfer Ratio	3.75	2.525	0.5021	0.5529
Limit of detection	92ppb	56ppb	538ppb	480ppb

The enhanced detector response due to the stacking and indirect detection thus gave rise to a hundredfold improvement in the limits of detection for arsenate and arsenobetaine and a fiftyfold improvement in the detection limits for MMA and DMA.

The exact contribution of both aspects of the technique, indirect detection and stacking were examined by injecting standard solutions of the arsenic species made up in run buffer as opposed to water. Findings proved that while indirect detection gave rise to a substantial improvement in detector sensitivity in its' own right, the technique was still restricted to small injection plug volumes due to overloading effects. The incorporation of stacking thus allowed for a greater amount of sample to be introduced onto the column, further enhancing the limit of detection.

3.3.15. Investigation of potential interferants in the analysis arsenic species by the DETA/PMA stacking method

Due to the universal nature of indirect detection, high concentrations of anions such as carbonates or chlorides may interfere and mask the peaks due to the arsenic species. Five of the most commonly occurring anions in environmental matrices; chloride, carbonate, nitrate, phosphate and sulphate, were assessed as potential interferants for this DETA/PMA method of arsenic speciation. The migration times of each of these anions and those of each of the arsenic species separated under the optimum separation conditions are listed in table 3.10.

Table 3.10; Migration times of most commonly occurring interferants

Anion	Migration Time (min)
Arsenobetaine	2.33
Chloride	3.32
Arsenate	4.64
Carbonate	4.66
Nitrate	5.33
Phosphate	5.55
Sulphate	5.74
MMA	6.96
DMA	8.91

Interferences form a high level of chlorides is often problematic with ion exchange chromatographic arsenic speciation methods, however chloride does not co,-migrate with any of the arsenic compounds separated by this alternative capillary electrophoresis procedure. As the majority of arsenic speciation studies deal with sea water and marine organisms, this ability to separate arsenicals from chloride is an important advantage. In spite of this, chloride removal is still advisable for the analysis of sea water samples, where the level greatly surpasses that of any arsenic species. Samples containing high levels of carbonates or nitrates will also have to undergo a pre-treatment step to eliminate these interferants before arsenic determination can proceed.

In order to test the method for its' suitability for "real life" applications, a number of fish tissue extracts were spiked with a 5ppm standard solution of arsenic species and injected onto the column. These extracts had been prepared by sonicating each tissue in methanol for one hour. The resulting supernatant solution was then evaporated to dryness under nitrogen and reconstituted in water.

These solutions were not subjected to any further sample clean up procedures and hence were in a very crude state when injected onto the column, this would account

for the large number of matrix peaks appearing on the electropherograms displayed in fig 3.32. The arsenic compounds nevertheless are clearly distinguishable.

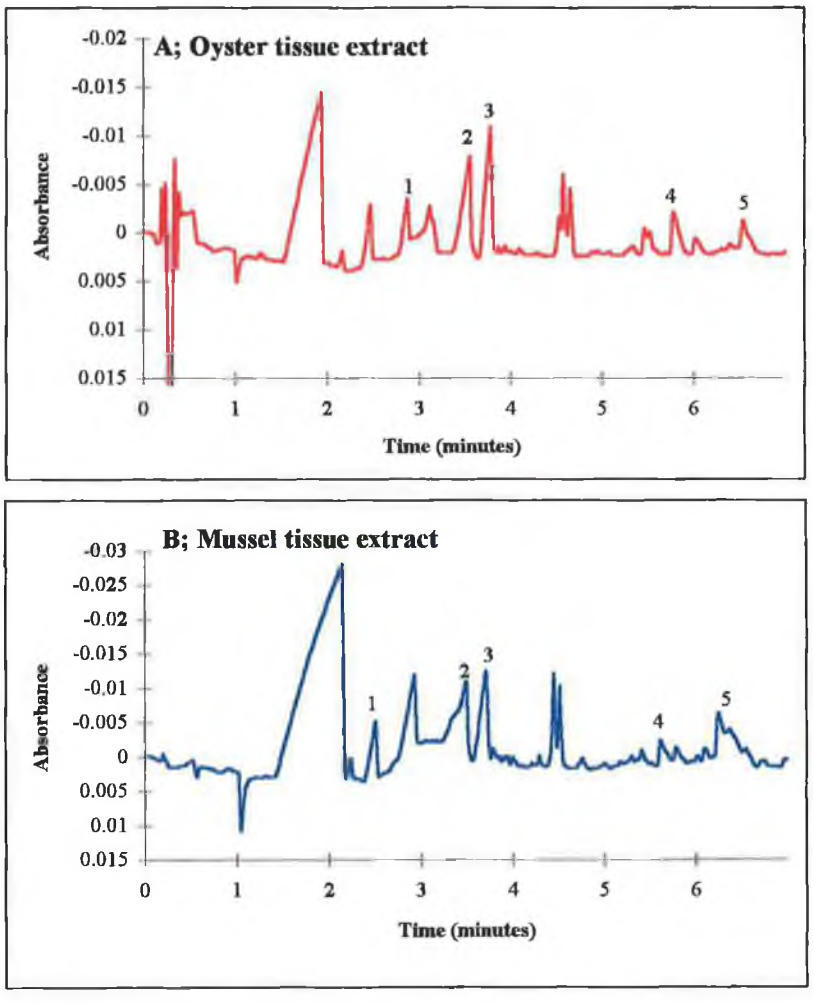


Fig 3.30; Electropherograms of A; Oyster and B; Mussel tissue extracts spiked with a 5ppm dilution of arsenic species Separations were carried out using a 15mM DETA/5.5mMPMA run buffer at pH 8.50 at 40°C with a 60 second injection time and a separation voltage of 30kV. Migration order; 1, Arsenobetaine; 2. Arsenate; 3. MMA and 4. DMA.

3.3.16. Conclusions

The modification of Burgis flow modified stacking method, for the purposes of arsenic speciation has thus met with a great deal of success. Using a 15mM DETA/5mMPMA run buffer at pH8.50, it was possible to separate arsenobetaine, arsenate, MMA and DMA in under 10 minutes in a single straightforward step. This contrasts with existing HPLC-HGAAS methods which involve at least one derivatisation step between the separation and detection modules. In addition to this, the calculated efficiencies for each arsenic species in terms of theoretical plate numbers separated by capillary electrophoresis exceed the previously reported values for a corresponding HPLC based separation by up to a factor of fifteen.

The main advantage of the flow modified stacking technique however, was the considerable enhancement in detection sensitivity obtained compared to that obtained with the borate based direct UV protocol. This is most effectively illustrated in fig 3.37 which compares the separation of a 100ppm standard mixture using the borate method with the separation of a 1ppm mixture using the DETA/PMA method. In both the electropherograms the peaks due to MMA, DMA and arsenobetaine (peaks 3, 4 and 5 respectively) are of similar area in spite of the fact that the concentration of the solution analysed by the DETA/PMA method is one hundred times more dilute than that analysed by the borate method. Furthermore, the size and symmetry of the peak due to arsenate (peak 6) is substantially improved with the DETA/PMA separation.

The calculated limits of detection for arsenobetaine, arsenate, MMA and DMA with the DETA/PMA based separation are 92ppb, 56ppb, 538ppb and 480ppb respectively. This represents a hundredfold improvement in the detection sensitivity for arsenate and arsenobetaine and a fiftyfold improvement in sensitivity for MMA and DMA over the standard capillary electrophoretic separation in the absence of stacking and indirect detection. These detection limits require further reduction if this capillary electrophoretic technique is to be used for the determination of arsenic species in environmental samples, this could be achieved by the incorporation of a chromatographic pre-concentration step prior to the electrophoretic separation.

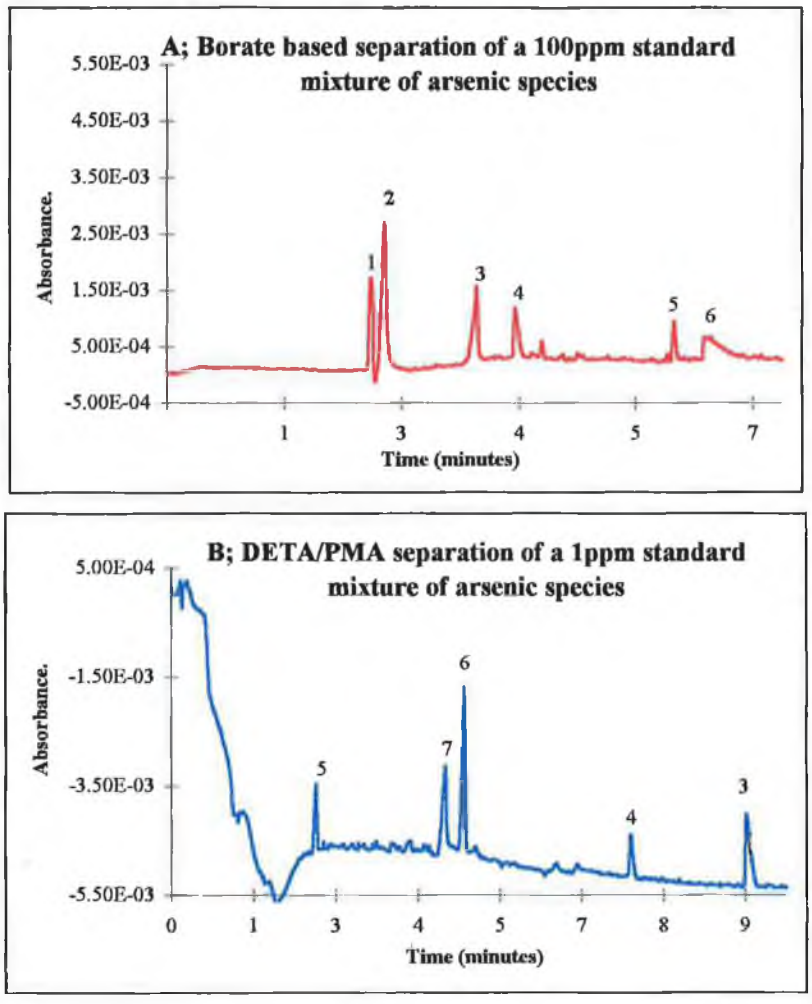


Fig 3.37; Comparison of the separation methods, in terms of detection sensitivity. 100ppm standard mixture separated in 25mM borate pH 8.50 employing a 9 second injection time and 1ppm standard mixture separated following stacking in 15mM DETA/5.5mM PMA pH 8.50 with a 45 second injection time. A separation potential of 30kV was employed in both cases. The peaks are as follows; 1. water matrix peak, 2. arsenite, 3. DMA, 4., MMA, 5., Arsenobetaine, 6. arsenate, 7 system peak.

The DETA/PMA capillary electrophoretic separation method provides a simple and relatively rapid method of separating and detecting arsenic species in the ppb range with minimal manual input and solvent consumption. When used in combination with chromatographic pre-concentration techniques, this method would thus be highly amenable for the purposes of routine arsenic speciation in environmental samples.

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Chapter Four

Separation of methylated tin compounds by Capillary Electrophoresis

4.1 Introduction

4.1.1. The origin of organometallic tin compounds in the environment.

Organometallic tin compounds are widely used in both industrial and agricultural contexts. In the 1980s it was estimated that 14% of the 1.8×10^8 kg of tin mined each year was converted into organometallic derivatives for further use with mono and di-butyltin compounds accounting for two thirds of this production.[1] These butyl tin compounds are extensively used in the polymer industry where they act as heat stabilising agents in PVC and as catalysts for the vulcanisation and cross linking processes during the manufacture of polyurethane foam and silicone rubbers.

The biocidal property of certain trialkyl and triaryl tin compounds has also led to their use in agriculture as fungicides, wood preservatives and as antiparasitc agents in poultry foodstuffs with tributyl tin compounds being used extensively as the active ingredient in marine antifoulant paints[2]. These applications have in turn given rise to the introduction of organotin compounds as pollutants into the environment. Byrd and Andrae[3] have identified tin as being the third most important environmental pollutant after lead and tellurium based on a comparison between the concentration in atmospheric particulates and its' natural abundance in the earths' crust. Leaching of trialkyltins from antifoulant paint on the hulls of ships and yachts into the marine environment are the primary source of this contamination and concentrations as high as 120ppb have been recorded in sea water and sediment samples from harbour areas in various locations[4,5].

4.1.2. Toxicity of Organometallic Tin Compounds

These organotin compounds are considerably more toxic than elemental tin, however the nature of this toxic effect varies between individual species. While both tri and di alkyl tins exert an inhibitory effect on oxidative metabolism, trialkylated forms target the enzyme system involved in the synthesis of ATP thus resulting in a decline in cellular energy. Diaklytins however inhibit the biological activity of pyruvate and α-keto-glutarate dehydrogenase and also interfere with the synthesis of RNA[6].

Methyltins have been shown to be potent neurotoxins in mammals; Bouldin et al.[7] reported the necrosis of neurons in specific areas of the hippocampus in rats after a 5mg/kg dose of trimethyltin had been administered orally. Stanton et al.[8] also observed that trimethyltin damaged the spatial working memory in rat pups. The acute toxicity of methyl tins in terms of LD_{50} values for mammals have been calculated to be 9, 40, and 1261mg/kg for tri, di and mono methyltin respectively[9]. The fate of organotin compounds with respect to environmental cycling and bio-accumulation thus needs to be rigidly monitored in order to ensure that human exposure to these highly toxic species is kept to a minimum.

4.1.3. Environmental transformations of organometallic tin compounds

Studies into the exact nature of these organotin species in the environment have identified the prevalence of methylated tin compounds in addition to the expected tri-butyl and phenyl species more commonly used in marine antifoulant paints and agrichemicals[4,5,10,11]. Subsequent research has revealed a number of successive degradative and methylating pathways that cause inter-conversion between tin species.

Degradation of organotin compounds under environmental conditions has been achieved in laboratory based experiments by photochemical reactions or biologically mediated cleavage of the tin-carbon bond. Sonderquist and Crosby[12] demonstrated that tirphenyltin hydroxide commonly used in fungicide preparations could be completely degraded in aqueous solutions upon exposure to sunlight and that the photolysis rate was substantially increased in the presence of 3% acetone. The proposed mechanism of this photolysis was homolytic cleavage of the C-Sn bond to form phenyl and hydroxydiphenyltin radicals. A subsequent reaction between this hydroxydiphenyltin radical and dissolved oxygen ultimately yielding diphenyltin oxide that would be degraded further to inorganic tin(IV)oxide. This degradation was expected to be accompanied by the gradually formation of benzene and biphenyl compounds. Analysis of aqueous solutions, after photo-degradation revealed the presence of low levels of diphenyltin, tin(IV)oxide, phenyltin, benzene and biphenyl. These findings were consistent with this theory of homolysis. There was a discrepancy

however in the total quantity of tin compounds recovered after photolysis and the initial concentration of triphenyltin before irradiation. This apparent loss in extractable tin species was accounted for by the formation of a phenytin polymer.

Maguire et al.[13] have subsequently demonstrated the photolytic decomposition of bis(tri-n-butyltin oxide) when placed in sunlight with a half life of 89 days. Furthermore, the reaction was promoted by the presence of fulvic acids commonly found in environmental water samples. It was suggested that a free radical mechanism was also the driving force behind this degradation, with dibutyltin, monobutyltin and inorganic tin accounting for 75% of the total loss of the originally tributyltin compound.

A number of biodegradative pathways also exist for the successive dealkylation of organotin compounds. Maguire at al.[14] have reported the debutylation of tri butyltin to mono and di butyl analogues by the green alga *Ankistrodesmus falatus* with a half life of 4 weeks. It should be noted however that experimental concentrations of the organism were up to one hundred times higher than that found in the natural environment. A related study demonstrated the capacity of oligochaetes for the uptake and debutylation of tributyltin species following their adsorption onto sediment material[15]. This degradative process was slow with a half life of several months being reported.

Microbial activity can also account for the formation of the majority of methyltin species that exist in the marine environment. Biological methylation of inorganic tin compounds was initially demonstrated by Huey et al.[16] who used a tin resistant strain of pseudomonas bacteria to produce dimethyltin upon incubation with tin chloride. The biotransformation of tin species has since been demonstrated using mixed inoculums of bacteria isolated from a number of sediment samples[17,18]. Guard and Cobet[17] reported the conversion of trimethyltin hydroxide to volatile tetramethyltin by estuarine sediments. The extent of $(CH_3)_4Sn$ formation was low however with a maximum of 2.4% conversion being achieved after an 80 day incubation period. In a further series of experiments, sediments taken from Chesapeake Bay transformed inorganic tin salts to di and trimethylated forms[18]. Monomethyl tin was also detected

in minute quantities but not in every case. Sterile controls and inoculums poisoned with sodium azide did not produce any methylated compounds.

Methylation of tin has also been shown to occur by an abiotic process. Methylcobalamin, a coenzyme of vitamin B_{12}, has been identified as one of the principal naturally occurring methylating agents and is capable of interacting with a number of metal ions under aerobic conditions to give alkylated derivatives[19]. In the case of tin, methylation occurs only for Sn(II) species in the presence of an oxidising agent such as iron(III). The reaction proceeds by a free radical attack on the Co-C bond by an intermediate Sn(III) species which results in the release of a CH_3 radical followed by the formation of a Sn-CH_3 bond[20]. The rate of reaction was enhanced in the presence of a high chloride ion concentration.

Methylation of inorganic tin (II) is also possible in the presence of methyl iodide[21,22]. This compound is secreted by macroalgae and is found in high concentrations in sea-water in area rich in algal populations. The reaction is brought about by the attack of the carbocation species (CH_3^+) on the high electron density region of the Sn(II) atom and its' subsequent oxidation to Sn(IV). Ring and Weber[23] reported that this reaction was inhibited in the presence of fulvic acid under simulated estuary conditions and that the reaction was more effective in an anaerobic environment.

Transmethylation reactions between a number organometallic and inorganic species can also contribute to the overall environmental cycling of tin compounds. Huey et al.[16] demonstrated the formation of methyl mercury when an inorganic mercury (II) salt was added to a solution of methyltin compounds incubated with sediment. A similar phenomenon was observed by Chau et al.[24] for the transmethylation of inorganic tin by trimethylated and dimethylated lead compounds. The reaction was significantly enhanced in the presence of sediment, and although methylation of both inorganic tin species was possible, the extent was greater for the divalent state.

It is therefore evident that the environmental cycling of tin is mediated by a series of biological and abiological processes. Studies into the nature of tin species in environmental samples have revealed the prevalence of various methyl tin compounds.

Although anthropologic inputs may account to a certain extent for the presence of these compounds, the demonstration of various demethylation and methylation reactions under simulated environmental conditions provides sufficient proof that these process occur naturally and have a governing influence on the cycling of tin species in the environment.

4.1.4. Analytical techniques for the determination of environmental tin species

The development of reliable analytical methodology is central to improved understanding of the exact fate of organometallic tin compounds in the environment and necessary for the accurate determination of the potential exposure to humans.

In the majority of studies, analytical speciation of tin has been characterised by a three step process in which the compounds are first converted to more volatile derivatives after which they are separated on a gas chromatographic stationary phase followed by element specific detection using atomic absorption, emission or flame photometric techniques.

4.1.5. Analytical methods based on the generation of tin hydride species

Reduction of acidified samples with sodium borohydride has proved to be one of the more popular methods of derivatisation. This approach was pioneered by Hodge et al.[25] who employed a hydride generation selective volatilisation technique with detection by atomic absorption spectrometry for the separation and determination of inorganic Sn(IV), mono, di and tri methyltin, di and tri ethyltin, mono, di and tri n-butyltin and phenyltin. The tin hydrides were carried from the reaction vessel by the passage of helium and collected in a liquid nitrogen cooled U-tube packed with glass wool. This U tube was then heated gradually to allow the evolution of trapped hydrides in order of increasing boiling point. The construction of the apparatus is discussed in greater detail in section 1.2 of chapter one. This method was successfully applied to the determination of tin species in water samples from San Diego Bay and

Lake Michigan at the ppt. level. This method was later adapted by Seligman et al.[26] for the purposes of measuring the degree of rate of photolysis of tributyltin and the formation of its mono and tributyl analogues.

A similar system employed by Braman and Tompkins[27] has also been discussed in section 1.2 of chapter one. In this instance a gas chromatographic stationary phase, OV-3 on Chromasorb W, replaced the glass wool in the U-Tube trap and a flame emission detector measured the eluting tin hydride species. Interferences from other metal cations and organic solvents were found to be negligible with detection limits ranging from 0.65pg Sn for dimethyltin to 1.6pg Sn for methyltin. The high sensitivity on the method coupled to the absence of interference made it possible to determine the concentrations of inorganic and methylated tin compounds in fresh, saline and estuarine water samples taken from areas near Tampa Bay Florida and in human urine. The highest methyl tin concentration was measured in the sea-water sample representing 60% of the total content. This was one of the first reports confirming the presence of methyltins in the environment and therefore had important implications for further research into the processes involved in natural tin cycling. The method was subsequently utilised by Hallas et al.[18] for their study into the production of methyltins from inorganic forms through the actions of estuarine micro-organisms.

The technique was further developed by Donard et al. to include an electrothermally heated quartz furnace as a means of atomising the tin hydrides[28]. The furnace was designed to include a single inlet located in the middle and a gas premixing chamber. It was enclosed in a 1mm layer of asbestos, coiled with a double strand of nichrome wire (7 Ω resistance) insulated by asbestos cord and mounted on a custom built stainless steel frame on the burner head of an AAS instrument. The interface between the hydride trap and furnace inlet was provided by 2 PTFE transfer tubes, one placed inside the other which were also wrapped in nichrome wire and insulated with asbestos tubing. The temperature of these transfer lines was maintained at 95°C. Organotin hydrides were trapped on a Chromasorb G AW_DMCS (45-60 mesh) stationary support coated with 3% SP-2100 with the trap being again wrapped in nichrome wire. Following hydride generation and collection this trap was heated at a rate of

4.2°C/min. for 3 minutes followed by 7.2°C/min. until a maximum temperature of 200°C had been reached. Helium gas flowing at a rate of 400ml./min. was used both for stripping of the hydrides from solution and their separation by GC. These conditions permitted the collection and separation of methyl and butyltin hydride species within 8 minutes. The method was linear in the range 0.1-30ng Sn for all methyl and butyl species, the sensitivity was greater for methyl tin species however with limits of detection as low as 30pg Sn being achieved in all cases. The sensitivity for butyl tins was slightly lower, ranging from 50-200pg Sn with the tendency to decrease with increasing numbers of butyl groups. This was due to the fact that atomisation of the hydride species was impeded in the presence of larger alkyl groups. In spite of this, the use of electrothermal atomisation did significantly improve the sensitivity of the tin speciation technique and detection limits obtained were substantially lower than those reported in previous studies.

Valkirs and co.-workers[29] compared hydride generation followed by cryogenic trapping - selective volatilisation and detection by atomic absorption spectroscopy with a procedure in which hydrides were separated by gas chromatography with flame photometric detection for the determination of di and tri butyl compounds. The cryogenic trap method was adapted from Braman and Tompkins[27]. For the GC method, hydrides were first extracted into dichloromethane and evaporated to 200µl before separation on a 1.83m glass column packed with 1.5% OV-101 on Chromasorb G HP (100-200 mesh). A thermal gradient was employed in which the column, after an initial holding period of 2 min, was heated from 23°C - 170°C at a rate of 32°C/min. An excellent correlation between the two methods was reported with close agreements between the slopes obtained from the respective calibration plots. In the majority of cases, values obtained from the analysis of standard reference materials by both methods were within 20% of their concentration means. The strong similarities in the results obtained from two different detection and separation systems were a demonstration of the precision and reliability of the hydride generation method for the determination of tin species.

The majority of analytical speciation studies of tin involving hydride generation deal with water samples, however Tsuda et al. applied the technique to the determination of tin species in sediment and fish tissue samples[30]. The generation and collection of hydrides proceeded as follows. Firstly, samples were treated with conc. HCl and extracted into ethyl acetate, the organic fraction was then evaporated to dryness, reconstituted in ethanol and reacted for 10 minutes with 2ml of 2.5% sodium borohydride. To this reaction mixture, 15ml. of water and 5g of sodium chloride were added and the organotin hydride compounds were subsequently extracted into hexane. Sample clean up was achieved by passing the hexane extract down a silica gel packed column followed by elution with 5ml. aliquots of hexane. Analysis was carried out by gas chromatography with an electron capture detection system.

This extraction proved to be highly efficient with reported recoveries of 86.2-99.8% for butyl tin species and 75-88% for phenyltin species from fish tissue samples and 70.3-96.6% and 71.7-85.7% respectively for butyltin and phenyltin species from sediment. Spiking levels of 1μg and 10μg per 10g fish tissue and 1μg and 10μg per 20g sediment were employed for all recovery trials. Detection limits ranged from 0.01-0.05ng Sn which were comparable with those obtained with atomic absorption detection systems .

4.1.6. Grignard derivatisation techniques for analytical tin speciation schemes.

The volatility of tin species can also be increased if converted to tetraalkylderivatives by the action of a grignard reagent. This reaction is carried out by extraction of acidified tin compounds into a solvent such as benzene or toluene containing 0.1-0.5% of tropolone which acts as a metal co-ordinating ligand. Alkylation then proceeds with the addition of grignard reagent, and the derivatives can then be separated by gas chromatography coupled to a tin specific detector. The addition of butyl[5], ethyl[31] and pentyl[4] groups have all been investigated with pentylation proving the most popular as it provides a means of distinguishing alkyltins of environmental origin from those generated as part of the analytical process[32].

A singular drawback of this derivatisation procedure is the difficulty of extracting methyltins due to their relatively high polarity compared to corresponding butyl compounds. Chau and co.-workers addressed this problem and investigated the effects of hydrochloric, hydrobromic and acetic acids and sodium chloride salt on the efficiency of a 0.1% tropalone in benzene extraction of methyl tins[5]. Extracts were subsequently analysed as butylated derivatives by gas chromatography-atomic absorption spectrometry. Although a small improvement in recovery of mono and di methyl tin was observed in the presence of acetic acid, it did not exhibit a beneficial effect on the extraction of trimethyltin. An acceptable consistent recovery of the trimethylated species could only be achieved in the presence of a saturated solution of sodium chloride. The detection limit of the method was defined by the volume of sample used for extraction. The derivatised tin species had higher boiling points than the benzene solvent and thus could be concentrated by evaporation. A limit of detection of 0.04 ppb was reported when a five litre water sample was used for the extraction.

Reader and Pelletier[33] have reported the use of hexylmagnesium bromide for the grignard derivatisation of butyltin species. Analysis was carried out by gas chromatography with detection by ion trap-mass spectrometry. This provided structural information on the derivatised compounds in addition to highly sensitive detection. The mass spectra confirmed the formation of Bu_3SnHex, Bu_2SnHex_2 and $BuSnHex_3$ respectively. A limit of detection of 5ppt was reported assuming 100% extraction efficiency for organotins.

The multiple steps involved in the grignard derivatisation approach however may lead to considerable losses and furthermore substantially increase the total analysis time required. In addition to this, side reactions with matrix constituents may introduce interferences which may not be resolved from the tin species by gas chromatography. These limitations would discourage the use of this technique for the purposes of routine tin speciation by gas chromatography, in spite of the high extraction efficiency and low limits of detection which have been reported.

4.1.7. Liquid Chromatographic approaches to tin speciation.

While HPLC coupled to AAS is recognised as one of the most versatile and efficient methods of trace metal speciation, the technique has not been extensively researched for the separation of tin species, gas chromatography tending to be the preferred analytical technique in the majority of tin speciation studies. The technique however, offers the advantages of the absence of a derivatisation requirement and a diverse array of mobile and stationary phases to suit the particular separation.

The coupling of liquid chromatography with graphite furnace atomic absorption spectroscopy described by Brinkman et al.[34], was the earliest report of the separation of organometallic tin compounds by HPLC. The construction and operation of the system have been discussed in section 1.7 of chapter one. Separation of triphenyl, tributyl and tripropyl tin species was achieved on a C2 reverse bonded phase column with isocratic elution with 100% methanol. Complete elution of the species requires twenty two minutes. The limit of detection was reported to be 111ng Sn.

The slightly polar nature of the majority of organotin compounds permits their separation on strong cation exchange columns. Ebdon and co.- workers[35] employed a Partisil silica based cation exchange column to facilitate the separation of tributyltin from inorganic Sn(II) and Sn(IV). Resolution of the three species was achieved with an 80/20 V/V methanol/0.1M ammonium acetate mobile phase. Column eluant was transferred to the flame atomic absorption detector via a discrete volume nebuliser, the construction of which has been discussed in section 1.6 of chapter one. This system was successfully applied to the determination of tributyltin in sea water samples following pre-concentration by extraction into chloroform.

A more advanced pre-concentration scheme was investigated by Ebdon and Garcia Alonso[36]. In this case solvent extraction was replaced by on-line chromatographic pre-concentration on a non-polar ODS packed column. Complete elution of tributyltin from these columns was achieved with an 80% methanolic mobile phase followed by separation on a Partisil SCX column as described previously[35]. In this instance, tributyl tin was detected flourimetrically following post column derivatisation with 0.0025%

Morin reagent (2,3,4,5,7-pentahydroxyflavanone). The high sensitivity of this detection system gave rise to detection limits as low as 16ng Sn for tributyltin compounds. This made possible the direct determination of tributyltin in estuarine water samples.

Pobozy and co.-workers have compared the cation exchange ion chromatography with capillary electrophoresis for the determination of trimethyl, triethyl, tributyl and triphenyltin compounds[37]. In both cases an indirect detection scheme using benzyl-trimethylammonium chloride (BTMA) as the probe, was employed for the determination of trimethyl-, triethyl- and tributyl-tin with triphenyl tin determination by direct UV absorbance at 262nm. The HPLC separation was carried out on a Whatman Partisil SCX-10 strong cation exchange column. Tin species were eluted isocratically with a 70 : 30 methanol : 10mM acetate mobile phase at pH 5.9 and containing the BTMA indirect detection agent at a concentration of 2mM. With this system, the peaks due to tributyl and triphenyl tin were not baseline resolved however quantitation was possible when peak height measurements were used instead of peak area and detection limits ranged from 150ppb for triphenyl tin to 2.5ppm for tri-methyl tin.

A more satisfactory separation of the four organotin compounds was achieved by capillary electrophoresis with an electrolyte composition of 20mM tartaric acid-20% methanol and 4mM BTMA. Separations were carried out in fused silica capillaries 60cm in length with the detector window situated 35cm from the point of injection with an applied voltage of 20kV. The peaks due to tributyl and triphenyl tin compounds were completely resolved from one another and in all cases an increase in peak sharpness was observed. Improved limits of detection were obtained for all tin species with the capillary electrophoresis approach. In this case detection limits ranged from 9ppb for triphenyl tin to 290 ppb for tributyl tin which accounts for a 16 fold improvement in HPLC methods.

4.1.8. Conclusions

The widespread use of organotin compounds in both industry and agriculture coupled with growing concern over their fate in the environment and potential hazard to the general population has prompted the development of a number of highly sensitive analytical speciation schemes for the purposes of their regulation. These techniques serve a dual purpose of monitoring the levels of individual tin species in the environment while serving to increase understanding of the various biological and abiological processes which control the overall natural cycling of the element. Gas chromatography preceded by derivatisation with borohydride and coupled to atomic absorption spectroscopy has proved particularly successful in this regard and is capable of measuring individual tin species at concentrations in the sub ppb range. The liquid chromatographic approach has met with varying degrees of success; while the requirement of complicated derivatisation has been eliminated, difficulties in resolving certain organotin species limits the application of the technique. The potential of capillary electrophoresis for use for tin speciation has recently been explored. This technique is capable of overcoming the problems of resolution incurred by analogous HPLC approached and when an indirect detection scheme is employed limits of detection on the ppb range have been recorded.

4.2; Experimental

4.2.1. Introduction

The superior resolving power of capillary electrophoresis coupled with the high sensitivity of indirect detection has been demonstrated as a reliable and efficient method for the determination of trimethyl-,triethyl-,tributyl-and triphenyl tin species with minimum sample preparation requirements[37]. In spite of these advantages, the use of capillary electrophoresis for this purpose has received little attention. Further expansion of the technique is required to include other environmental tin compounds before it can be established as an alternative to the existing chromatography - atomic absorption spectroscopy methods for the purposes of environmental tin speciation.

In the course of this study, the application of capillary electrophoresis to the separation of methyl tin species was evaluated. The highly polar nature of these compounds has often proved to be a hindrance to their analysis due to the difficulty in quantitatively extracting them for grignard derivatisation[5] or their strong interaction with liquid chromatographic stationary phases[37]. This property however makes them amenable to electrophoretic separations as ionic charge is one of the governing factors for migration in an electric field. The CE method was optimised to achieve maximum separation efficiency and resolution. The precision of the optimised method could then be assessed by the standard validation procedures. Finally, the separation method was applied to the measurement of methyltin mixtures sampled from a transmethylation reaction between tetramethyltin and mercury chloride irradiated by UV light.

4.2.2. Apparatus.

Separations were carried out a Beckman P/ACE system as described in section 3.10 of chapter three. In this case however fused silica capillaries 50μm x 37cm (total length) were employed with the detection window burned 30cm from the injector end. Conditioning of the column between each set of runs was carried out as described in chapter three.

4.2.3. Reagents

The following methyl tin halides were investigated; monomethyl tin (CH_3SnCl_3); dimethyltin ($(CH_3)_2SnCl_2$) and trimethyltin ($(CH_3)_3SnCl$); all of which were obtained from Aldrich. The phosphate, acetate and borate buffer systems were prepared from sodium-dihydrogen phosphate (Merck) adjusted to the required pH with disodium-hydrogen phosphate (Reidel de Haan), ammonium acetate (BDH-Chem. Co.) adjusted with acetic acid (Reidel de Haan) and disodium tetraborate (BDH-Chem.Co.) adjusted with boric acid (Merck). Additional pH adjustments were made with hydrochloric (HCl) and phosphoric (H_3PO_4) acids both purchased from Reidel de Haan. Diethylenetriamine(DETA), 1,3-diaminopropane (DAP) (both from Aldrich) and cetyltrimethylammonium bromide (CTAB) (Sigma Chem. Co.) were all investigated as buffer additives. The complexing ligands examined were 8-hydoxyquinoline-5-sulfonic acid from Sigma Chem. Co., ethylenediaminetetraacetic acid and pyridylazoresorcinol, both obtained from Aldrich.

Sample reaction mixtures were provided by Deirdre Brennan, School of Chemical Sciences, Dublin City University.

As for previous CE work, all solutions were prepared in distilled-deionised water and were filtered and degassed before use.

4.2.4. The development of a separation scheme for methyltins by capillary electrophoreses.

The capillary electrophoretic separation was developed for mono-, di- and tri-methyl tin chlorides, tetramethyl tin was not considered due to its insolubility in aqueous solutions and the incompatibility of the CE system to high levels of organic solvents. At the outset of this research the methyl tin compounds were assumed to behave as cations in aqueous solution and their separation was investigated accordingly.

Initially, the ability of each of the organotin compounds to form complexes with various chelating ligands was examined. Complexation is a commonly used approach

for the separation of metal cations by capillary electrophoresis and has been discussed in detail in section 3.6 of chapter three. Experiments were carried out with 8-hydroxyquinoline (HQS^-), pyridylazoresorcinol (PAR), and ethylenediaminetetraacetic acid (EDTA). These were added to solutions of each methyltin compound to give a concentration ratio of 1:1. An organotin concentration of 2.5ppm was used for all experiments which corresponded to an added ligand concentration of $1x10^{-5}M$, $1.17x10^{-5}M$ and $1.29x10^{-5}M$ for mono-,di- and trimethyl-tin solutions respectively. In order to investigate the effect of pH on complexation, solutions were prepared in distilled deionised water (pH 7.0), acetate acid buffer (pH4.5) and borate buffer (pH 8.5) and were left to stand for 30 minutes before analysis. The formation of a complex was determined spectrophotometrically by the generation of a UV-Vis spectrum of each methyltin-ligand mixture in the region 190-400nm and comparison with the spectra of the individual compounds in the different buffer media.

Alternative techniques of separating cationic substances by capillary electrophoresis were also investigated for their suitability as methods for the separation of methyl tins. For the purposes of these experiments, a standard mixture containing each compound at a concentration of 100ppm was used, 100ppm solutions of individual compounds were also prepared for peak identification. In addition to this, solutions of 1% acetone in run buffer were employed as electroosmotic flow markers. Injection was carried out hydrodynamically for a duration of 5 seconds and a potential of 20kV was applied across the capillary. Migrating compounds were monitored by direct UV detection at 190nm.

The use of support electrolytes below a pH of 2, has been reported to suppress the ionisation of the surface silanol groups thereby preventing their interaction with strongly cationic analytes[38]. The electrophoretic behaviour of the methyltin compounds was therefore examined using a 20mM phosphate buffer in the pH range 2.0-4.0 as the run buffer.

Suppression of wall effects can also be achieved through the incorporation of an amine additive into the buffer system. The ability of diethylenetriamine (DETA) to suppress the electroosmotic flow has been demonstrated in chapter three. In this instance,

DETA based electrolytes at concentrations; 1mM, 2.5mM and 10.0mM in the pH range 2.75-7.00 (adjusted with HCl or H_3PO_4) were evaluated in terms of the effect on the bulk electroosmotic flow and the apparent mobility of the methyltin compounds. The use of 1,3-diaminopropane was similarly investigated. This additive has proved highly effective in minimising wall adsorption effects for the separation of positively charged protein molecules at low pH levels [39]. Solutions of 3, 6 and 15mM, 1,3-diaminopropane were chosen for this study, these were adjusted the required pH with H_3PO_4.

The interaction between cationic analytes and the capillary wall can be completely eliminated if the charge on the capillary surface is reversed. As outlined in section 3.5 of chapter three, this can be accomplished through the incorporation of a cationic surfactant in the support electrolyte. Reversal of the wall charge is accompanied by a reversal in the direction of the electroosmotic flow and hence the instrument must be operated in reverse polarity mode to allow for detection of migrating ions. In this case, cetyltrimethylammonium bromide (CTAB) was chosen as the flow modifying agent, this was added to a 20mM phosphate buffer to give an overall concentration of 0.1mM. The pH of the electrolyte was then varied from 7.00-8.00. As the magnitude of the electroosmotic flow tends to exceed that of electrophoretic migration, detection of cations is possible with this system even though their inherent migration is in the opposite direction.

When a suitable buffer system had been chosen, the separation of methyl tins was further optimised in terms of pH, ionic strength of buffer constituents, voltage and injection time by the criteria outlined in chapter three. The effect of the presence of methanol and acetonitrile solvents in the run buffer was also examined.

4.2.5. Validation of separation method.

The reproducibility and linearity of the optimised method were assessed as before by intra and inter variability assays[40]. In this case, calibration curves were prepared in the concentration range 20-50ppm with each standard mixture being injected six times. A

50ppm standard mixture was used for all between day reproducibility experiments. In order to accurately evaluate the between day reproducibility, the same standard was used for analysis of each day of the trials.

4.3; Results and Discussion

4.3.1. Complexation experiments

The use of complexing agents to aid separation and improve detection of methyltins proved to be unsuitable due to the inability of the ligands examined to interact with all three compounds. In the case of EDTA, the absorption maximum was in the region of 190-200nm and therefore coincided with that of each of the methyltins. Additional absorbance peaks were not observed when mixtures of EDTA and methyltins were examined and it was impossible to ascertain whether the observed absorbance increase observed at 190nm was due to a metal-ligand complex or merely the cumulative effects of the individual components of the mixture.

For mixtures containing methyltin compounds and 8-hydroxyquinoline, complex formation could be observed for mono- and dimethyltins at pH 7.0 and pH 4.5. In solutions of monomethyltin, the presence of a complex was indicted by the appearance of a shoulder at 255nm on the side of the absorbance peak of 8 hydroxyquinoline in three replicate solutions. This 255nm absorbance was considerably stronger in the case of dimethyltin compounds appearing as an individual peak in the corresponding spectra. Spectra generated from mixtures containing trimethyltin however exhibited no significant differences from those obtained from 8 hydroxyquinoline solutions at any of the pH levels examined.

Similar results were observed when pyridylazoresorcinol was used as the complexing agent. In this case the formation of a metal-ligand complex was manifested by a colour change from yellow to pink. Solutions were also examined spectrophotometrically; a shift in absorbance maxima to a higher wavelength was observed only for those solutions in which a colour change had occurred. Complexation of monomethyltin by PAR occurred only at pH 4.5, whereas the formation of dimethyltin -PAR complexes were observed at pH 4.5 and 7.0. As in previous experiments, solutions of trimethyltin were unaffected by the presence of the PAR reagent this was possibly due to the fact that the three methyl groups around the central tin atom sterically hindered any

interaction with a ligand. In all cases, there was no interaction between the methyltin compounds and complexing ligand at a pH of 8.5

As complexation occurred for only two of the methyltin compounds under restricted conditions, capillary electrophoretic separation of the complexes was not examined further.

4.3.2. Initial experiments on capillary electrophoresis of methyltins; choice of buffer system.

As outlined in section 4.12, at this stage of research the methylated tin compounds were assumed to be cationic in nature and consequently the development of a CE separation method was approached from this perspective.

The use of low pH buffers as support electrolytes proved ineffectual for the separation of the three methyltin compounds. The electroosmotic flow was so slow with phosphate run buffers of pH 2.5 and below, that the peak due to the acetone neutral marker did not appear within 20 minutes of injection. Subsequent injections of a 100ppm standard mixture of methyltins yielded a similar result and no peaks were observed above the noise level within 30 minutes of injection. Raising the pH to 4.0 using an acetate based buffer led to a marked increase in the electroosmotic flow as indicated by the appearance of the acetone peak at 2.37 minutes. Injection of the 100ppm standard mixture under these conditions however yielded a single sharp peak at 4.41 minutes. A peak at this migration time was also observed following injection of standard solutions of each of the methyl tin compounds under investigation.

The lack of success of these simple low pH buffer systems for the separation of methylated tins prompted investigation into the use of amine buffer additives such as diethylenetriamine (DETA) and diaminopropane (DAP). These function by masking the negative charge on the capillary inner surface thus minimising the retentive effect caused by interaction between the methyltin compounds and the capillary wall. In the previous chapter, DETA has been shown to be capable of completely suppressing the

electroosmotic flow at concentrations as low as 2.5mM when used in combination with pyromellatic acid. In this case, the use of hydrochloric or phosphoric acid to adjust the pH of the DETA solutions served merely to reduce the electroosmotic flow rather than eliminate it completely. Run buffers composed of 1mM solutions of DETA adjusted to pH 2.5, 4.5 and 6.5 with 0.1M HCl were initially examined. As expected, an increase in the electroosmotic flow was observed with increasing pH. In each case however, all three methylated tin compounds remained unresolved, migrating with equal apparent mobilities which were slower than that of the electroosmotic flow. Similar observations were made when the DETA concentration was increased to 2.5 and 10mM.

A possible explanation for this was that the relatively high levels of chloride ions in the run buffer from the addition of HCl would suppress the dissociation of the methyltin chloride compounds thus causing them to migrate with equivalent electrophoretic velocities. The use of phosphoric acid for pH adjustments did not result in an improvement in resolution with injection of methyltin standard mixtures still yielding a single peak and therefore this theory of ionisation suppression was disproved.

Diaminopropane buffers proved similarly incapable of separating the three methyltin compounds. When a 3mM solution adjusted to pH 8.0 with phosphoric acid was employed as the run buffer, the peak due to the acetone marker appeared at 6.69 minutes. Subsequent injection of the 100ppm standard mixture resulted in a single broad peak which had its' maximum at 8.28 minutes. Increasing the diaminopropane concentration to 6mM at pH 8.0 afforded the separation of Me_3Sn and Me_2Sn which appeared at 9.10 and 9.94 minutes respectively, MeSn however, did not migrate under these conditions. Variation of the pH of this run buffer in the range 5.0-9.0 simply influenced the migration and resolution of the tri and di methylated species with no peaks being obtained from the injection of monomethyltin standards in all cases. Analogous results were obtained when the diaminopropane concentration was further increased to 15mM with the exception that in this case the migration times of Me_3Sn and Me_2Sn were increased to 12.46 and 13.0 minutes respectively at pH 8.0 due to the inverse relationship between viscosity and electrophoretic mobility.

It was thus concluded that the use of these amine modifiers was ineffective in eliminating electrostatic attraction between the methylated tin species and the charged silica surface which was assumed to be the chief factor in impeding electrophoretic migration. Therefore, the complete reversal of surface charge through the incorporation of a cationic surfactant into the run buffer was explored. Cetyltrimethylammonium bromide (CTAB) was chosen as the surfactant species for the purposes of this investigation. It has been reported that this compound is capable of inducing surface charge reversal with a resultant reversal in electroosmotic flow at concentrations as low as 0.05mM[41]. This ultimately proved to be the most successful approach to the capillary electrophoretic separation of methylated tin species. Electrophoresis was initially carried out in the presence of 20mM phosphate buffer pH 7.00 containing 0.05mM CTAB under conditions of reversed polarity. With this system, injection of the acetone flow marker yielded a peak at 3.59 minutes and electrophoresis of a methyltin standard mixture produced 3 peaks at 0.99, 2.29 and 2.73 minutes respectively. When standards of mono, di and tri methyltin species were injected separately, each electropherogram consisted of 2 peaks. The initial peak at 0.99 minutes was present in all cases, in addition to this, injection of monomethyltin produced a second peak at 2.31 minutes with a peak at 2.75 minutes resulting from the injection of both Me_2Sn and Me_3Sn. The area of this later peak was much greater following the injection of Me_3Sn than for corresponding Me_2Sn injections.

Injection of very dilute solutions of hydrochloric acid and sodium chloride onto the column under these conditions served to identify the early migrating species at 0.99 minutes as chloride ions. A subsequent increase in the pH of the phosphate buffer to 7.25 gave rise to a partial separation of Me_2Sn and Me_3Sn. The feasibility of this CTAB/phosphate system for the separation of the three methylated tin species was thus demonstrated and all further investigations focused on optimising the conditions to achieve maximum efficiency and resolution.

The behaviour of each of the tin species under these conditions suggested that the original assumption that they acted as cations in aqueous solution was incorrect. The migration order of MeSn, Me_2Sn and Me_3Sn respectively was opposite to that expected under conditions of negative polarity and electroosmotic flow reversal.

Furthermore, the migration of all species towards the positive electrode ahead of the electroosmotic flow was indicative of compounds which were anionic in character. Organotin halides are known to behave as lewis acids in solution[42]. It is therefore possible that interaction between the methylated tin and water molecules lead to the formation of hydroxide compounds which subsequently lose an acidic hydrogen to yield an anionic oxide species. Confirmation of this theory was not possible in the course of this project.

4.3.3. Optimisation of Buffer pH

The governing factor for these pH optimisation experiments was the resolution between the di- and tri- methyltin species. Data concerning the acid dissociation constants for each of the methylated tin compounds was not available and therefore the influence of pH on their electrophoretic behaviour could not be predicted as had been possible with the arsenic species in chapter three. The run buffer pH was examined in the range 7.00 to 8.75. Phosphate buffer systems at a concentration of 20mM were used for all analyses, borate based systems examined over the a similar pH range at the same concentration were ineffective in separating the methylated tins. The concentration of the CTAB flow modifier was held at 0.05mM throughout the experiment.

The variation in migration times of each of the methylated tin compounds with increasing pH is illustrated in fig 4.1. The migration of methyltin decreases very marginally in a linear fashion as the pH is increased. This is an indication that the actual mobility of the ion is invariant over this pH range and the gradual drop in migration time is a consequence of the slight increase in electroosmotic flow which was also observed with increasing pH.

The migration of di and trimethylated compounds initially follow the opposite trend and steadily increased as the pH was raised from 7.00 to 7.75. As can be seen from table 4.1, the resolution between these compounds is poor within this pH range and co.-elution occurs at the lower end of the scale. These observations would suggest

that ionisation of the solvated di and tri methylated tin compounds is incomplete below pH 7.75 and the difference in the ionic charges of the respective species is insufficient to allow them to migrate independently of each other. This pattern of increasing migration time is the opposite of what was expected and its cause is poorly understood. It is possible that the partially charged di- and tri-methylated compounds may interact with the positively charged surfactant bilayer causing a retardation in their migration, though this was not investigated further in the course of this experiment. The degree of separation improves as the pH approaches 8.00, and the migration times of the two ions remain relatively invariant in the range 7.75 to 8.50, this is followed by a drop in both migration times at pH 8.75 possibly due to an increase in the degree of ionisation of di-methyltin or both species at this pH. In accordance with the Kohlraush regulating function[43], the migration of trimethyltin is strongly influenced by the behaviour of the preceding dimethyltin zone with the respective mobilities following identical trends with increasing pH.

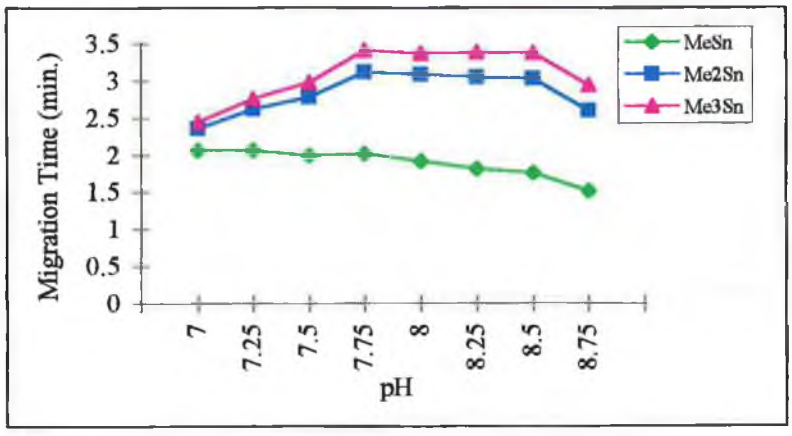
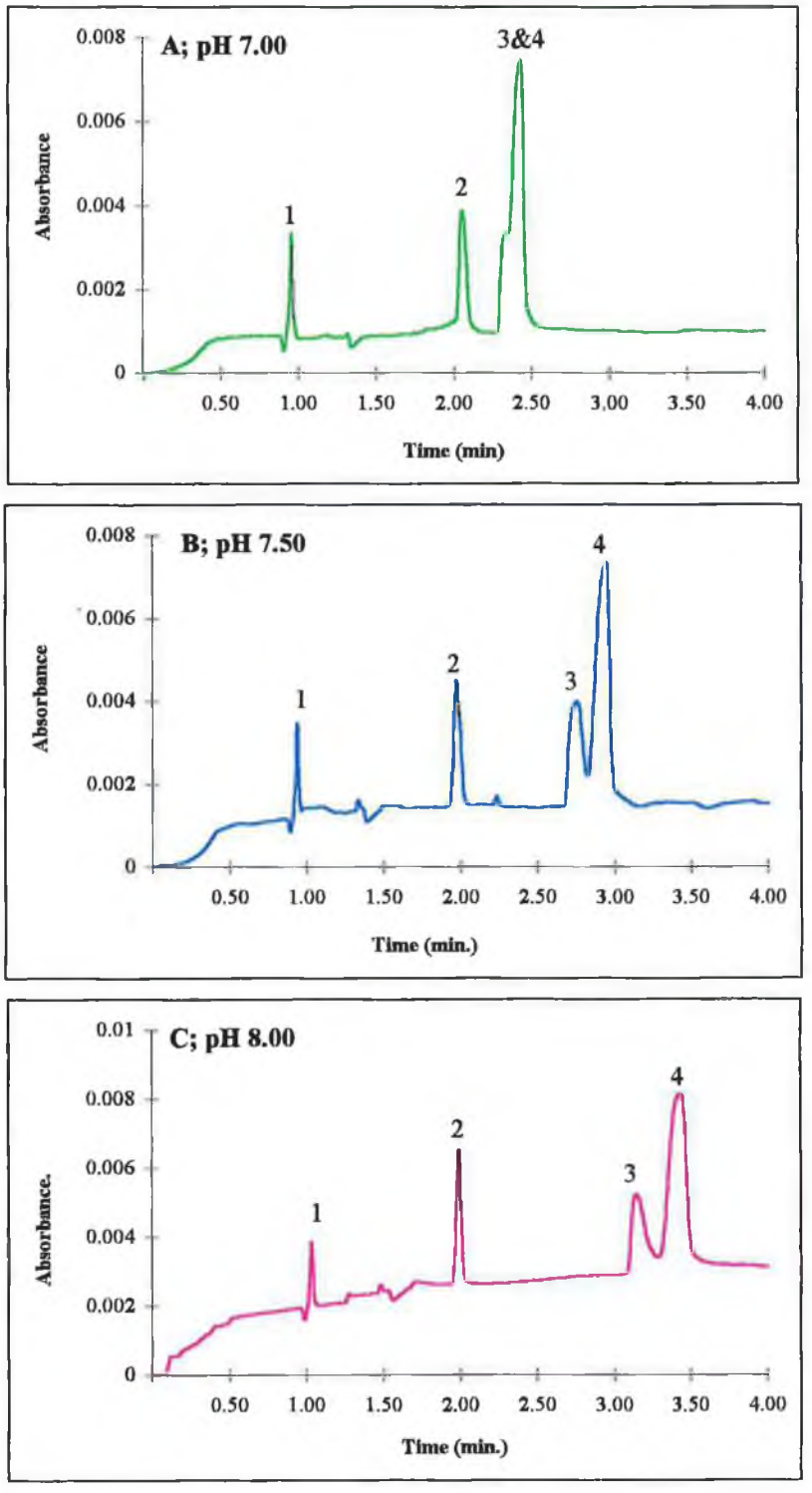


Fig 4.1; Effect of pH of run buffer on migration times of methyl-, dimethyl- and trimethyltin compounds.

Table 4.1; Resolution between Me_2Sn and Me_3Sn with increasing pH.

pH	Resolution
7.00	0.628
7.25	0.812
7.50	0.967
7.75	1.265
8.00	1.293
8.25	1.358
8.50	1.343
8.75	1.378

The impact of increasing pH on the separation of Me_2Sn and Me_3Sn is more clearly illustrated in fig. 4.2 where electropherograms recorded at pH 7.00, 7.50 and 8.00 are compared. At pH 7.00 both species practically co.-migrate and the peak due to Me_2Sn merely appears as a shoulder on the Me_3Sn peak. Two distinct peaks are evident at pH 7.50 however these are not baseline resolved. A substantial improvement in separation can be seen at pH 8.00. although baseline resolution is still incomplete. Separation did not improve significantly beyond this pH. The migration time of MeSn is not notably altered with increasing pH.



Fig; 4.2 Comparison of electropherograms of methyltin mixtures generated using a 20mM phosphate/0.05mM CTAB run buffer at A; pH 7.00, B; pH 7.50, C; pH 8.00. Elution order; 1. Cl^-, 2. MeSn, 3. Me_2Sn & 4. Me_3Sn.

The separation efficiency (N) for each methylated tin compound was calculated as before using the formula

$$N = 5.54\sqrt{\frac{t_m}{w_{\frac{1}{2}}}}$$

Where t_m is the migration time and $w_{1/2}$ is the peak width at half height. The variation in calculated efficiencies with pH for each methylated tin species is illustrated in fig 4.3. As was the case with migration times, the efficiencies for Me_2Sn and Me_3Sn follow exactly the opposite trend to those calculated for MeSn and decrease steadily with increasing pH. This decline in efficiencies arises due to the increase in peak width which accompanies the longer migration times incurred at higher pH levels. This is particularly apparent for Me_3Sn, the least mobile of the three species, where longitudinal diffusion of the migrating zone leads to significant bandbroadening as the migration time increases with pH. The increase in efficiencies calculated for Me_2Sn and Me_3Sn at pH 8.75 arises as a consequence of the increase in apparent mobility which occurs for these species at this pH.

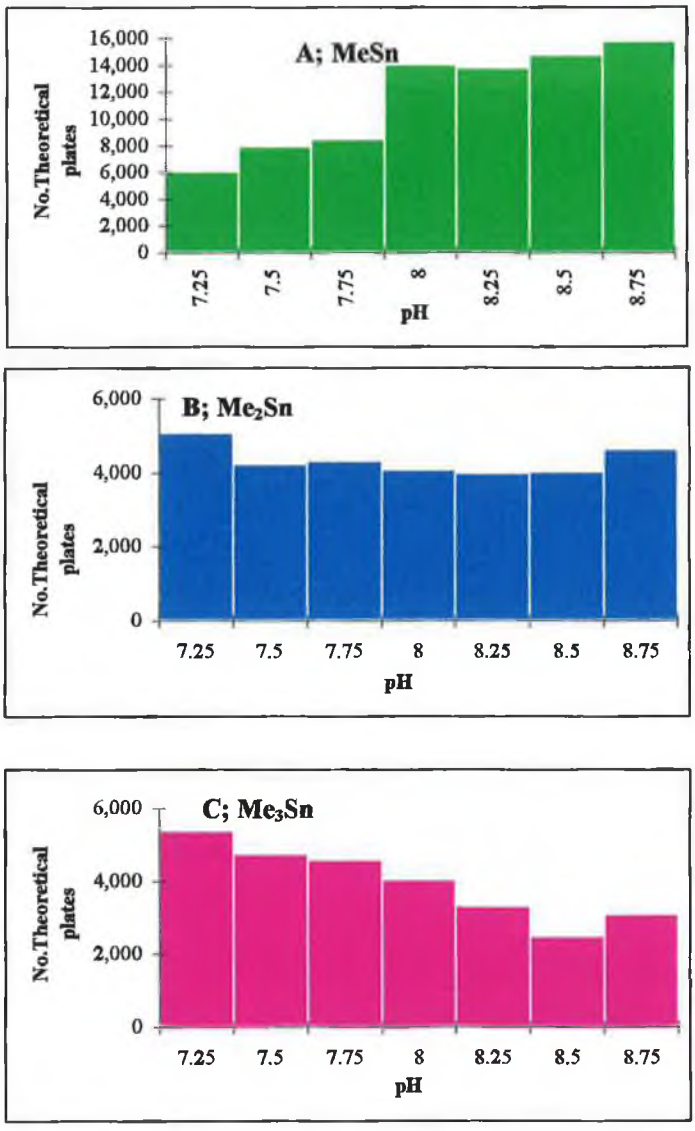


fig 4.3; Variation in calculated efficiencies with pH for each methylated tin species

The resolution between Me_2Sn and Me_3Sn ultimately dictated the choice of optimum pH and a compromise between high efficiency and adequate separation was required. The minimal variation in separation efficiencies in the pH region 8.00-8.75 made this the most suitable range to carry out all further electrophoresis experiments. In order to discriminate between the pH values 8.00, 8.25, 8.50 and 8.75, a multi level optimisation protocol was employed for the remaining run buffer conditions in which one of the properties such as CTAB concentration was held constant and the remaining properties i.e. pH and phosphate concentration were varied.

4.3.4. Optimisation of phosphate buffer concentration

Experiments on the influence of phosphate concentration on the separation of methylated tin compounds were carried out at pH 8.25, 8.50 and 8.75. In each case. the phosphate concentration was varied in the range 10 to 20mM and the CTAB concentration was held constant at 0.05mM.

The variation in migration time with phosphate concentration at each pH examined is illustrated in fig. 4.4 As expected, an increase in migration times with increasing phosphate concentration was observed in all instances due to the inverse relationship which exists between electrophoretic mobility and viscosity of the buffer medium.

The bulk electroosmotic flow was relatively unaffected by the concentration of the run buffer and injection of a 1% acetone solution yielded a peak at approximately 5.00, 4.85 and 4.50 minutes at pH 8.25. 8.50 and 8.75 respectively regardless of the concentration of the phosphate. This was due to the fact that the strength of the EOF was primarily controlled by the absorption of CTAB molecules onto the silica surface which was insensitive to changes in ionic strength of the buffer medium.

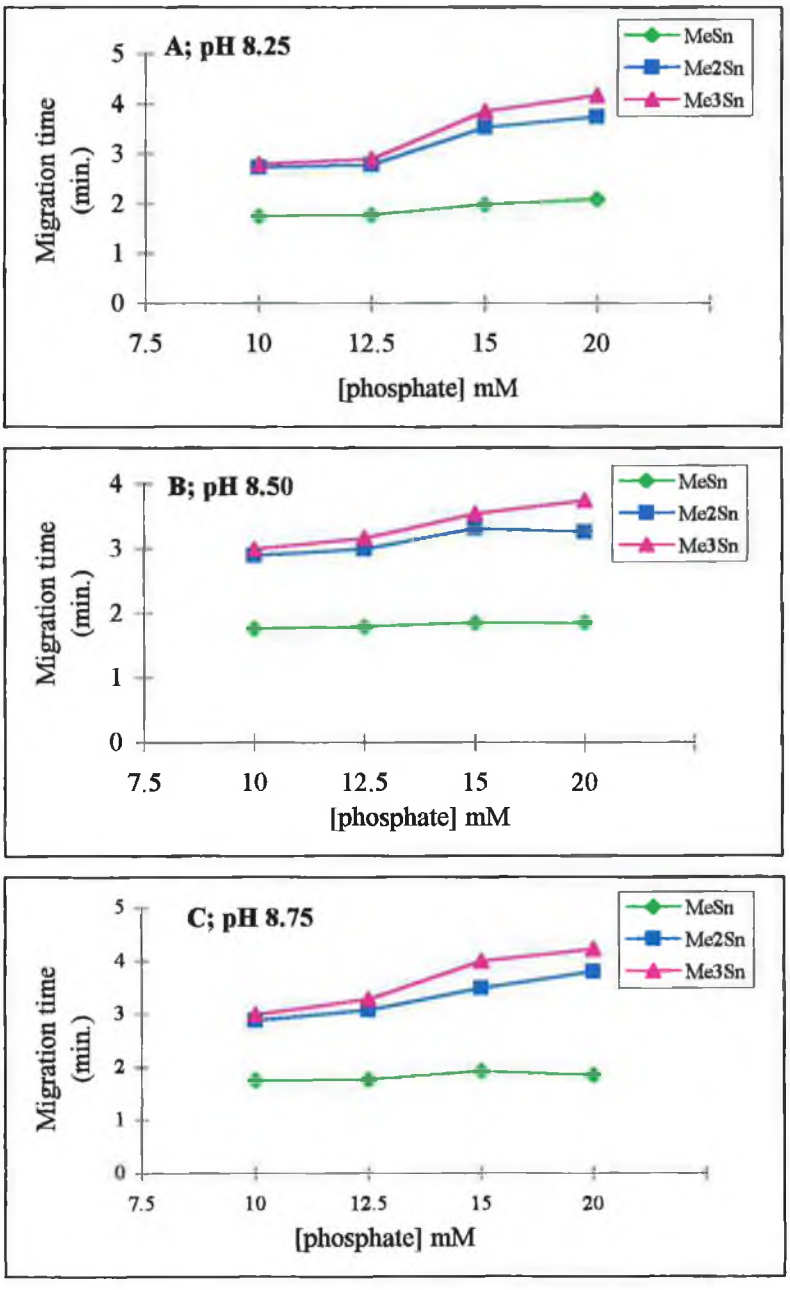


Fig 4.4; Variation of migration times of methylated tin compounds with phosphate buffer concentration at A; pH 8.25, B; pH 8.50, C; pH 8.75. The concentration of CTAB was held at 0.1mM in all cases.

As was the case in pH optimisation experiments, the resolution between Me_2Sn and Me_3Sn was the deciding factor in choosing the most suitable phosphate concentration. The effect of increasing phosphate concentration on the separation of these two species is clearly illustrated by comparing electropherograms run with 10mM, 12.5mM and 15mM phosphate at pH 8.50 (fig 4.5). The more dilute buffer systems are incapable of resolving these two species; the use of a 10mM phosphate buffer results in co.-migration of Me_2Sn and Me_3Sn and only a partial separation occurs with a

12.5mM buffer concentration. In order to attain an acceptable degree of separation, a minimum phosphate concentration of 15mM was required.

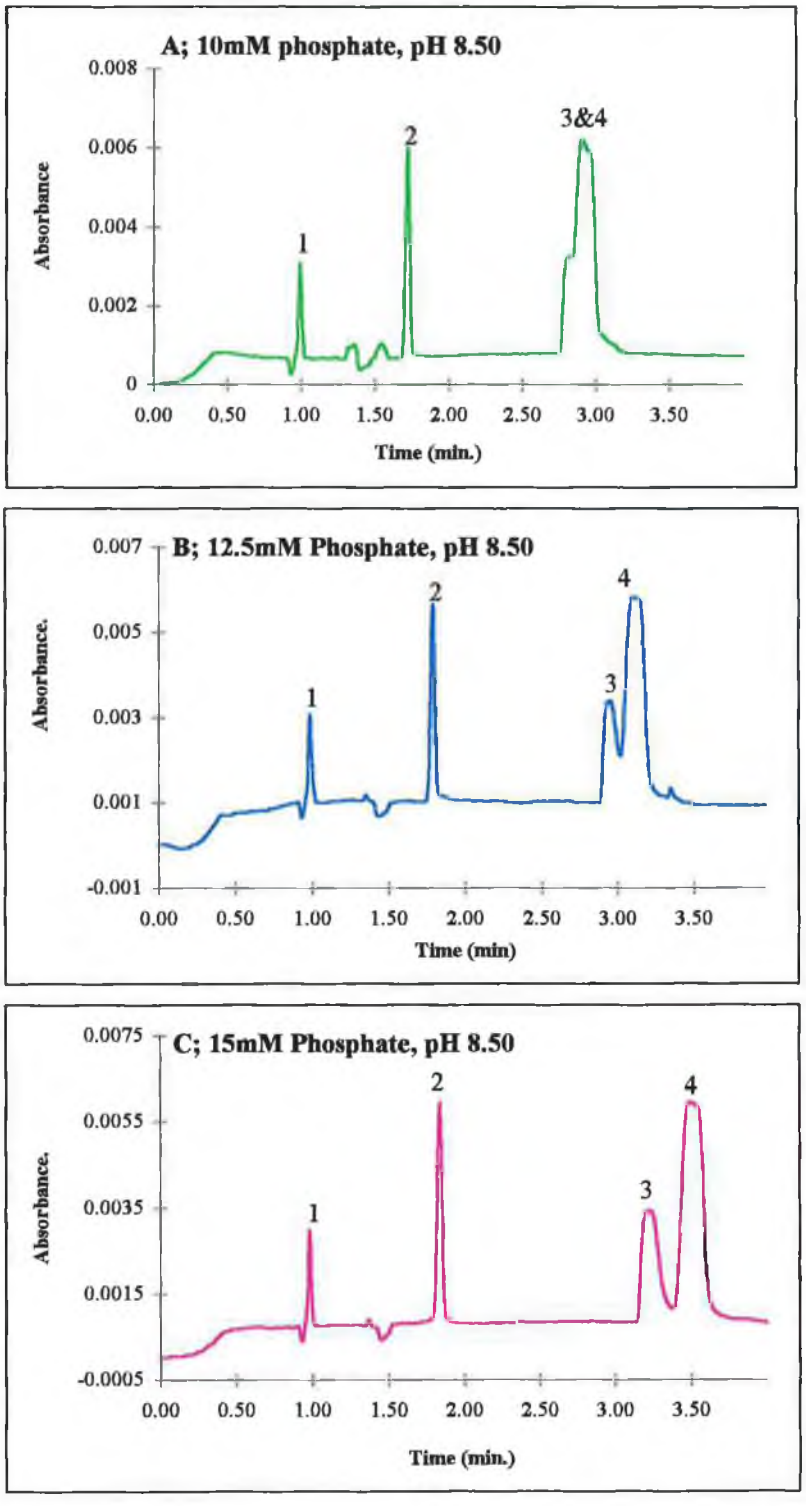


Fig. 4.5; Comparison of electropherograms of methylated tin compounds generated using run buffers of A; 10mM, B; 12.5mM and C; 15mM phosphate at pH 8.50. The concentration of CTAB was maintained at 0.1mM for all experiments. Elution order; 1. Cl^-, 2. MeSn, 3. Me_2Sn & 4. Me_3Sn.

These observations can be further confirmed by calculating the resolution between the Me_2Sn and Me_3Sn peaks for each concentration and pH level examined.

Table 4.2; Calculated resolution between Me_2Sn and Me_3Sn peaks with increasing phosphate concentration at pH 8.25, 8.50 and 8.75.

Phosphate conc. (mM)	pH 8.25	pH 8.50	pH 8.75
10.00	0.4012	0.5005	0.4434
12.50	0.7228	0.7023	0.6838
15.00	1.1397	1.1997	2.197
20.00	1.505	1.7751	1.899

It can be seen from table 4.2, that the calculated resolution values for all species are consistently below 1.00 at phosphate concentrations of 10 and 12.5mM, indicting that separation is incomplete at these levels. With more concentrated run buffers, the resolution increases steadily with increasing pH. This increase is most pronounced with 15mM buffer solutions and when the run buffer concentration was increased to 20mM, the variation in migration times and separation of the Me_2Sn and Me_3Sn is minimal. The increased joule heating leading to reduced separation efficiency and the longer analysis times which arise as a consequence of higher ionic strength buffer systems are necessary drawbacks for achieving maximum separation of Me_2Sn and Me_3Sn. The use of 15mM phosphate solutions provides a compromise between efficiency and resolution and was thus deemed to be the most suitable ionic strength for the separation procedure.

4.3.5. Optimisation of CTAB concentration.

The influence of the CTAB concentration on bulk electroosmotic flow and the migration of the methylated tin species was investigated at concentrations of 0.025mM, 0.05mM and 0.1mM in both 15mM and 20mM phosphate buffer systems at pH 8.50. In all cases, an increase in the electroosmotic flow and apparent mobility of each ion was observed with increasing CTAB concentration. This is in accordance with the findings of Lucy and Underhill[41] who interpreted this effect as being due to the gradual neutralisation of the silica surface charge through the adsorption of surfactant monomers followed by bilayer formation. At lower CTAB concentrations not all the surface sites are taken up by surfactant adsorbents and hence a local EOF may exist in the opposite direction which consequently results in a net reduction in the EOF towards the cathode. As the CTAB concentration increases less surface sites are exposed and thus the EOF continues to increase until the silica surface becomes completely saturated after which the EOF remains constant.

It can be seen from fig. 4.6 that the effect of increasing the CTAB concentration was greatest for slower migrating compounds such as Me_3Sn, where the apparent mobility was similar to the electroosmotic flow. The migration of MeSn is largely independent of the EOF, and therefore the variation in migration time and calculated efficiency (fig 4.8) is minimal.

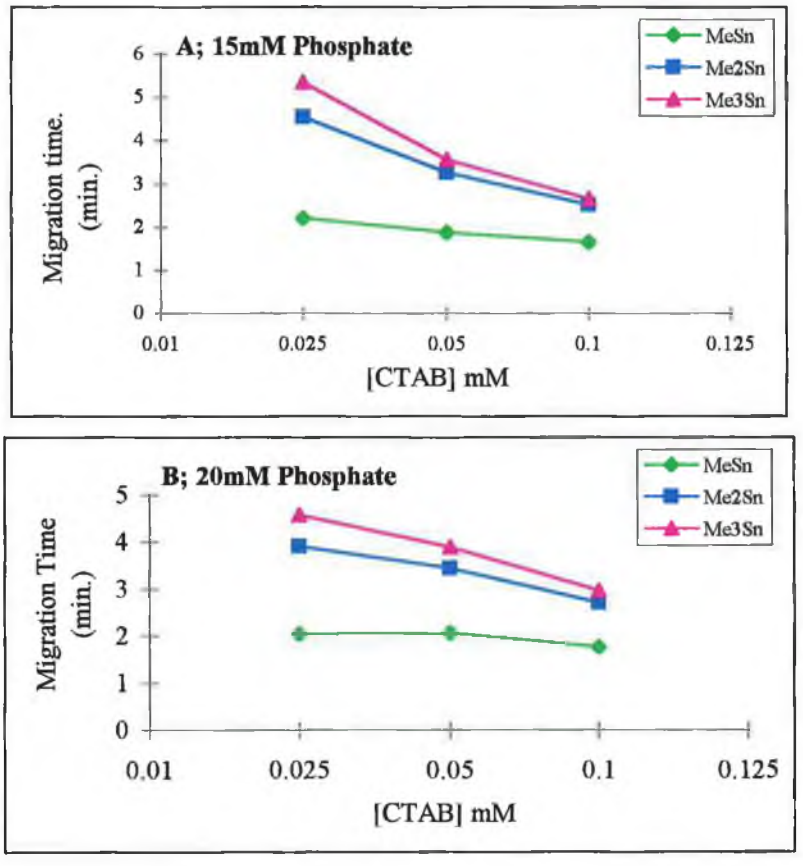


Fig 4.6; Effect of CTAB concentration on migration times of methyltins in A; 15mM phosphate, B; 20mM phosphate. The pH of the run buffer was maintained at 8.50 for all experiments.

A consequence of decreasing migration times with increasing CTAB concentration is that the degree of separation between Me_2Sn and Me_3Sn deteriorates. This is particularly notable when 15mM phosphate run buffers are employed as the lower ionic strength buffer further reduces the length of time required for the ions to migrate through the capillary. The calculated resolution values between Me_2Sn and Me_3Sn for each CTAB and phosphate concentration are listed in table 4.3. For 15mM phosphate solutions, resolution drops by 54% as the CTAB concentration is doubled from 0.025mM to 0.05mM with a further 25% decrease occurring when this concentration is doubled a second time to 0.1mM.

Table 4.3; Calculated resolution between Me_2Sn and Me_3Sn peaks with increasing CTAB concentration at phosphate concentrations of 15mM and 20mM

Phosphate conc.	0.025mM CTAB	0.05mM CTAB	0.10mM CTAB
15mM	2.02	1.10	0.44
20mM	2.16	1.81	1.24

Comparison of electropherograms run with successively increasing CTAB concentrations illustrates this effect on the migration and resolution of these methylated tin species. (fig 4.7). With buffers containing 0.025mM CTAB, all peaks are well separated but the required analysis time is almost 6 minutes and the peaks due to the later migrating species are quite broad. Resolution is maintained when the CTAB concentration is increased to 0.05mM but all species migrate within 4 minutes. Finally with 0.1mM CTAB solutions, the peaks due to Me_2Sn and Me_3Sn remain unresolved.

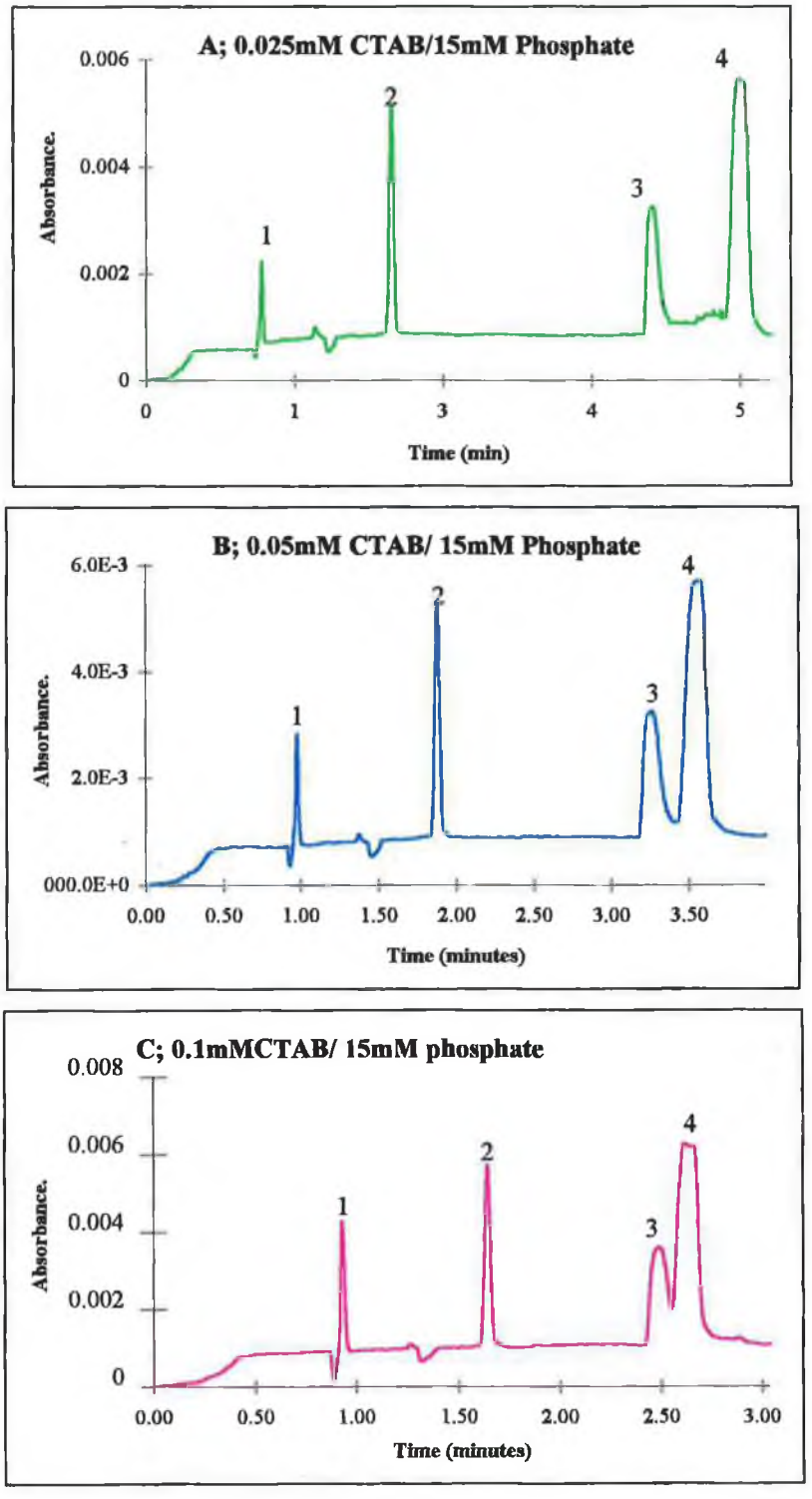


Fig.4.7; Comparison of electropherograms generated with A; 0.025mM CTAB/15mM Phosphate pH 8.50, B; 0.05mM CTAB/15mM Phosphate pH 8.50, and C; 0.1mM CTAB/15mM Phosphate pH 8.50.

Figure 4.8 compares the efficiency values calculated using 15mM and 20mM phosphate buffers in the presence of 0.025mM and 0.05mM CTAB flow modifier respectively. The efficiency data for separations carried out in the presence of 0.1mM CTAB is not included in this comparison due to the fact that co.-migration of Me_2Sn and Me_3Sn occurred at this level.

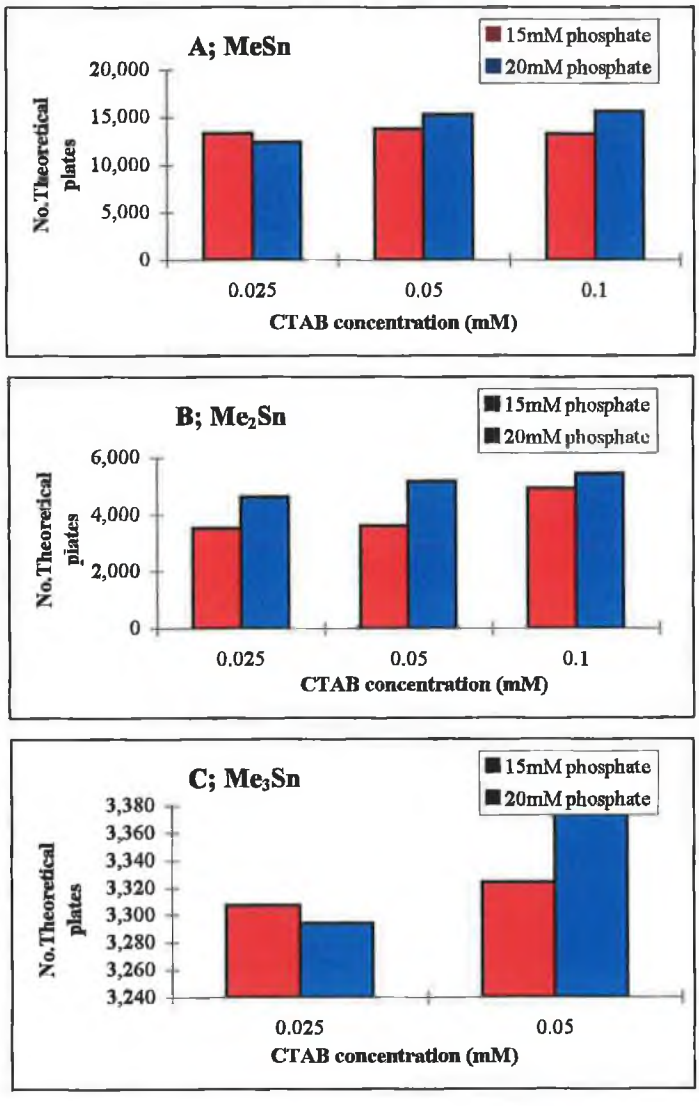


Fig 4.8; Comparison in calculated efficiency values for each methyltin species at each CTAB and phosphate concentration investigated.

As would be expected, the separation efficiency in terms of theoretical plate numbers increase with the shorter migration times afforded by higher CTAB concentrations. Therefore the choice of optimum CTAB concentration was a compromise between the conflicting parameters of maximum resolution and efficiency. A CTAB concentration of 0.05mM added to a 15mM phosphate buffer at pH 8.50 was ultimately chosen as the most suitable run buffer for this separation.

4. 3.6. Optimisation of separation voltage

As outlined in section 3.17 of Chapter three, the amount of heat generated due to electrical conduction through the capillary and the ability of the system to remove it, dictates the maximum operating voltage possible for the separation. Voltage optimisation experiments were carried out using a 15mM phosphate run buffer at pH 8.50 containing 0.05mM CTAB. The Ohms law plot of current (μA) against voltage (kV) is illustrated in fig. 4.9. It can be seen that there was a marked deviation from linearity at an operating voltage of 20kV as the generated current began to rise steeply.

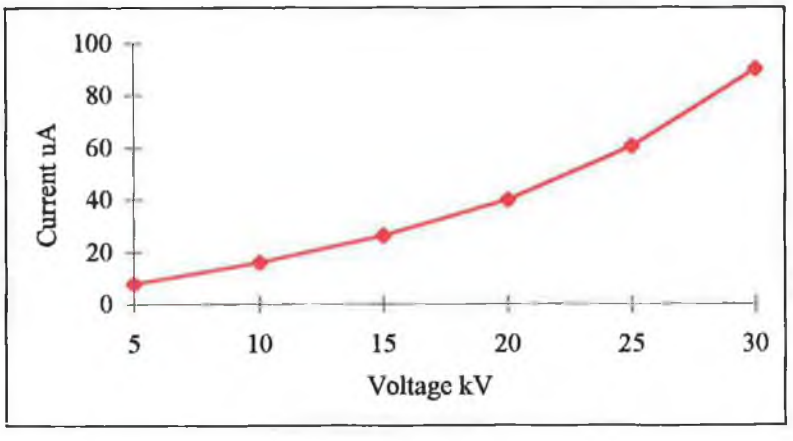


Fig 4.9; Ohms Law plot for 15mM phosphate buffer system pH 8.50 containing 0.05mM CTAB

An operating voltage of 20kV was thus used for all subsequent optimisation and validation experiments.

4.3.7. Optimisation of injection time.

The primary concern in optimising injection time was to maximise the amount of sample introduced to the column while keeping efficiency losses to a minimum.

The influence of injection time on the respective peak areas is represented in fig.4.10 For MeSn and Me_2Sn, the increase in peak area follows an approximately linear trend. This increase is very slight for MeSn, as the compounds does not absorb appreciably at 190nm. With increased methyl group substitution, the molar extinction coefficient of the compounds and hence the absorbance difference between increasing concentrations, becomes greater.

For the trimethylated tin species, the peak area rises steeply between 7.00 and 10.00 seconds after which it begins to stabilise. This corresponds to a loss in peak symmetry and increased broadening which is indicative of column overloading.

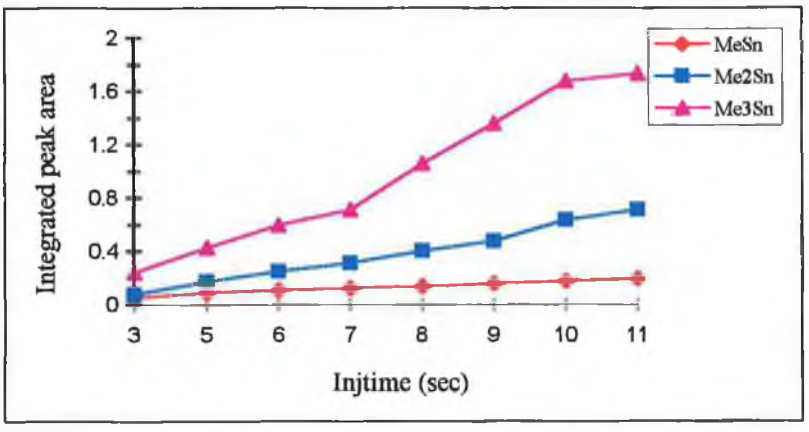


Fig 4.10; Effect of increasing injection time of peak areas of mono-, di- and trimethyltin compounds.

As expected, increasing the length of the injection plug results in a deterioration in the efficiency of the separation. The calculated theoretical plates numbers for each peak at increasing injection times are compared in fig 4.11.

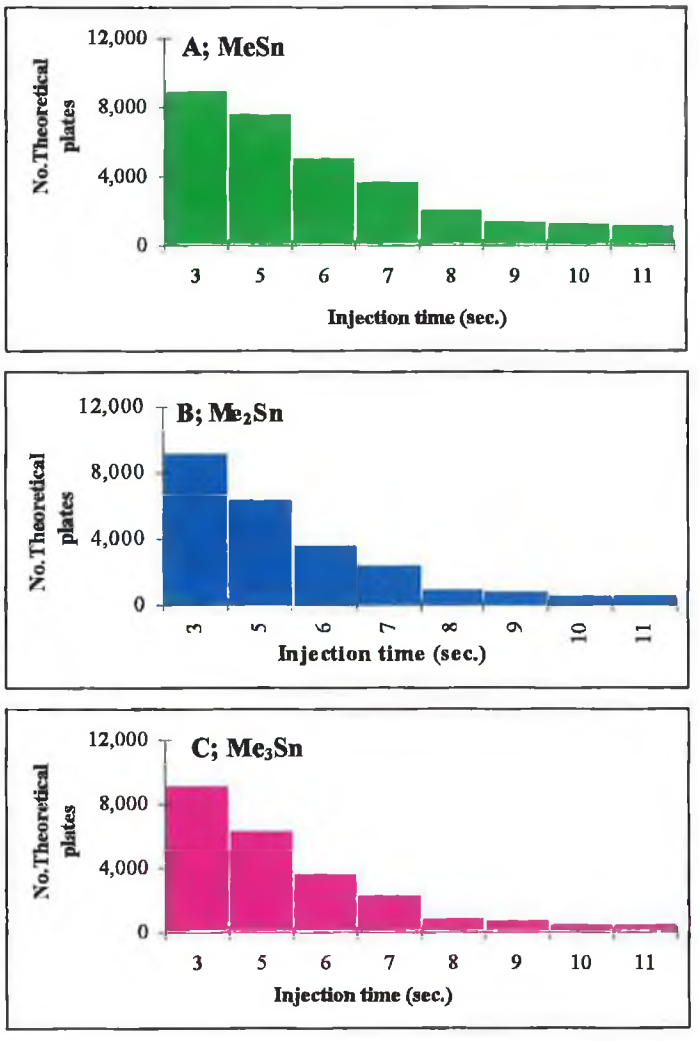


Fig 4.11; Effect of increasing injection time on calculated separation efficiencies (no. Theorctical plates).

For all compounds, there is a significant drop in efficiency as the injection time is increased from 3.00 to 7.00 seconds followed by a more gradual decline as this is increased to 11.00 seconds. These results would indicate that the maximum injection plug length is exceeded with a five second injection time. This is particularly noticeable for Me_3Sn where there is a 35% drop in efficiency as the injection time is increased from 5.00 to 6.00 seconds.

To a certain extent high efficiency could be compromised in favour of increased peak areas however the resolution between Me_2Sn and Me_3Sn also proved to be an

important consideration for determining the optimum injection time. The increase in respective peak widths brought about by increased sample loading had an adverse effect on the separation of these two species. It can be seen from table 4.4, that there is a steady decline in resolution and when injection times greater than 7.00 seconds are employed, these two peaks become unresolved.

Table 4.4 Calculated resolution between Me2Sn and Me3Sn peaks with increasing injection time.

Injection time (sec.)	Resolution
3.00	1.978
5.00	1.611
6.00	1.265
7.00	1.068
8.00	0.702
9.00	0.627
10.00	0.547
11.00	0.541

These resolution considerations ultimately dictated the maximum injection plug length which could be employed. A 7.00 second injection time was chosen as optimum, as this produced reasonably sized peaks while maintaining separation between the later migrating species and an acceptable level of efficiency.

4.3.8. Validation of separation method.

Validation of the separation method was carried out using the conditions listed below;

Table 4.5; Optimum conditions for separation of methylated tin species

Column dimensions	50μm(i.d.) X 37cm
pH	8.50
Phosphate concentration	15.00mM
CTAB concentration	0.05mM
Voltage	-20kV
Injection time	7.00 sec.

It can be seen from fig.4.12, that the detector response was linear in the range 20-50ppm for each of the methylated tin species with R^2 values consistently exceeding 0.99 (listed in table 4.9). Below 20ppm, there was insufficient detector sensitivity to discriminate between solutions of similar concentrations and a marked deviation from linearity was observed when concentrations greater than 50ppm were injected.

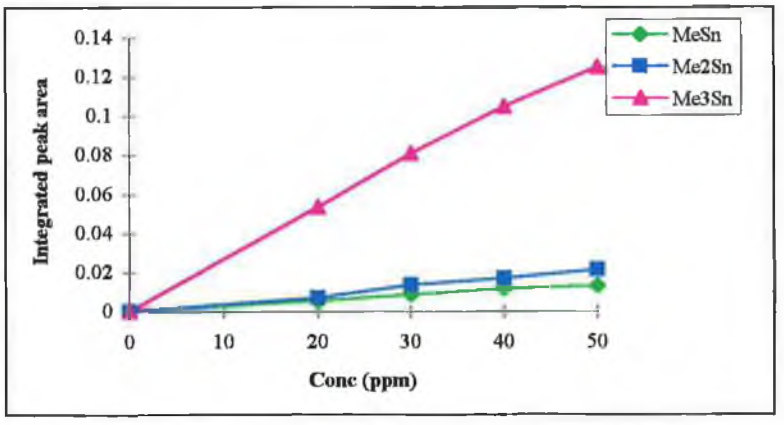


Fig. 4.12; Calibration graphs of integrated peak area against concentration (ppm) for MeSn, Me_2Sn and Me_3Sn.

The limit of detection for each species was defined as the concentration which produced a response equal to three times the noise level. Calculated values are listed in table 4.6, these values are theoretical however and in a practical situation it was not possible to identify individual peaks at concentration below 5ppm.

Table 4.6; Limits of detection and standard curve regression values for each methylated tin species separated under optimum conditions

Tin species	R^2	Limit of Detection (ppm)
MeSn	0.9934	0.69
Me_2Sn	0.9922	2.20
Me_3Sn	0.9969	0.60

Table 4.7; Statistical validation for the separation method.

	within day variability (n = 6)	between day variability (n = 6)	mean no. theoretical plates
MeSn	8.08%	9.35%	18,882
Me_2Sn	7.40%	10.67%	6,173
Me_3Sn	9.52%	10.17%	3,170

The statistical validation figures for within and between day variations are summarised in table 4.7. The variation in migration times within a single run is quite high when compared with analogous studies with arsenic species discussed in chapter three. These values however, are all below 10% and therefore satisfy the criteria for a valid separation method. The between day reproducibility was poor, with the % RSD in the migration times of Me_2Sn and Me_3Sn exceeding 10%. The relatively large fluctuation in migration times over a period of five days was accompanied by a similar variation in the electroosmotic flow of the carrier electrolyte and therefore was possibly due to incomplete and irreproducible coating of the capillary inner surface with the CTAB modifier.

The reproducibility of this method therefore needs to be improved if it is to be used as a viable and accurate approach to the determination of methylated tins. In this regard alternative surfactant modifiers or wider bore capillaries need to be investigated.

4.3.9. Application of the CE method to the analysis of samples containing methyltin compounds.

As outlined in the previous section, the detection limits of this method are too high for environmental applications and investigations into the feasibility of stacking and/or indirect detection are required.

The method in its current format however provided a rapid and simple means of monitoring the variation in the methyltin content over a period of time in a number of "in house" reaction mixtures. This task has been previously carried out by proton nuclear magnetic resonance spectroscopy, however this approach is hampered by poor sensitivity and the fact that samples must be prepared in expensive deuterated solvents.

Two reactions were monitored; the photo-degradation of tetramethyl tin in acetonitrile and the formation of methylmercury and trimethyltin following transmethylation between tetramethyltin and mercuric chloride, also in acetonitrile. In both cases, a 22.5µl sample was removed from the reaction mixture and transferred to 4.5ml. of run buffer before injecting into the capillary this corresponded to a 1/200 dilution of the original sample.

Examples of electropherograms obtained are illustrated in fig. 4.13. Electropherogram A related to a sample taken during the photo-degradation of Me_4Sn. It can be seen that conversion to Me_3Sn proceeds rapidly however further degradation to Me_2Sn occurs only to a slight extent. The formation of MeHg is evident in electropherogram B by the appearance of an extra peak due to this compound at 3.25 minutes. In this case only Me_3Sn is formed

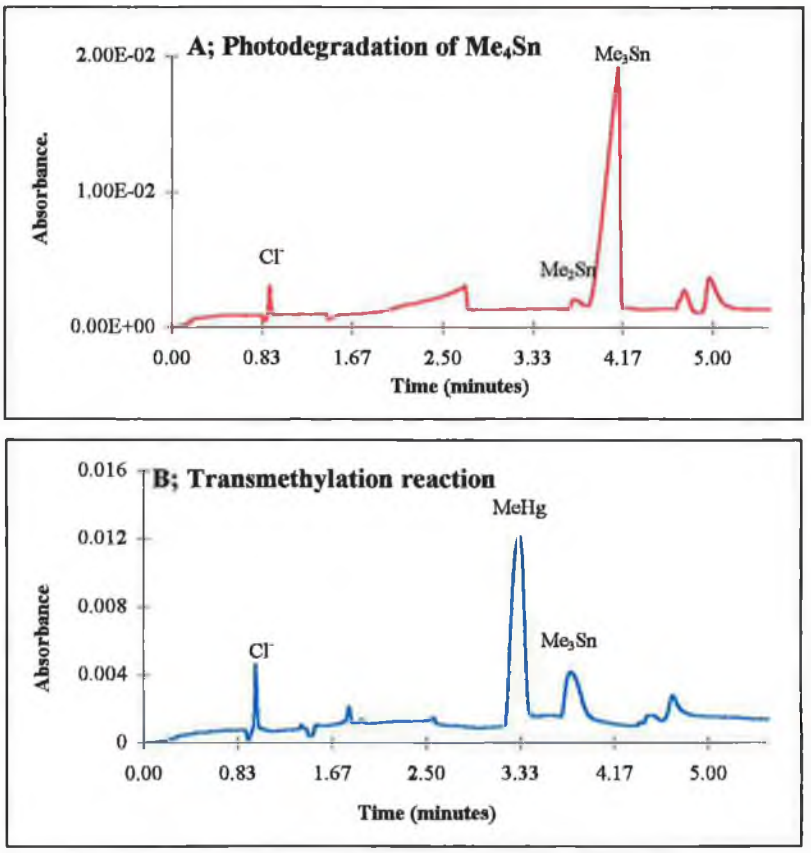


Fig. 4.13; Electropherograms of samples taken from A; the photo-degradation of Me4Sn and B; the formation of MeHg by transmethylation between Me_4Sn and $HgCl_2$.

4.3.10. Conclusions.

The feasibility of capillary electrophoresis for the separation and determination of methylated tin species has thus been demonstrated. The initial assumption that these compounds were cationic in character under the conditions studied proved to be incorrect and a reversed polarity/flow modified approach was necessary to facilitate the separation. The use of CTAB as the surfactant flow modifier gave rise to reproducibility problems with respect to electroosmotic flow and migration times and future work should focus on alternative modifier molecules and or wider bore capillaries as a means of achieving a lower within and between day variation.

In this instance a direct UV detection scheme was employed. This gave rise to detection limits too high for environmental applications however the technique proved to be a viable and method of monitoring the formation of certain methylated tin compounds in a number of reactions carried out in this department. To date this task has been carried out by proton nuclear magnetic resonance spectroscopy (NMR). This technique however provided only qualititative information on the nature of the methylated compounds being formed. With capillary electrophoresis it is now possible to determine the concentrations of each methylated tin compound present in the reaction mixture thus enabling the rate of each reaction to be determined. In addition to this, the capillary electrophoresis approach is consideralby more economical then proton NMR which requires the sample to be prepared in expensive deuterated solvents.

Future work should also focus on the development of indirect detection and stacking methods which would improve detection limits making the capillary electrophoresis approach amenable to environmental determinations.

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Chapter Five

Conclusions and recommendations

5.1. Conclusions and recommendations

It is now universally recognised that metals exist in the environment not as a single entity but in a wide array of inorganic and organometallic species each with individual physicochemical properties. The use of analytical techniques which can accurately measure each species is therefore vital for evaluating the true toxicological hazard and environmental impact of a particular metal.

The primary objective of analytical research in this field is the development of a method with the capability of discriminating between species and the sensitivity to detect these species at sub ppb levels while maintaining the integrity of speciation in the original sample. To date, hybrid techniques involving chromatographic separation with detection by atomic absorption spectroscopy has proved most successful in this regard. While gas chromatography offers superior resolution and compatibility for interfacing with flame, furnace and plasma based atomic absorption spectrometers, it is limited in its application to more volatile species such as the alkylleads and metallic compounds which are susceptible to derivatisation with sodium borohydride or grignard reagents. It is liquid chromatography therefore, with its broad range of available stationary and mobile phases that has found the widest application in the area of trace metal speciation. The development of liquid chromatography-atomic absorption systems for routine metal speciation work has been hampered however due to difficulties with interfacing the chromatography system to the atomic absorption detector. The incompatibility of a flowing liquid system with the nebulisation process of a flame atomic absorption spectrometer leads to low sensitivity as only a fraction of the column eluant is introduced to the analytical flame. Sensitivity of flame based techniques can be significantly increased if the metal species are converted to volatile hydrides prior to reaching the flame, however this approach is restricted to those metal species which are capable of reacting with the sodium borohydride reagent. In addition to this, the hydride generation interface involves a complex network of rubber tubing, pumps and valves with requires constant attention and maintenance. The use of furnace based methods of atomisation although providing the necessary sensitivity to detect species at environmental levels, requires a complicated fraction collection

process and manual transfer system making the technique a very time consuming process and labour intensive process. The high cost of the instrumentation and graphite atomisation tubes which have to be replaced on a regular basis would also discourage the use of this technique for routine metal speciation studies.

In the course of this study a number of alternative techniques for metal speciation were examined. The criteria for these techniques were that each should be straightforward in design and operation, requiring a minimal level of manual input while remaining cost effective.

Although analytical methods for trace metal speciation are a widely researched topic, an area which has received little attention is the actual preparation of the sample prior to analysis. For water samples, sample handling merely involves a simple filtration step, however for more complex matrices such as tissue samples, isolation of the metal species involves a complex series of liquid-liquid extraction steps often using toxic and corrosive solvents such as phenol. The use of a solid phase extraction technique known as matrix solid phase dispersion (MSPD) as an alternative to liquid-liquid extraction for the isolation of arsenic species from fish tissue is examined in chapter 2. With this MSPD technique, disruption of the tissue matrix is brought about by physically grinding it up with a quantity of chromatographic packing. The resultant mixture is then packed into a solid phase extraction cartridge where cellular components can be washed away with a non-polar solvent followed by elution of the analytes with the chosen mobile phase. This technique has already proved to be a highly effective method in isolating a number of antibiotics, pesticides and growth promoter compounds from a wide range of tissue samples. In the course of this study the parameters of the extraction procedure were modified to adapt the approach for the extraction of arsenic species. With the exception of arsenic (III), the technique was highly successful for this purpose, the highest percentage recoveries of arsenic species were obtained when the tissues were blended with an anion exchange chromatography packing and the resultant columns were eluted with 8ml. of 0.2M phosphate buffer at pH 5.82. The percentage recoveries for arsenic(V), MMA, DMA and arsenobetaine ranged from 74.30% to 80.23% with all relative standard deviation values representing within day and between day variations below 10%. Comparison of this modified

MSPD extraction with a liquid-liquid extraction protocol commonly used for the isolation of arsenic species from fish tissue showed that the later approach was extremely time consuming and labour intensive. The large number of steps involved inevitably lead to substantial losses so the final yield was an inaccurate reflection of the concentration of arsenic species in the original sample. The MSPD approach on the other hand was a straightforward clean technique requiring only four separate steps and yielding a high recovery of analyte and a final extract which was relatively free from organic interferants.

Further work is required however to adapt the MSPD approach so that the elution step can be carried out on-line with the analytical column. This would further enhance the reproducibility of the method by reducing variation due to manual input. In addition to this the effectiveness of the technique for extraction very low concnetrations (< 50ppb) of arsenic species from fish tissues has yet to be evaluated.

The suitability of capillary electrophoresis as an analytical technique for the determination of trace metal species is examined in chapters 3 and 4 with regard to arsenic and methylated tin species respectively.

Capillary electrophoresis involves the separation of analytes in a buffer filled narrow bore capillary based on differences in the rate of their migration in an electric field. This migration is brought about by two factors, the bulk electroosmotic flow of the support electrolyte and the intrinsic electophoretic flow of the analyte. The principal merit of this technique lies in the fact that separation efficiencies comparable with those obtained with capillary gas chromatography are possible. The separation of complex mixtures of non-volatile analytes which conventionally require lengthy analysis times or a column switching approach is thus possible within short period of time in a single step. The fused silica capillary tubing used for the separations is inexpensive, this combined with the low reagent consumption makes capillary electrophoresis an attractive and economical alternative to HPLC for the separation of metal species. The chief drawback of capillary electrophoresis is that the low injection volumes and narrow optical path lengths' results in low sensitivity for on-line UV absorbance systems conventionally used for detecting the migrating analytes. For the purposes of

trace metal speciation, this problem is further compounded by the fact that metallic species do not absorb appreciably in the UV region. Sensitivity problems from capillary electrophoretic determinations can be overcome in two ways; firstly the quantity of sample introduced onto the column can be increased through the use of a technique known as stacking. This approach exploits the fact that an analyte will migrate faster in a low ionic strength buffer as it experiences a higher electric field strength. If a sample is prepared in water or highly dilute buffer and subsequently injected onto the column its initial migration will be rapid until it reaches the boundary between injection buffer and the more concentrated support electrolyte where the analyte velocity is abruptly decreased. The resulting effect is a compression of the analyte zone thus enabling larger volumes of sample to be injected without loss in efficiency and peak symmetry.

The second approach for sensitivity enhancement involves the inclusion of a high UV absorbing molecule known as "an indirect detection probe" into the run buffer. Migrating analytes cause displacement of the probe and this will appear as a decrease in absorbance at the detector. The sensitivity of this technique is based on the background signal to noise ratio, the concentration of the probe and the ability of the analyte to displace it. The development of a method which combines these two techniques for the separation and determination of arsenic species by capillary electrophoresis is discussed in chapter 3. The method employed was an adaptation of a technique used for the separation and determination of inorganic anions such as chlorides and nitrates and involved the use of an electroosmotic flow modifier diethylenetriamine (DETA) and an indirect detection probe pyromellatic acid (PMA) with the polarity of the instrument electrodes configured such that the positive terminal is at the detector end of the capillary. The DETA modifier interacts with the inner capillary surface and causes suppression of the electroosmotic flow. Introduction of an aqueous solution of sample into the column results in the generation of a local electroosmotic flow in the direction on the injection due to the redissolution of the DETA into the water matrix. Anionic constituents will migrate in the opposite direction to this flow due to the enhanced electric field and stack at the sample/electrolyte boundary. The water plug therefore effectively pumps itself out of

the column while the anionic analytes are compressed into a narrow plug, this enables injection volumes of up to 90% of the capacity of the capillary to be employed. Due to the anionic nature of the four most commonly occurring environmental arsenic species; arsenic(V), MMA, DMA and arsenobetaine, the use of this flow modified stacking procedure with indirect detection proved to be a highly effective method of determining these compounds by capillary electrophoresis. The use of a 15mM DETA/5.5mM PMA run buffer at pH 8.50 and a separation potential of 30kV afforded the separation of these four arsenic species in a 57cm X 50μm capillary column in a single step within 10 minutes with separation efficiencies ranging from 4,613 to 57,766 theoretical plates. In terms of simplicity of operation this is a considerable improvement on existing HPLC-HGAAS methods which require at least one derivitisation step to enable detection of the eluting arsenicals which in turn necessitates the inclusion of cumbersome tubing, pumps and reaction coils into the analytical system. The combined effects of large volume stacking and indirect detection gave rise to limits of detection of 480ppb, 538ppb, 92ppb and 58ppb for DMA, MMA, arsenobetaine and arsenic (V) respectively. This represented a hundred fold improvement in sensitivity over a conventional capillary electrophoretic separation using direct UV absorbance detection. In order to further reduce the limits of detection for these arsenic species to environmental levels, a chromatographic pre-concentration step is required.

The application of capillary electrophoresis to the separation and determination of mono-, di- and tri-methylated tin compounds is investigated in chapter 4. In this instance, the focus of the experimental design is not detection sensitivity but rather the development of a straightforward and inexpensive alternative to nuclear magnetic resonance spectroscopy for the purpose of monitoring these compounds in a number of reaction mixtures. Initial experiments revealed that these methylated tin compounds behaved as anions in aqueous buffer solutions which was contrary to the original assumption that they were cationic in nature. Separation of the three methylated tin species was thus achieved in a 37cm X 50μm capillary column filled with a 15mM phosphate buffer at pH 8.50 which contained 0.05mM of the cationic surfactant CTAB. This compound interacted with the negatively charged capillary wall such that the direction of the electroosmotic flow was towards the positive electrode which was

situated at the detector end of the capillary. A potential of 20kV was selected as the optimum for the separation, this ensured complete separation of the methylated tin compounds within 4 minutes. This separation method was successfully applied to the determination trimethyltin and dimethyltin in a solution following the photodegradation of tetramethyltin. The formation of trimethyltin as a result of a transmethylation reaction between tetramethyltin and mercury chloride and its subsequent degradation to dimethyltin could also be monitored. This analysis had formerly been carried out by nuclear magnetic resonance spectroscopy which was considerably more time consuming and necessitated the use of expensive deuterated solvents.

The development of a capillary electrophoresis method for the separation of methylated tin compounds is however still in its preliminary stages. The degree of reproducibility of the separation using a phosphate/CTAB buffer is low possibly due to an uneven coating of the capillary inner surface by the surfactant modifier. Alternative modifier molecules therefore need to be examined in order to achieve a lower degree of variability between analysis. Furthermore, in order to extend the use of capillary electrophoresis to the determination of these methylated tin compounds in environmental samples, the use of the sensitivity enhancing techniques of stacking and indirect detection need to be examined.

The majority of metal speciation methods developed in the past two decades, while capable of determining individual metallic species and extremely low levels have the disadvantage of being time consuming and labour intensive, often requiring large volumes of solvents and expensive instrumentation. This may make them unsuitable for routine analyses thus preventing their transition from research to more practical applications. In the course of this study, the use of matrix solid phase dispersion extraction and capillary electrophoresis analysis were evaluated as feasible alternatives to existing techniques for the analysis of arsenic and methyltin species. Each technique investigated met with a considerable degree of success and in all cases provided a more rapid and simple method of carrying out the extraction or analysis with a lower consumption of solvents and other reagents. Future work should focus on improving the sensitivity of these techniques so as to make them more amenable to routine environmental applications.